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MOLECULAR WEIGHTS OF GLYCOLLATE OXIDASE FROM C_3 AND C_4 PLANTS DETERMINED DURING EARLY STAGES OF PURIFICATION

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Abstract—The M_rs of glycollate oxidase (EC 1.1.3.1) (GAO) determined soon after extraction from the leaves of several C_3 and C_4 plants are reported. The enzyme isolated from the C_3 plants wheat, barley, spinach, pea and tobacco has M_r in the range 160–180 000 and is probably a homotetramer. GAO purified from pea was previously reported as a dimer and as an octamer from spinach leaves. Therefore the quaternary structure of these GAOs soon after extraction differs from that of the purified proteins. The enzymes from the C_4 plants maize and sugar cane have M_rs *ca* twice this value in the range 290–310 000, whilst that of the C_4 grass *Panicum maximum* has an M_r of 162 000. An improved spectrophotometric assay for GAO, using a non-carcinogenic dye, is described.

INTRODUCTION

Glycollate oxidase (EC 1.1.3.1) (GAO) catalyses the conversion of glycollate to glyoxylate in the second reaction of the photosynthetic carbon oxidation cycle [1]. The quaternary structure of the GAO molecule has been reported to vary between plants and is a matter of controversy. Thus, the enzyme purified from pea leaves is a dimer [2], from cucumber cotyledons a tetramer [3] and from spinach leaves an octamer [4]. Forms of GAO with up to 16 subunits have been described [3]. The reason for this apparent diversity of structure is unclear. Instability during purification and a tendency to form aggregates has been suggested [3]. Another possibility is that different molecular forms of the enzyme have been selected during the purification procedures or that there is real diversity in the molecular structure of the enzyme.

Native M_rs of GAO from C_3 and C_4 plants are reported in this paper. Our findings using this enzyme at the early stages of purification are compared with M_rs reported for the purified glycollate oxidase.

RESULTS

M_rs *by exclusion chromatography*

The profile from a Sephacryl S300 column of a typical experiment using a spinach leaf extract showed that only one form of the enzyme is present with an M_r of *ca* 160 000 (Fig. 1). The bulk of GAO activity was found between fractions 215 and 233. The arrow at fraction 205 marks the predicted position of the octamer (M_r 300 000); only a trace of activity was present in this fraction. Similarly, the activity found in fractions 238–245, where the dimer would be expected, was very low. From this we conclude that in crude and partially purified extracts, only one form of GAO is present. This form is a homotetramer, based on published subunit weights of 37 000 [4, 5]. In all experiments, four internal markers of known M_rs were included (Fig. 1).

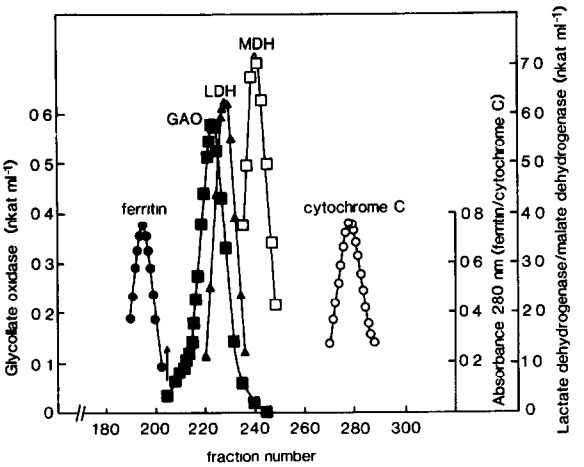

Fig. 1. Profile from a typical gel exclusion chromatography experiment. A crude extract of spinach leaf (prepared by method 2) was run on Sephacryl S300, eluted at 4 ml/hr. The regression coefficients for $Y = A + B \log X$ was 0.998 using the four markers. Fractions of 0.5 ml were collected, ↑ shows where an M_r of 300 000 would be found on this column.

M_rs *by sucrose gradient centrifugation*

Ultracentrifugation of crude extracts of spinach, pea, wheat and barley leaves gave M_rs between 153 000 to 199 000 (Table 1). In all experiments three internal markers were included. The profile from a typical experiment using spinach leaf extracts gave a M_r for GAO of 176 500 (Fig. 2). No GAO activity was detected in fractions where an octamer of this enzyme would be found; i.e. between ribulose 1,5-bisphosphate carboxylase (RuBisCO) and catalase.

Our results in Fig. 2 can be contrasted with Fig. 2 in ref. [4] which shows purified spinach GAO between catalase and RuBisCO on the 5–20% sucrose gradient.

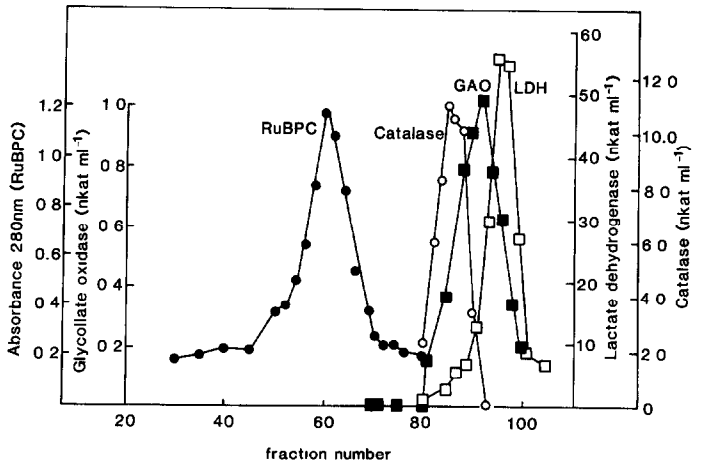

Fig. 2. Profile of a typical M_r determination using sucrose gradient centrifugation. A crude extract of spinach leaf (prepared by method 2) was centrifuged on a 5.5–20% w/v linear sucrose gradient. Assays were as described in the Experimental section.

Table 1. M_rs of glycollate oxidase determined by sucrose gradient centrifugation

Source of enzyme	M_r	Sample preparation
Wheat	161 500	Method 2
Spinach	176 500	Method 2
Barley	153 000	Method 2
Pea	199 000	Method 2

Enzyme sample (1 ml) containing GAO, 4 mg spinach RuBP carboxylase ($S_{20,w}$ = 18 0), 0.42 mg rabbit muscle lactate dehydrogenase ($S_{20,w}$ = 7 0) and catalase ($S_{20,w}$ = 11.4) was layered on top of a 30 ml linear sucrose gradient (5.5–20% w/v) and centrifuged in a Beckman 70-Ti rotor at 70 000 rpm for 2 hr. At the end of centrifugation 0 3 ml fractions were collected.

Enzyme from C_3 and C_4 plants

The GAO isolated from leaves of the C_3 plants wheat, spinach, pea, tobacco and barley had an M_r of 160–180 000 (Table 2). The enzyme from two of the C_4 plants studied had an M_r of 290–310 000 (Table 2), whilst that of *Panicum maximum* had an M_r of 162 000. GAO from *Spartina anglica* had an M_r of 93 000 (Table 2). It is not possible to ascribe one type of GAO to C_3 plants and another form of the enzyme to C_4 plants. However our results (Table 2) show that many C_3 plants have a form of GAO which is presumably a homotetramer. SDS gel electrophoresis of the wheat enzyme revealed one major band of M_r 43 000 (data not shown).

Changes of experimental conditions

The experimental conditions were changed to see if this had any affect on the M_r of the wheat enzyme; samples used were either crude extracts or partially purified according to method 1. The M_r was measured on Sephacryl S200, S300 and S400. Gradient gel electrophoresis indicated an M_r of 160 000 (data not shown). Changing the column buffer from 50 mM HEPES/50 mM NaCl pH 7.4, to 0.3 M Tris–HCl (pH 10, 4°) did not change the M_r. Chromatofocusing of the wheat enzyme showed the presence of only one peak (Fig. 3), although a shoulder of activity similar to that reported in ref. [3], was present.

Assay for glycollate oxidase

A new assay for GAO was developed. The assay for glucose [6] was modified so that the glycollate-dependent production of H_2O_2 was linked through peroxidase to the yellow dye antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one). During the reaction, this dye was oxidized to a red product (in the presence of phenol) with an increase in absorbance at 510 nm.

A change in absorbance of 0.1 corresponded to the production of 63 nmol H_2O_2 in the 3 ml reaction mixture. The assay was linear between 0–300 nmol H_2O_2. Although another spectrophotometric method for GAO using horseradish peroxidase has been reported [7], this used the suspected carcinogen *o*-dianisidine, which is avoided in the present technique. The new assay was more sensitive and gave more stable rates than other methods [2]. Enzyme activity measured in the antipyrine/peroxidase assay was very similar to that using the oxygen electrode assay [2].

DISCUSSION

Although there are reported differences in the M_r of native GAO, the subunit size is generally within the range of 37 000–48 000 [2–4, 5, 8]. Allowing for the error in SDS electrophoresis, which is about ± 10% [9], this suggests a similar subunit weight regardless of the source of the enzyme. The equivalent enzyme in animal tissues, which preferentially oxidises short chain aliphatic hydroxyacids (hydroxyacid oxidase A), also has a subunit weight of 40 000 [6]. Our data indicate that the subunit weight of the wheat leaf enzyme is 43 000 and the M_r of the active native enzyme is *ca* 160 000 (Tables 1 and 2) suggesting that GAO from this C_3 species is a homotetramer. If the

Table 2. M_rs of glycollate oxidase from C_3 and C_4 plants determined by gel exclusion chromatography

Source of enzyme	M_r	Gel exclusion medium	Sample preparation
Wheat	166000	S200, S300, S400	Methods 1 and 2
Spinach	158000	S300	Methods 1 and 2
Barley	175000	S300	Method 2
Tobacco	167000	S300	Method 1
Pea	184000	S300	Method 2
Maize	308000	S300	Method 3
Sugar cane	287000	S300	Method 3
Spartina anglica	93000	S300	Method 3
Panicum maximum	162000	S300	Method 3

Values shown are the mean from at least two experiments; for wheat the figure is a mean from seven experiments.

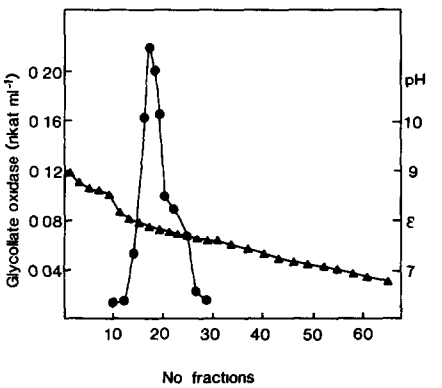

Fig. 3. Chromatofocusing of wheat leaf GAO on PBE 94. The enzyme sample, prepared by method 1, was centrifuged at 10000 *g* for 5 min, redissolved in 0.5 ml of 25 mM ethanolamine–acetic acid, pH 9.4, and desalted [20] into the same buffer. Separation was achieved at a flow rate of 15 ml/hr and 65 fractions of 4 ml were collected. Activity (●) was measured by the antipyrine assay; ▲, pH.

subunit weights reported in the literature [2–4, 5, 8] apply to the other enzymes studied, then GAO proteins of M_r 160–185000 would presumably be tetramers and those with weights of 290–310000 would be octamers.

Our findings that GAO from both pea and spinach crude extracts is a tetramer contrast with reports on the purified enzyme. Pea GAO is a dimer [2], although a trace of higher M_r form was observed. Purified spinach leaf GAO is established as an octamer [4, 5, 8]. It seems possible that the degree of polymerization or aggregation of subunits of GAO may change during purification. Several lines of evidence support this view; Firstly it was shown that the spinach enzyme can exist both as a tetramer and an octamer under conditions of high ionic strength [10]. Secondly, a hydroxyacid oxidase A from animal tissues was conclusively shown to self-associate at concentrations of 4–6 mg/ml [6]. Thirdly, Behrends *et al.* [3] reported that the enzyme from cucumber cotyledons changed from an M_r of 700000 to 150000 during purification. These authors stressed the importance of studying the enzyme at the initial stages of purification, which may represent a form of GAO similar to that found in the intact cell.

In this paper we reported the existence of dimeric, tetrameric and octameric forms of GAO according to the source of the enzyme. Why the enzyme has such variation in quaternary structure is unclear. A recent report [11] showed that the M_r of GAO from *Lemna minor* grown on NH_4^+ is double that of GAO from *Lemna* grown on NO_3^-. Interestingly these changes in M_r were associated with changes in the specific activity of the molecule.

EXPERIMENTAL

Plant materials. Wheat, *Triticum aestivum* (L. cv Maris Dove); barley, *Hordeum vulgare* (L. cv Golden Promise); maize, *Zea mays* (cv. Eta); tobacco, *Nicotiana tabacum* (cv xanthi-nc); pea, *Pisum sativum* (cv. Progreta); sugar cane, (*Saccharum officinarum*); *Panicum maximum* and *Spartina anglica* were grown in a greenhouse under natural daylight.

Preparation of samples for M_r *determinations* Three methods were used: 1. GAO extracted from wheat, spinach and tobacco leaves was partially purified by $(NH_4)_2SO_4$ precipitation and sucrose gradient centrifugation as far as step 3 described in ref. [12]. After RuBisCO was collected from the bottom of the gradient, the top fractions containing GAO were precipitated with 60% satd $(NH_4)_2SO_4$ A 10–15 ml aliquot was centrifuged at 10000 *g* for 15 min and redissolved in 1 ml of 50 mM HEPES–KOH containing 50 mM NaCl (pH 7 4). 2. Leaf tissue (0.5 g fr wt) was ground at 4° with 2 ml of 50 mM HEPES–KOH containing 50 mM NaCl (pH 7.4). The extract was filtered and centrifuged at 10000 *g* for 30 min. One ml of the supernatant was used for M_r determination. 3. Leaf tissue (5 g fr wt) was ground at 4° with 20 ml 50 mM HEPES–KOH containing 50 mM NaCl (pH 7.4). The homogenate was filtered and centrifuged at 100000 *g* for 30 min. The supernatant was precipitated with 60% satd $(NH_4)_2SO_4$, and then centrifuged for 15 min at 10000 *g*. The pellet was redissolved in 1 ml of the HEPES buffer (pH 7 4).

Gel exclusion chromatography. The M_r was determined using Sephacryl S200, S300 and S400 (Pharmacia) following the method of ref. [13]. The enzyme sample (1 ml, 3–4 mg protein) containing four marker proteins was centrifuged at 10000 *g* for 5 min at 4° before use The sample was applied to a 90 × 1.6 cm column and eluted at 4 ml/hr in 50 mM HEPES–KOH containing 50 mM NaCl (pH 7 4 at 4°). Fractions of 0.5 ml were collected and assayed for GAO activity.

Internal M_r standards for the S300 column were ferritin (440 000), rabbit muscle lactate dehydrogenase (LDH; 142 000), bovine heart malate dehydrogenase (MDH; 70 000), and horse heart cytochrome *c* (12 400). Lactate dehydrogenase [14] and malate dehydrogenase [15] were assayed using spectrophotometric methods Ferritin and cytochrome *c* were detected by *A* at 280 nm.

For the S200 and S400 columns, additional markers were: thyroglobulin (670 000); catalase (250 000); BSA (67 000); egg albumin (43 000) and carbonic anhydrase (31 000). The origins, assays and values for M_rs were derived from ref. [13]

Sucrose density gradient centrifugation. The enzyme sample (1 ml) was centrifuged in a Beckman L8 70 ultracentrifuge at 215 000 *g* for 2 hr using a 70Ti rotor according to the method of ref. [16]. Marker proteins added to the sample were spinach RuBisCO (4 mg) and rabbit muscle LDH (0.42 mg protein). Catalase already present in the sample was used as another marker. Linear sucrose gradients (5.5 to 20% w/v) prepared in 50 mM Tris–HCl buffer (pH 7.75 at 5°) were used. After centrifugation 0.3 ml fractions were collected and diluted with 0.6 ml 50 mM Tris–HCl buffer (pH 7.75 at 5°). Fractions were assayed for GAO, catalase [17] and LDH activities [14]. RuBisCO was detected by *A* at 280 nm.

Assay for glycollate oxidase. H_2O_2 produced in the reaction between glycollate and GAO was measured using a modification of the assay for glucose [18]. The reaction mixture (3 ml final vol.) contained 50 mM Tris–HCl, pH 8.3; 0.66 mM antipyrine; 3 units of horseradish peroxidase (150 units/mg solid); 2.1 mM phenol; 0.04 mM FMN and 0.67 mM Na glycollate. Assays were initiated with glycollate The increase in *A* at 510 nm was followed at 30°. Interference by catalase present in the extracts was checked for by the addition of extra peroxidase.

The assay was calibrated by adding an aliquot of H_2O_2 to the assay mixture without glycollate of GAO being present. The H_2O_2 soln was first standardized assuming a molar absorptivity coefficient for peroxide of 61 M^{-1} cm^{-1} at 230 nm [19]. Enzyme activity was also measured in an oxygen electrode by determining glycollate-dependent O_2 uptake [2].

Further purification for SDS electrophoresis. A 1 ml sample was applied to a 90 × 1 6 cm gel filtration column of Sephacryl S300 equilibrated with 50 mM Tris–HCl, pH 8.3 (4°). The enzyme was eluted at 4 ml/hr and 0.5 ml fractions collected. The peak fractions (10 mg protein) were added to 1 ml of DEAE-Sephacel equilibrated in 50 mM Tris–HCl (pH 8.3 at 4°) and allowed to react for 1 hr at 4°. Under these conditions GAO did not bind to the DEAE-Sephacel. The supernatant was added to the SDS sample buffer and SDS polyacrylamide gel electrophoresis carried out using a 10% gel [20]. Gels were stained in 0.25% Coomassie Blue G250. M_r standards were lysozyme (13 400); carbonic anhydrase (31 000); ovalbumin (43 000) and serum albumin (67 000).

Chromatofocusing of glycollate oxidase. A 1 ml sample prepared by method 1 was centrifuged at 10 000 *g* for 5 min, redissolved in 0.5 ml starting buffer (25 mM ethanolamine–HOAc, pH 9.4) and desalted [21]. The 0.5 ml sample (10 mg protein) was applied to a 50 × 1 cm column (bed height 25 cm) filled with polybuffer exchanger (PBE 94; Pharmacia). The column was equilibrated with 1 l. starting buffer followed by 5 ml of elution buffer (polybuffer 96–HOAc, pH 6.0, diluted 1·10). Separation was achieved with elution buffer, pH 6, at a flow rate of 15 ml/hr and 65 fractions of 4 ml were collected. Enzyme activity was measured by the antipyrine assay.

Gradient gel electrophoresis. GAO from wheat, partially purified by method 1, was applied to a gradient gel (Pharmacia PAA 4/30) [22]. The same M_r markers were used as for Sephacryl S300 gel exclusion. GAO activity was detected using the method for oxidase enzymes [23] with glycollate as substrate.

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