

Purification and partial characterization of glyoxalase I from a higher plant *Brassica juncea*

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The glyoxalase I was purified from *Brassica juncea* by affinity chromatography on S-hexyl GSH sepharose 4B. Homogeneity of the protein was confirmed electrophoretically by a silver stained gel. Activity staining on a native starch gel also showed a single band. The effect of glutathione, methylglyoxal, and pH on enzyme kinetics was studied. Magnesium was found to stimulate the enzyme activity.

Glyoxalase I; Enzyme purification; Methylglyoxal; Glutathione; S-hexyl glutathione

1. INTRODUCTION

The glyoxalase enzyme system comprising of glyoxalase I and II was discovered 80 years ago [1] and it is found to be ubiquitous [2]. Although its widespread distribution and the presence of an active glyoxalase system throughout life from embryogenesis to cell death [3] suggests some very basic and important function [3], its exact biological role is still a mystery.

Of the two glyoxalase enzymes, glyoxalase I has been studied most extensively in animal and microbial systems [5]. Glyoxalase I (EC 4.4.1.5, lactoyl GSH lyase) catalyses the formation of S-D-lactoyl GSH from the hemithioacetal formed non-enzymatically from MG and GSH [4]. In plants, there are scant reports about the enzyme. In gymnosperms, the presence of MG and glyoxalase was reported [6]. In higher plants, activity of glyoxalase I was found in pea [7] and a correlation between the enzyme activity, as affected by various factors, and cell proliferation was noticed [8-10]. We now report on the purification of *Brassica* glyoxalase I to homogeneity. This to our knowledge is the first report on the purification of glyoxalase I from plants.

2. MATERIALS AND METHODS

2.1. Chemicals

Most of the chemicals used were of AR grade and purchased from Sigma chemical Co. except Sephadex G-50 and low molecular weight

markers which were purchased from Pharmacia fine chemicals, Uppsala, Sweden.

2.2. Growth of seedlings

Seeds of *Brassica juncea* Pusa bold were obtained from IARI, New Delhi. Seedlings were grown at $25 \pm 2^\circ\text{C}$ on germination paper and supplied with Hoagland's nutrient solution.

2.3. Preparation of matrix i.e. S-hexyl GSH Sepharose 4B

ECH Sepharose 4B (activated gel) was coupled to the ligand (S-hexyl GSH) in presence of carbodiimide, according to the instructions given in Pharmacia handbook [11].

2.4. Enzyme assay

Seedlings were extracted in 1:2 extraction buffer (0.1 M sodium phosphate buffer pH 7.0, 16 mM magnesium sulphate, 50% glycerol, PMSF (0.25%), 5 mM EGTA, 2% w/v PVP) and the extract was centrifuged ($20\,000 \times g$, 1 h). The supernatant was used for assay as described by Racker [12] with slight modifications [7]. Formation of thioester (absorbs at 240 nm) was measured. One enzyme unit is the amount of enzyme catalyzing the formation of one μmol of S-lactoyl-GSH per minute.

2.5. Protein Estimation

Protein estimation was done by Bradford's method [13]. BSA (fraction 5) was used as a standard.

2.6. Purification of glyoxalase I

All steps were carried out at 4°C and 0.1 M sodium phosphate buffer pH 7.0 was used throughout unless indicated. 260 g seedlings were extracted and centrifuged. The supernatant was saturated with pulverised ammonium sulphate to 40% w/v concentration and centrifuged ($10\,000 \times g$, 30 min). The supernatant was further precipitated to 80% ammonium sulphate concentration and centrifuged ($10\,000 \times g$, 30 min). The pellet was dissolved in a minimum volume of buffer and dialysed for 5 h against 10 mM buffer. The dialysed sample was loaded after addition of NaCl (0.2 M) to and equilibrated (buffer containing 0.2 M NaCl) column ($1.5 \times 8\text{ cm}$). The column was washed with buffer until absorbance at 280 nm was less than 0.02. Second washing was with buffer + NaCl (0.2 M) and final wash was with buffer + GSH (10 mM). For elution, same buffer with GSH (10 mM), S-hexyl GSH (2 mM) and NaCl (0.2 M) was used.

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Abbreviations: GSH, reduced glutathione; MG, methylglyoxal; PMSF, phenyl methylsulfonyl fluoride; PVP, polyvinylpyrrolidone; EGTA, ethylene glycol-bis(β -aminoethyl ether) N,N,N',N' -tetraacetic acid

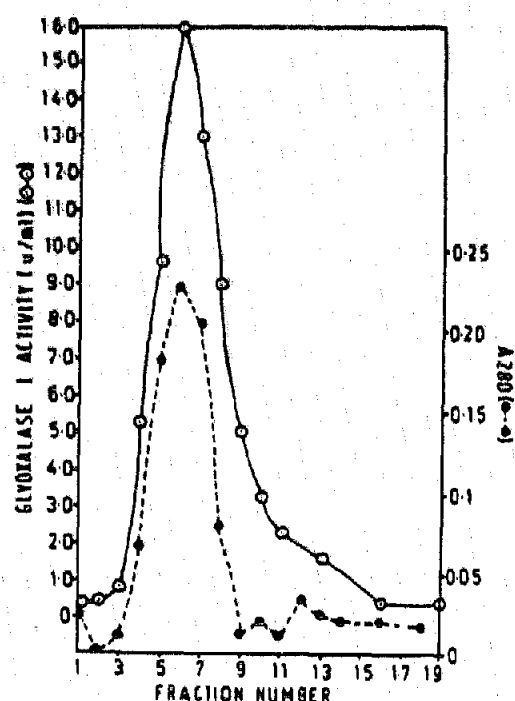


Fig. 1. Elution profile of glyoxalase I from S-hexyl GSH sepharose 4B.

The eluted peak was loaded on to an equilibrated (10 mM buffer) sephadex G-50 column (2.5 × 50 cm), to remove S-hexyl GSH. The elution was done with equilibration buffer.

2.7. Electrophoresis

SDS PAGE was carried out according to the previously described procedure [14]. A 10% gel was used and silver stained [15]. Sub-unit molecular weight was determined by running different standard proteins along with the enzyme on the gel. Rf vs log molecular weight was plotted. For activity the staining protocol of Bagster et al. [14] was modified and used. Purified enzyme was run on a 7% native gel containing hydrolysed starch (0.13%), at a constant current of 35 mA for 3.5 hours at 4°C. The gel was incubated for 30 min at 37°C in buffer containing 0.1 M sodium phosphate pH 7.0; 16 mM magnesium sulphate; 0.6% GSH and 4% v/v of (40% stock) methylglyoxal. The gel was rinsed with water and coloring reagent (1% KI and 0.05%

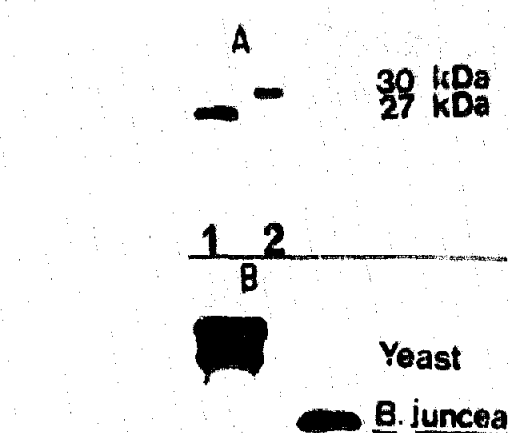


Fig. 2. (A) 10% silver stained gel of glyoxalase I. 1 ml fractions of enzyme were TCA precipitated, dissolved in SDS sample buffer and loaded on the gel. Lane 1, glyoxalase I 40 µl; lane 2, carbonic anhydrase 6 µl. (B) Activity staining of glyoxalase I after starch gel electrophoresis. Lane 1, yeast glyoxalase I; lane 2 *Br. juncea* glyoxalase I.

iodine solution) was added. Blue bands could be observed within a minute.

2.8. Densitometer scanning

Scanning of the gels was done with Hirschmann densitometer using 523 nm scanning light beam.

3. RESULTS AND DISCUSSION

3.1. Purification of Glyoxalase I

Enzyme was purified from 7-day-old seedlings as described in section 2. The enzyme was eluted specifically as a symmetrical peak from the affinity column (Fig. 1). The purification steps are summarised in Table I. With an overall recovery of 23% and 145-fold purification, the enzyme obtained was homogeneous. This method of purifying glyoxalase I is quick and simple.

Table I
Purification of glyoxalase I from *B. juncea*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification factor
Crude	375.00	476.25	622.50	1.30	100.00	1.00
0-40% AS(s)	380.00	410.40	718.20	1.70	115.37	1.30
40-80% AS(p)	57.00	100.32	310.65	3.09	49.90	2.37
40-80% AS(d)	90.00	148.50	236.00	2.87	37.91	1.70
S-hexyl GSH Sepharose	24.30	*	180.00	—	28.91	—
Sephadex G-50	57.00	0.93	145.70	188.33	23.39	144.86

AS, ammonium sulphate; (s), supernatant; (p) pellet; (d), dialyzed.

*Protein concentration was not determined due to interference from S-hexyl GSH.

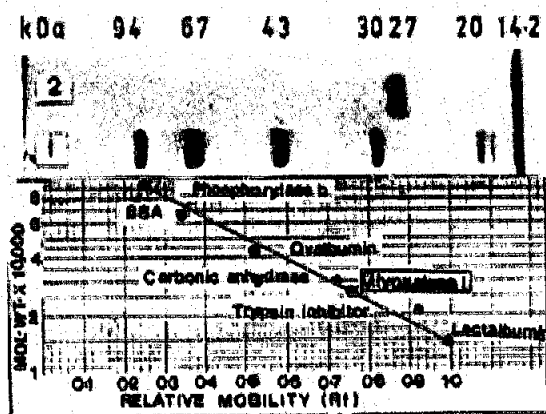


Fig. 3. Determination of the subunit molecular weight of the glyoxalase I by SDS electrophoresis. *Above*: Coomassie Brilliant Blue-R stained gel with standard protein markers and the enzyme. Lane 1, phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor, and α -Lactalbumin; Lane 2, glyoxalase I. *Below*: Rf vs log mol.wt plot of same gel.

3.2. Molecular properties of Glyoxalase I

The purified enzyme recovered from Sephadex G-50 was homogeneous as judged by SDS-PAGE (Fig. 2A). Densitometer tracing of the same gel showed a single symmetrical peak confirming the presence of a single band (not shown). The molecular weight was calculated to be 27 kDa (Fig. 3). It is slightly higher than the glyoxalase I purified from animal sources where it ranges from 21 kDa to 24 kDa [3].

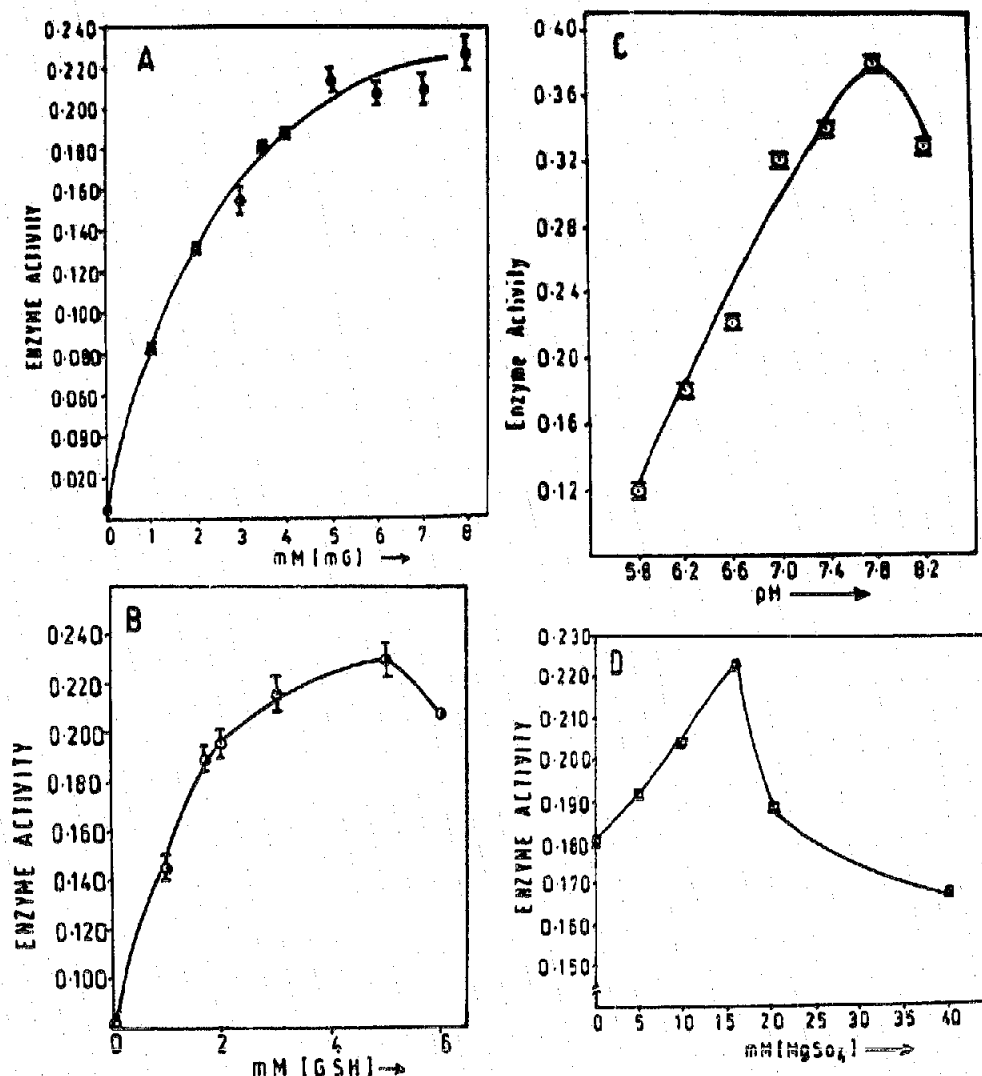


Fig. 4. (A) Glyoxalase I activity with varying concentration of methyl glyoxal (concentration of GSH is 1.7 mM). (B) Glyoxalase I activity with varying concentration of GSH (concentration of MG is 3.5 mM). (C) Effect of pH on glyoxalase I activity, buffers used were MES (pH range 5.5-6.7), MOPS (pH range 6.5-7.9), sodium phosphate (pH range 5-8) and HEPES (pH range 6.8-8.2). (D) Effect of different $[Mg^{2+}]$ on glyoxalase I.

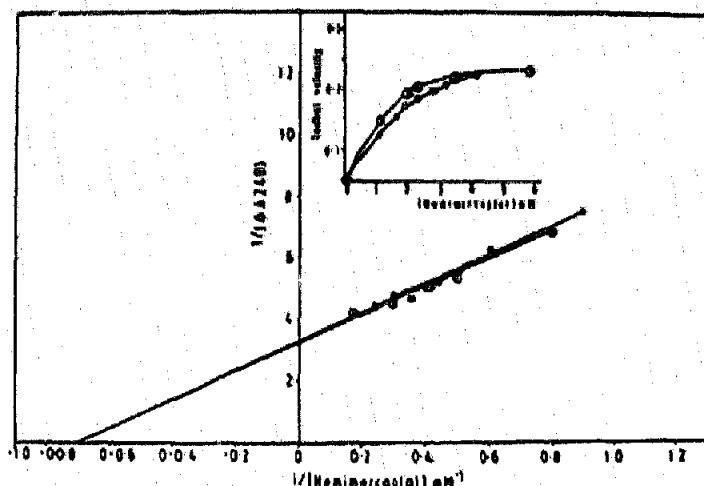


Fig. 5. Inset shows Glyoxalase I activity with varying concentration of hemimercaptal of methylglyoxal and GSH. The concentration of hemimercaptal for various concentrations of methyl glyoxal and GSH have been calculated using $K_d = 3.0 \times 10^{-3}$ M for equilibrium between methyl glyoxal, GSH and hemimercaptal. Replotted against their initial velocities. [Hemimercaptal] at fixed MG with varying GSH concentration ($\circ$ — $\circ$), and at fixed GSH with varying MG concentration ($\bullet$ — $\bullet$). Lineweaver-Burk plot of reciprocal of the initial velocity of glyoxalase I vs reciprocal of concentration of hemimercaptal. Values of hemimercaptal are taken from inset and meaning of symbols is retained.

Activity staining of Brassica glyoxalase I showed a single blue band in a native gel, indicating that there was no isoform (Fig. 2B). Yeast glyoxalase I, used as control, showed a number of bands. These may be due to isoforms or different oxidised forms [5]. There are reports of the existence of up to 3 isoforms in some animal systems [17].

3.3. Substrate Requirement

The extent of glyoxalase I activation both by GSH (at fixed MG concentration) and MG (at fixed GSH concentration) was studied (Fig. 4A, B). As already reported in animal systems hemimercaptal (the non-enzymically-formed adduct of MG and GSH) is the real substrate for glyoxalase I. If it is true, then when the velocity is plotted against adduct concentration, one should get a linear plot of $1/v$ vs $1/[S]$ where $[S]$ is hemimercaptal concentration [18]. When plotted we got results similar to those reported for animal glyoxalase I [18] indicating that in plants also the adduct is the real substrate (Fig. 5). For calculation of adduct concentration K_d was taken from Vander Jagt et al. [19].

The enzyme showed a pH optimum at 7.8 (Fig. 4C). As reported for the animal apoenzyme reactivation experiments, Mg^{2+} is the most potent stimulator of enzyme activity [5]. We also found stimulation of enzyme activity by Mg^{2+} from crude (data not given) to the purified preparation. The optimum $[Mg^{2+}]$ was found to be 16 mM (Fig. 4D).

The present report demonstrates conclusively the presence of glyoxalase I in plant systems. The purified enzyme shows properties similar to those of glyoxalase from other systems except that the size of the plant enzyme seems to be larger than that of the enzyme obtained from animal sources.

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