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BIOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF *BRASSICA JUNCEA* GLYOXALASE I

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Key Word Index—*Brassica juncea* L.; Brassicaceae; glyoxalase I; CD spectrum apoenzyme; amino acid modification; antibodies; cross reactivity.

Abstract—A homogenous preparation of glyoxalase I (*S*-lactoylglutathione-lyase, EC 4.4.1.5) was obtained from *Brassica juncea* seedlings. The enzyme is a heterodimer with 27,000 and 29,000 M_r subunits and native M_r of 56,000. The circular dichroic spectra of the protein showed characteristics of a distinctly helical protein, and magnesium affected the secondary structure. It is a zinc metalloenzyme. Amino acid modification studies suggested the involvement of histidine residues in catalysis. Apo-glyoxalase I was reactivated by divalent cations Mn^{2+} (0.5 Mm) > Mg^{2+} (5 Mm) > Zn^{2+} (0.05 Mm) and Ca^{2+} (0.01 Mm). Monospecific, polyclonal anti-glyoxalase I antibodies were raised, which showed its presence in seeds, roots, hypocotyl, cotyledon and different flower parts. They showed varied degree of cross reactivity with the extracts from various plants, yeast, bacteria and animal system. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Glyoxalase I and II are ubiquitous enzymes of the glyoxalase system. Both enzymes are involved in converting a variety of toxic 2-oxoaldehydes into less lethal 2-hydroxyacids. Methylglyoxal (MG), the physiological substrate for glyoxalase I, is toxic in free form as it reacts with proteins [1] and nucleic acids [2]. Glyoxalase I detoxifies the cells of the harmful effects of MG [3] by converting it to *S*–D lactoyl glutathione [4] which is then converted to D-lactic acid by glyoxalase II (EC 3.1.2.6).

Glyoxalase I has been purified and studied extensively in microorganisms [5] and animal systems [3, 6]. Its gene has been cloned from different systems [7, 8]. Besides detoxification it has also been proposed to have a role in cell division [9, 10]. The work on glyoxalase I in plants is still limited to a few systems only. Its presence was first reported in a gymnosperm, Douglas fir [11] and later in angiosperms [12]. Correlation between glyoxalase I and cell proliferation has been observed in callus cultures of soybean, *Datura innoxia*, pea, *Corchorus*, *Brassica oleracea*, and *Amaranthus* [13–15].

Glyoxalase I has been purified from *Aloë vera* [16], *B. juncea* [17] and soybean [10] however its detailed biochemical characterization is still lacking. Here we report its secondary structure, the metal ion associated with the native enzyme, the amino acid at the active site and some immunochemical studies, especially the cross reactivity of the antibodies with antigens from heterologous systems and its distribution in different (developing and differentiated) parts of the *Brassica* seedlings.

RESULTS AND DISCUSSION

Native M_r and subunit composition of glyoxalase I

Earlier a fast and simple protocol for the purification of *B. juncea* glyoxalase I [17] and some of its kinetic and molecular properties were reported. In the present study it was observed that for glyoxalase I purification either freshly harvested or frozen tissue could be used without any significant effect on the yields, and blue dextran or blue sepharose matrices were not as effective as *S*-hexyl glutathione agarose or Sepharose although they have been successfully used to purify the animal enzyme [18]. The native M_r was 58,000 as determined on a Sephadex G-75 column. The elution profile of the protein

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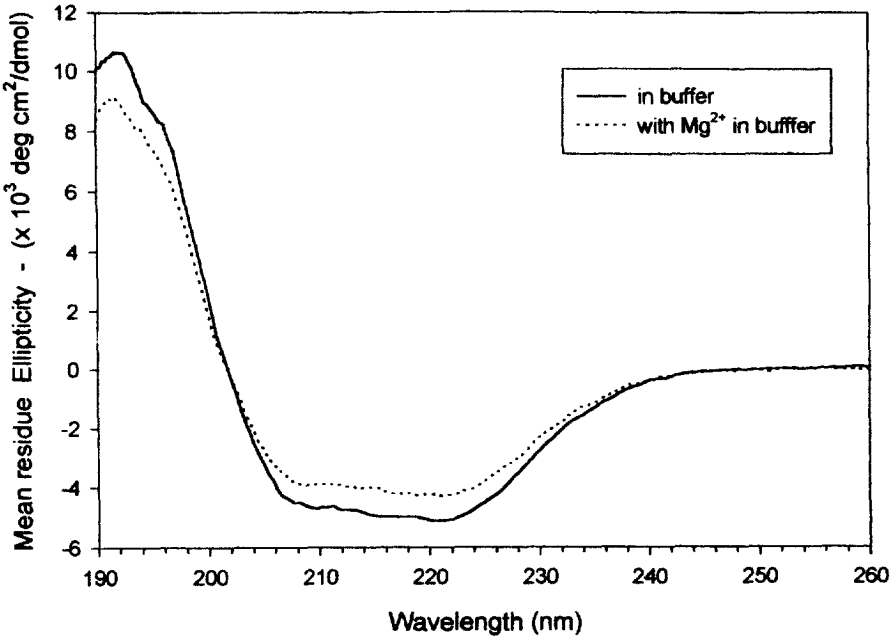

standards and glyoxalase I is shown in Fig. 1 and the inset shows the V_e/V_o versus M_r plot. On 10% denaturing gel, it migrated as a single band at M_r 27,000 (Fig. 2(A), lane 2) it was resolved into two polypeptides of M_r 27,000 and 29,000 on higher than 10% gel (Fig. 2(B), lane 2) suggesting that it is

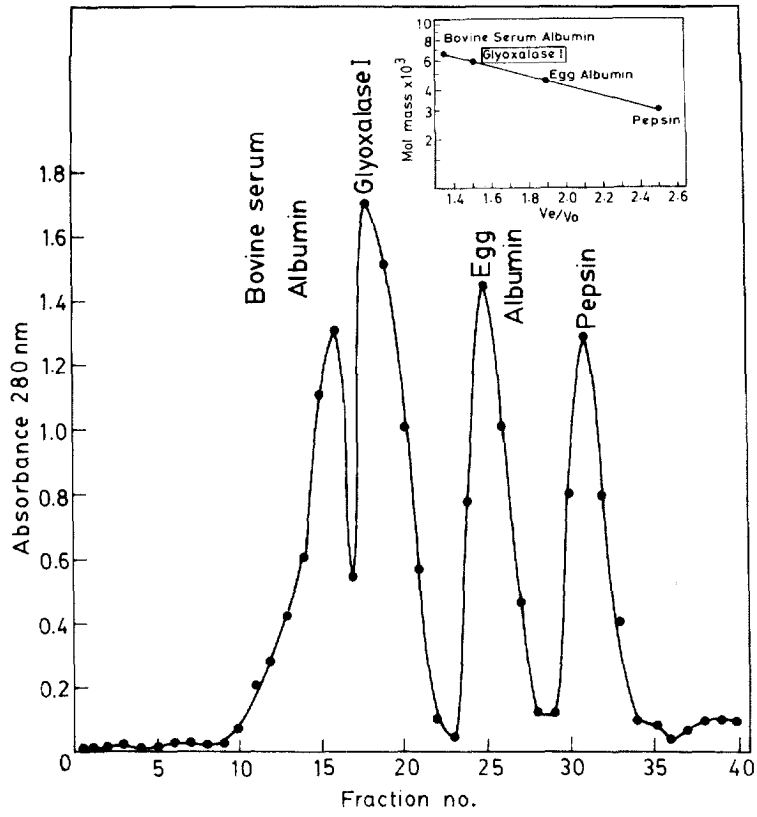

Fig. 1. Native M_r of *B. juncea* glyoxalase I determined using Sephadex G-75 column. Glyoxalase I (0.5 mg/ml) and standard proteins (1 mg/ml) 1 ml were loaded and eluted with 10 Mm buffer containing 0.2 M NaCl. The figure shows the elution profile of the enzyme and the standard proteins. Inset shows the $M_r \times 10^3$ versus V_e/V_o plot of the eluted proteins.

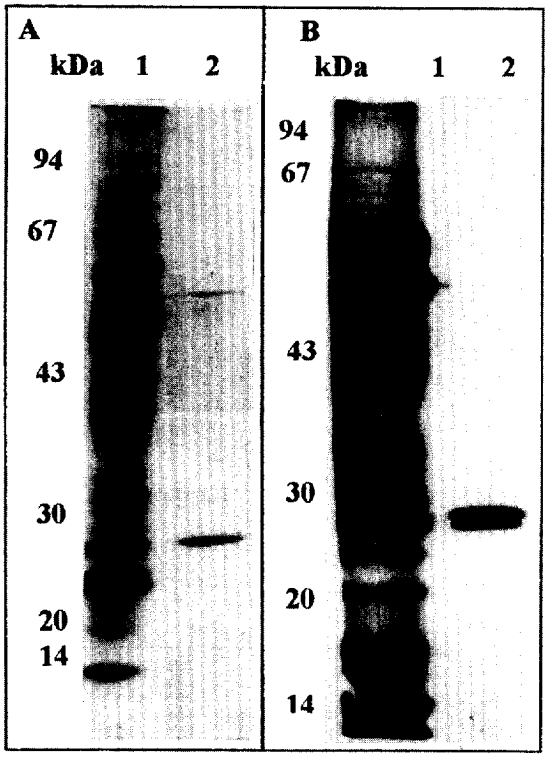

Fig. 2. Resolution of glyoxalase I on SDS–PAGE. The gels were stained with CBB (A) 10% gel (B) 12% gel. Lane 1, crude extract (120 and 150 μg protein in A and B respectively) lane 2, purified *B. juncea* glyoxalase I (1 and 2.5 μg in A and B).

a hetero-dimer. Later, both polypeptides could also be visualized on western blots. In *Aloë* (monocot) it is a monomer of M_r 44,000 as in yeast (*S. cerevisiae*, M_r 32,000) and bacteria (*E. coli* 34,000, *P. putida*, 20,000) whereas in soybean (26,000 and 29,000) and *Brassica*, it is a dimer like the mammalian enzyme [3].

Catalytic properties

Brassica apo-glyoxalase I had 5–6% residual activity and it was reactivated on the addition of Mn^{2+}, Mg^{2+}, Zn^{2+} and Ca^{2+} which were used at 2.5 μM to 15 Mm. Mn^{2+} was most effective with 162% stimulation (Fig. 3(A)) followed by 125% by Mg^{2+}, (Fig. 3(B)), 100% by Zn^{2+}, (Fig. 3(C)) and 55% by Ca^{2+} (Fig. 3(D)). Ca^{2+} restored 50% of the activity at 10 μM. The mammalian enzyme is significantly reactivated by Mg^{2+} Mn^{2+} and Zn^{2+} [19, 20] but the reactivation by Ca^{2+} was just 7% for sheep [19] and 25% for monkey intestinal mucosa glyoxalase I [20].

Atomic absorption studies with metal-free glyoxalase I showed 1.4 and 1.6 *g* atom of Zn^{2+} and Mg^{2+} respectively per mole of the enzyme (n = 3). The mammalian and yeast [3] glyoxalase I is also a Zn^{2+} metallozyme.

Amino acid modification studies were done using diethylpyrocarbonate (DEPC), which modifies histidine [21]. Treatment of 30 min decreased the activity by 60%, and by 1 h, the enzyme was completely inactivated (Table 1). The inactivated enzyme showed an increase in absorbance (A) at 242 nm, suggesting, presence of modified histidine residues. Hydroxylamine (0.1 M, pH 7.0) restored the activity to the untreated control levels. Tyrosine, tryptophan and lysine residues are present at active site of the mammalian [20], and cysteine, tyrosine and arginine residues at the active site of bacterial enzyme [3]. Involvement of arginine residues in glutathione binding to yeast glyoxalase I have also been shown [22].

CD studies with Brassica glyoxalase I

The CD spectrum of *Brassica* glyoxalase I showed a positive peak at 192 nm with two pronounced negative bands at 208 and 222 nm (Fig. 4, solid line), the characteristic feature of a helical protein. The spectrum studied in the presence of 16 mM Mg^{2+} [17] showed a shift in the positive peak and in the negative bands (Fig. 4, broken line) suggesting that Mg^{2+} is directly interacting with the protein and changing its conformation, which probably leads to its activation.

Immunochemical studies

Validation of Brassica glyoxalase I antibodies as an immunocytochemical probe. The titer of glyoxalase I antibodies was checked by ELISA and immuno double diffusion (IDD). ELISA could detect antibodies after second test bleeding at 1:5000 dilution and the titre improved by 3–4 fold after ammonium sulphate (0–50%) precipitation. After the fourth test bleeding IDD (at 1:25 dilution and 1 μg antigen) showed a precipitin line within 2 h at room temperature. The crude antiserum could precipitate 0.02 units of enzyme. Avidity of the antibodies was tested by immuno-titration and western blotting. The antiserum titrated out over 80% glyoxalase I activity (Fig. 5). On western blots, crude (inset, Fig. 5) as well as the purified protein (as seen in Fig. 8(A), last lane) showed two immuno-reactive bands (1:1000 dilution). The results showed presence of good titre, monospecific, anti-glyoxalase I antibodies. Earlier, antibodies

Table 1. Residual glyoxalase I activity after 30 and 60 min of DEPC treatment. Activity 100% = 3.4 μ kat./ml

DEPC (mM)	% residual glyoxalase I	
	30 min	60 min
0	100	100
5	96	90
15	96	80
25	77	10
50	56	0
100	39	0

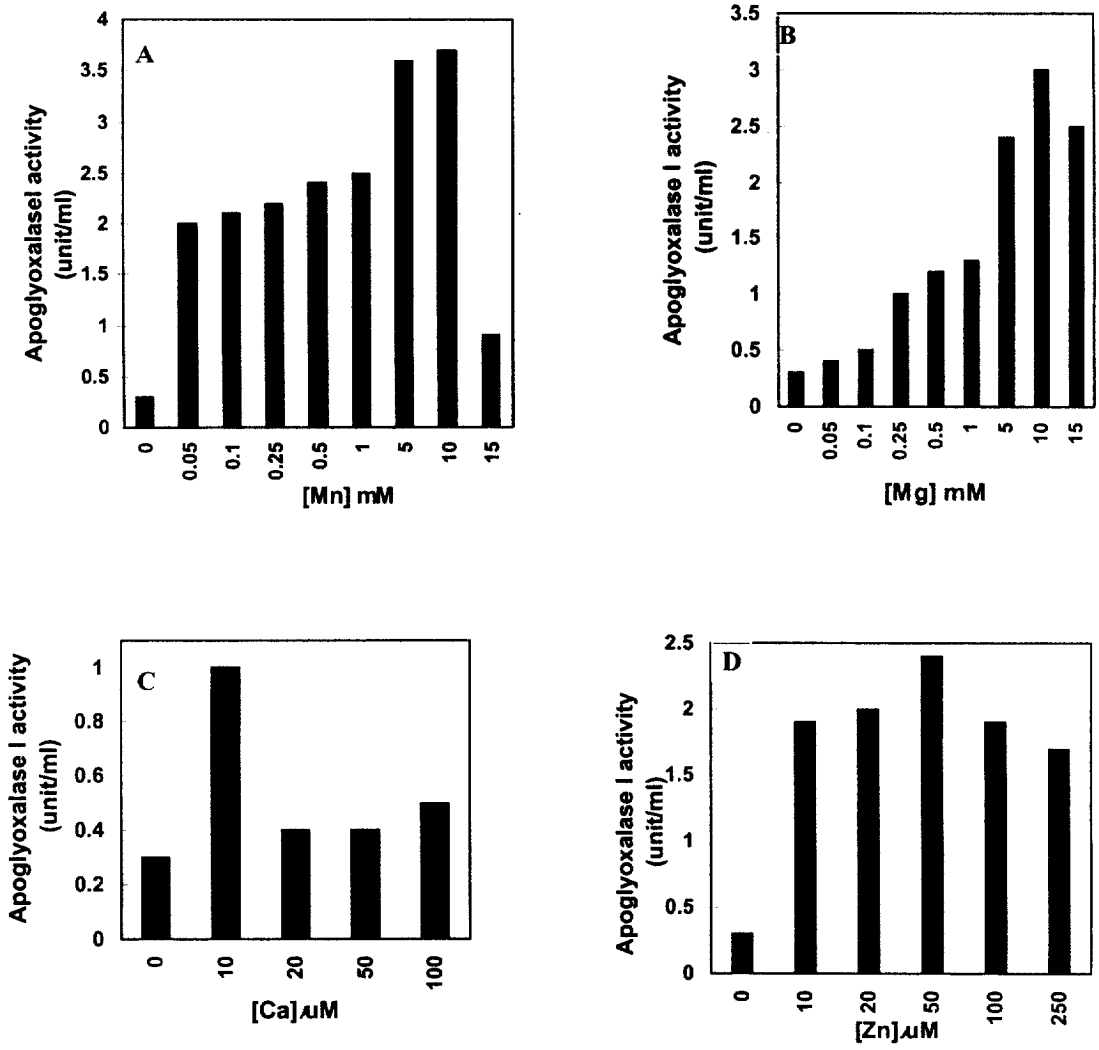

Fig. 3. Reactivation of apo-glyoxalase I with different cations. The activity was measured as described in Section 3. Fig A and B shows reactivation obtained by Mn^{2+} and Mg^{2+} at 10 μM–15 mM. C and D shows reactivation by Zn^{2+} and Ca^{2+} at 10 μM–1 mM. Data are means of three separate experiments.

have been obtained from yeast and mammalian sources [23].

Distribution studies

Glyoxalase I is essentially a cytosolic protein [3]. In *Brassica* also the catalytic activity (Fig. 6(A)) and the protein as shown in the immunoblot (Fig. 6(A) inset) was mostly confined to the soluble fraction. Distribution of glyoxalase I in different organs of *Brassica* showed 3-fold higher activity in the cotyledons in comparison with roots. The hypocotyl and total seedlings also showed higher activity than root tissue (not shown) and the immuno blot reflected the similar order, cotyledon > seedlings > hypocotyls > root of the protein profile (Fig. 6(B)). In *Brassica* flower (a differentiated organ), pistil and buds showed more protein and activity than petal, sepal and stamens (Fig. 7(B)). In stamens, glyoxalase I was very labile as a strong immuno-positive band was detected at the dye front, which might be due to protein degrading to smaller immuno-reactive peptides.

Immuno-reactivity with the extracts from different plants

Antigenic relationship among glyoxalase I in different plants was checked in native (by IDD) as well as in denatured form (by immuno blotting). Among dicots, *Brassica oleracea*, *B. juncea*, *B. campesteris* and *B. nigra* showed relatively higher cross reactivity, with immuno-positive polypeptides at M_r 27,000 and 29,000 (Fig. 8(A)). *Cajanus cajan* showed a very faint band at M_r 27,000 while cucumber and *Cicer* did not show any. *Arabidopsis* showed two bands at M_r 27,000 and 35,000. Wheat, maize and barley (monocots) showed cross reactiv-

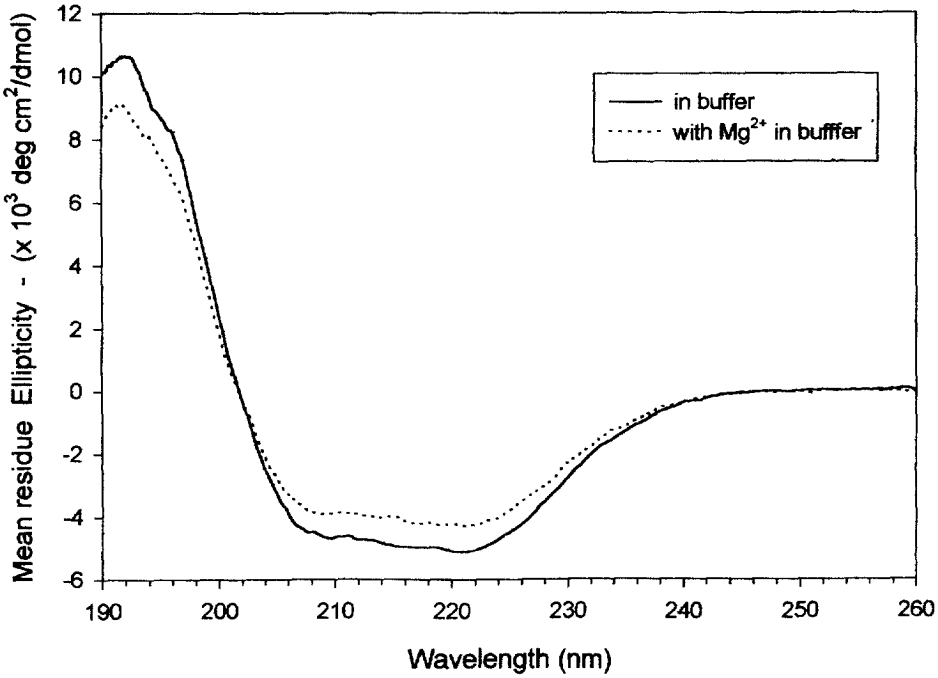

Fig. 4. Far UV CD spectra of purified *Brassica* glyoxalase I (30 μM) in Na-Pi Buffer (10 mM, pH 7.0) at 25°. Solid line (———) shows the spectrum in buffer alone and the broken line (- - -) is the spectrum obtained in presence of buffer and Mg^{2+} (16 mM).

ity only at higher (double) protein concentration while *Sorghum* protein did not cross react in spite of showing high glyoxalase I activity (Fig. 8(C)). In IDD only the extract from different *Brassica* species showed single precipitin lines but the extract from other plants did not suggesting that the enzyme may have quite different conformation in native form masking the antigenic epitopes.

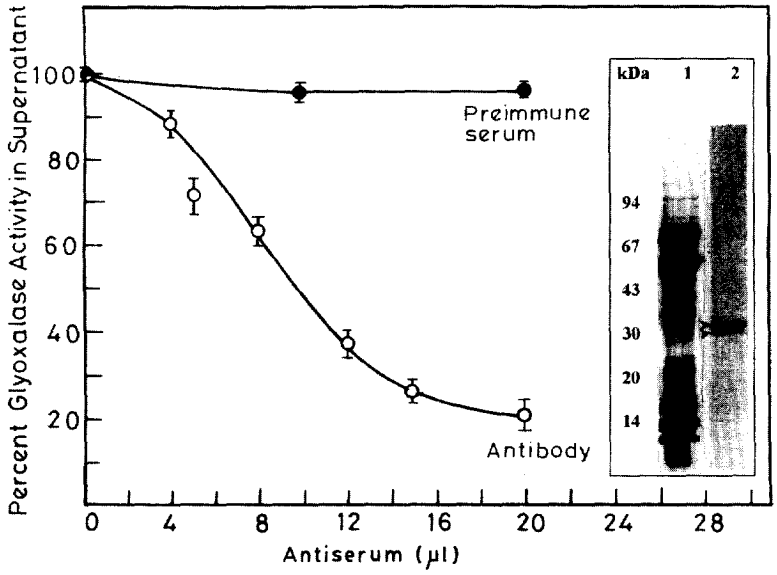

Fig. 5. Immuno-precipitation of *B. juncea* glyoxalase I activity. The protein (0.5 μg) was incubated with antiserum (4–20 μl) and pre-immune serum. Residual activity in presence of antiserum (○—○) and pre-immune serum (●—●). Inset shows CBB stained 12% SDS gel showing crude extract (200 μg protein, lane 1). Lane 2 — An immunoblot of lane 1 probed with anti-glyoxalase I antibodies.

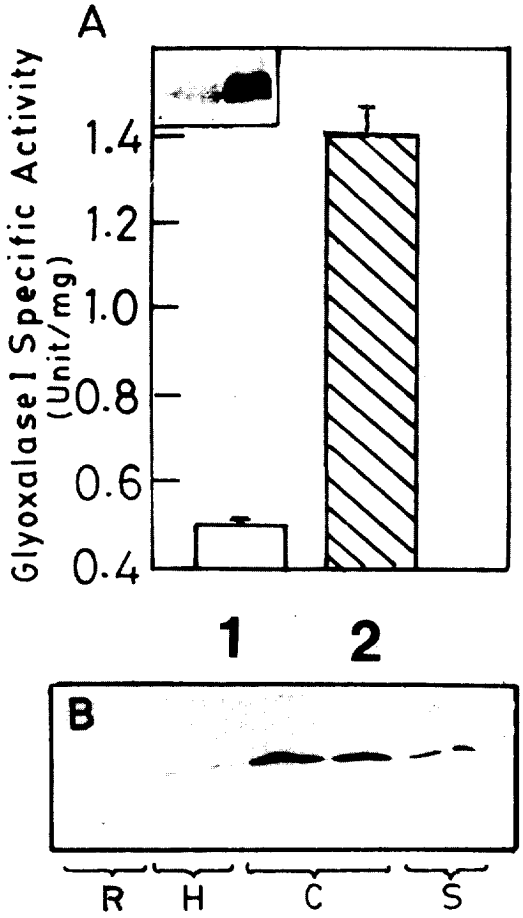

Fig. 6. Presence of glyoxalase I protein in different parts of *Brassica* seedlings. A Distribution of glyoxalase I activity in soluble (bar 1) and membrane enriched (bar 2) fractions. The inset shows an immuno blot of the same fractions. B Immuno-blot showing glyoxalase I protein in crude extract (150 μg) from root (lane R), hypocotyls (H), cotyledons (C) and seedlings (S). The proteins were separated on 10% gel, transferred to nitrocellulose membrane and was probed with the antibodies (1:1000).

Immuno-reactivity with the extracts from heterologous systems

Common antigenic determinants among plants and heterologous systems were checked using *Brassica* anti-glyoxalase I antibodies. (Fig. 9). The extract from mice liver and yeast showed a single band at M_r 22,000 while the *E. coli* extract showed two bands of M_r 34,000 corresponding to glyoxalase I and another at M_r 32,000 which is either the degradation product or another polypeptide sharing the epitopes. Mice liver glyoxalase I has a subunit M_r of 21,000–22,000. The *Candida albicans* (yeast) extract also showed a band at a similar position suggesting that glyoxalase I could be of that size in this particular yeast although in *Saccharomyces cerevisiae* it is a M_r 32,000 protein.

Earlier work in plants [10, 14, 15, 17] has suggested that glyoxalase I levels correlated with cell division status of the cells. However we observed it to be present in dormant seed, cotyledon, mature leaf and flower parts (non dividing parts) indicating that it has some other role too. Recently, its role under stress conditions has been emphasized [8].

The facts that the *Brassica* enzyme is a dimeric zinc metallozyme, has similar catalytic properties (K_m values, nature of substrate being utilized, holoenzyme is stimulated by Mg^{2+} [17] and the apoenzyme is reactivated by Mg^{2+}, Mn^{2+}, and Zn^{2+}) suggest a lot of similarity in *Brassica* and mammalian glyoxalase I. On the other hand its alkaline pH optima like the bacterial enzyme and its glycoproteic nature like the yeast enzyme [3] puts it close to the microbial glyoxalase I. Another important point of similarity to be noted is an inverse relationship between p*I* and pH of the enzyme. Mammalian [3], bacterial [5] and plant [10, 16] glyoxalase I has an acidic p*I* and neutral to alkaline

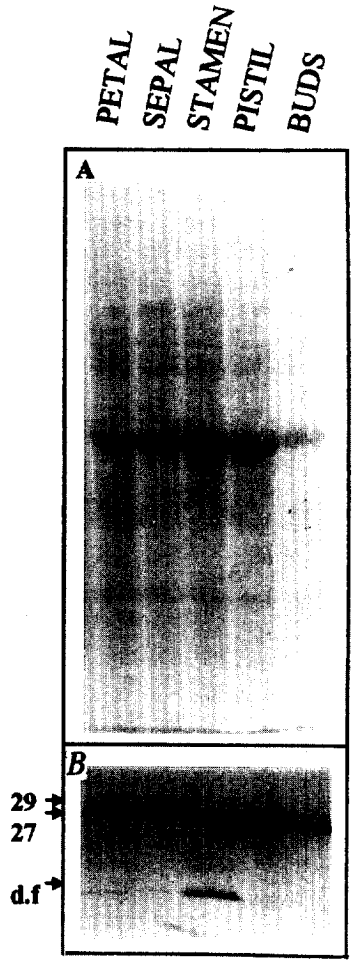

Fig. 7. Ponceau-S stained blot of extracts (120 μg protein) from different flower parts petal, sepal, stamen, pistil and buds of *Brassica* flower, separated on 12% gel. Immunoblot of the same extracts probed with anti-glyoxalase I antibodies (1:1000).

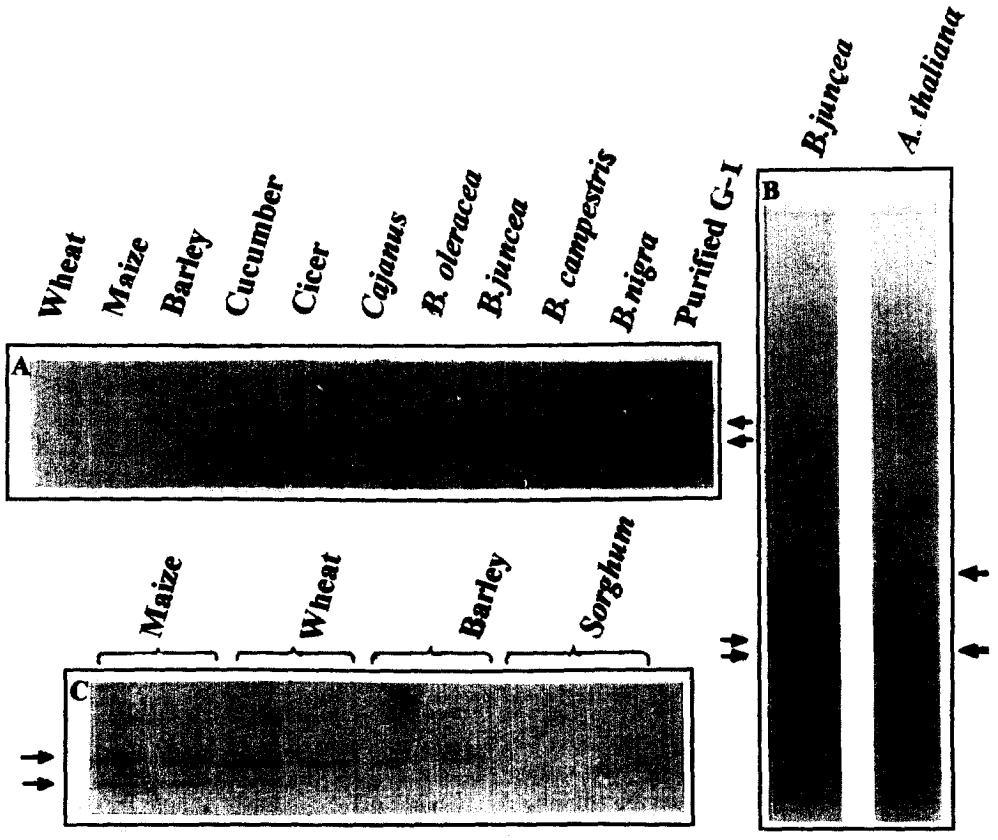

Fig. 8. (A) Immunoblot showing the immunochemical reactivity of anti-glyoxalase I antibodies with extracts from different plant sources. Equal protein (120 μg) was resolved on 12% SDS gel, transferred to nitrocellulose membrane. (B) Immuno-blot decorated with anti-glyoxalase I antibodies (1) *B. juncea* crude extract (2) the extract from *Arabadopsis*. (C) Cross reactivity of glyoxalase I antibodies with extracts from monocot at double (240 μg) protein concentration.

pH optima while the yeast enzyme has the values in a reverse order. *Brassica* glyoxalase I also showed some novel properties like the involvement of histidine in catalysis, reactivation of *Brassica* apoenzyme by Ca^{2+} and presence of almost equal Mg^{2+} and Zn^{2+} in the native enzyme. *Brassica* anti-glyoxalase I antibodies recognized the heterologous antigens while the mammalian antibodies did not cross react [23].

EXPERIMENTAL

Plant materials and growth conditions

Seeds of *Triticum aestivum* (HD-2329), *Zea mays* (Ganga-5), *Sorghum bicolor* (PC-6), *Hordeum vulgare* (IB-65), *Cajanus cajan* (Pusa 84), *Cicer arietinum*, *Brassica* napus (706), *B. campestris* (DYS-28), *B. oleracea* (Botrytis), *B. nigra*, *B. carinata* (226), *B. juncea* (Pusa bold) and *Cucumis sativus* (cultivar poinsette) were obtained from the Indian Agricultural Research Institute, New Delhi. The seeds were sterilized with 0.02% $HgCl_2$ were washed and soaked in H_2O for 12 h. They were plated on germination paper in plastic trays and were kept at $25 \pm 2^{\circ}$ under white light (1200 μW) obtained from fluorescent tubes at the plant level. Different parts of the plants were from 7 day old plants and the flower were taken from the field.

Purification of MG

A 40% aqueous solution of MG was steam distilled in the fume hood as described [24]. It was passed through a Dowex-1 (OH^- form) column to remove acidic impurities and was stored aliquoted at -20° after determining the concentration of the stock using yeast glyoxalase I as described in Ref. [25].

Extraction and enzyme assay

Extraction and assay was done as described earlier [17] except that the assay mixture (1 ml) contained 0.8 mM MG and 3 mM reduced glutathione (GSH) (the new optimums) determined using distilled MG. The change in A at 240 nm was converted to enzyme units using the extinction coefficient (3370) of the *S*-lactoyl GSH [26]. For

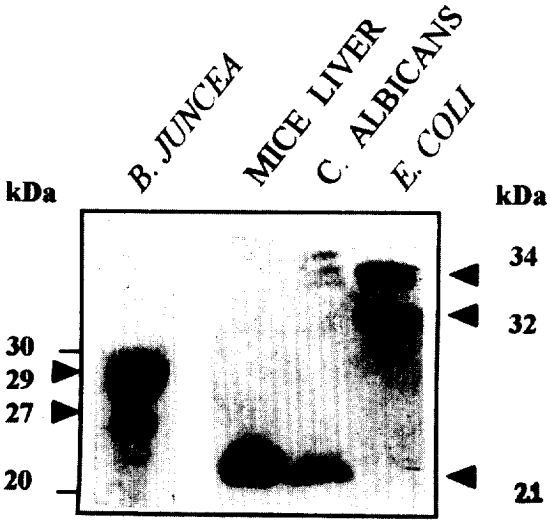

Fig. 9. Immunoblot showing immuno reactivity of the extracts of *Brassica juncea*, mouse liver, *Candida albicans* and *E. coli*, probed with anti-glyoxalase I antibodies (1:250 dilution). A 12.5% gel was run to separate the proteins (200 μg).

apoenzyme reactivation experiments Hepes buffer (100 mM, pH 7.0) was used. Change in A was recorded for at least 10 min at intervals of 30 s. The supernatant obtained after centrifugation at 20,000 g, was centrifuged at 100,000g in the Beckman ultracentrifuge at 4° for one hour. The pellet was designated as membrane enriched fraction and the supernatant as the soluble fraction. The pellet was washed (twice) and dissolved in 200 μl of extraction buffer.

Purification and native M_r *determination*

The enzyme was purified as described earlier [17]. For native M_r determination gel filtration (Sephadex G-75) column equilibrated with 10 mM Na–Pi buffer was used. The buffer was passed through the column for 2 days in order to stabilize the matrix bed. Standard proteins-bovine serum albumin (67,000), Egg albumin (43,000) and pepsin (30,000) (1 mg/ml) with or without blue dextran (1 mg) were processed either separately or as a mixture. Elution was done with the buffer containing 0.2 M NaCl.

Apoenzyme preparation

Apoenzyme was prepared by dialyzing the enzyme (5 ml, 0.25 mg/ml) against EDTA (1 mM) in 2 l, 50 mM Hepes buffer, pH 7.0 for 24 h and finally against 1 l buffer containing chelex-100 (2 g/l) to remove any residual cations. Glassware and dialysis tubing were treated as mentioned [19]. All the buffers and solutions were prepared in metal-free H_2O was prepared by passing through a chelex-100 column.

Polyacrylamide gel electrophoresis

For denaturing SDS–PAGE Laemmli's [27] protocol was followed. Both 10% or 12% gels were used for separating proteins and the gels were stained with Coomassie brilliant blue (CBB, R-250). Protein concentrations were estimated according to Bradford's micro (1–10 μg) and macro (10–100 μg) assay [28] using BSA (fraction v) as a standard.

Circular dichroism spectroscopy

The CD spectrum was measured in a JASCO J-720 spectropolarimeter in a 0.1 cm Quartz cuvette from 190–250 nm with glyoxalase concentration at 30 μM. The results are expressed as the mean residue molar ellipticity, (O), which is defined as $O = 1000\,O/LC$ where O is observed ellipticity, C is the concentration in mol/l and L is the path length. The spectrum was corrected for the contribution of the buffer. The spectrum was recorded in the presence of 16 mM Mg^{2+} (as determined earlier, [17]) with the same amount of protein.

Amino acid modification studies

Purified glyoxalase I (0.1 ml containing 70 μg protein) was incubated with 5–100 mM diethylpyrocarbonate (DEPC) at 22° for 30 and 60 min in Na–Pi (10 mM, pH 7.5). Each sample was dialyzed over night at 4° against the incubation buffer and the catalytic activity was checked. A control sample was processed in a similar way omitting the DEPC. A of the samples was also checked at 242 and 278 nm to detect the N-carbethoxy histidine and tyrosine residues. The DEPC treated fractions which had lost all the activity were treated with hydroxylamine (0.1 M, pH 7.0) for 30 min at room temperature, dialyzed and checked for the enzyme activity.

Atomic absorption spectrophotometric studies

Spectrophotometer model GBC 902 was used and samples were measured in A mode in triplicate. Before analysis, the samples were dialyzed for 30 h against 10 mM Hepes buffer containing EDTA (1 mM) prepared in metal free H_2O and finally against buffer alone to remove EDTA. The dialysis bags were boiled in 5 mM EDTA and 50 mM Na–Pi and were washed with metal-free H_2O. Concentration of metal ions was determined with reference to previously constructed standard curves for Mg^{2+} and Zn^{2+} salts, and samples were diluted to bring them into linear range of 1–20 μM for Zn^{2-} and Mg^{2+}.

Immunization

New Zealand white rabbits were used for raising antibodies. The protein (250 μg) was emulsified in Freund's adjuvant. The antiserum was purified by $(NH_4)_2SO_4$ precipitation (50%) and was stored ali-

quoted at −80° after overnight dialysis against phosphate buffer saline (PBS).

Western blotting, IDD, ELISA and immunotitration

The proteins were transferred onto the nitro-cellulose membrane after SDS–PAGE, in absence of SDS at 100 mA for 1–2 h at room temperature [29], using semidry electroblot apparatus. For developing immunoblots, *Brassica* glyoxalase I antibodies were used either at 1:250 dilution (for heterologous systems); otherwise at 1:1000 dilution. Blocking was done with bovine serum albumin (3% w/v in PBS) for 2 h at 37°. The blots were incubated in the primary antibodies for 2 h and for 30 min in the secondary antibodies (either horse radish peroxidase or alkaline phosphatase). 3 washes of 5 min each were given with PBS + Tween 20 (0.05%) after each incubation. For immunotitration different amounts of antiserum were added to 1 μg of glyoxalase I. The mixture was incubated for 1 h, centrifuged at 12,000*g* for 10 min and the activity was checked in the supernatant.

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