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Salinity and drought-induced methylglyoxal detoxification in *Brassica* spp. and purification of a high active glyoxalase I from tolerant genotype

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Abstract

This experiment was conducted to study the role of glyoxalase system in conferring salinity and drought stress in *Brassica* spp. Two *Brassic*a genotypes viz. BARI Sharisha16 (tolerant) and Tori7 (susceptible) were exposed to salt (16 dS m^{-1}) and drought for 2, 4 and 6 days. The comparative study of two *Brassica* genotypes under salinity and drought stresses revealed that BARI Sharisha16 is more tolerant than Tori7 in both stresses. Under drought stress and salinity stress, Gly I activity increased significantly in both genotypes. Notably, concomitant increased activities of Gly I and Gly II with increased methylglyoxal (MG) suggested their role in MG detoxification in *Brassica* Spp. At six-day of salt stress, it was remarkable that Gly I and Gly II activities were 49 and 36 % higher in BARI Sharisha16 than Tori7. In addition, Gly I and Gly II activities were 24 and 21 % higher in BARI Sharisha16 than Tori7 after sixth day of drought, and hence, using different column chromatography Gly I was purified from BARI Sharisha16 seedlings. In purification, the fraction eluted from affinity chromatography showed specific activity of 173.51 µM $min^{-1}mg^{-1}$ protein. In SDS-PAGE, the purified Gly I protein migrated as a single band on with an apparent molecular mass of 27 kDa. In final purification, the recovery of Gly I activity was 0.38% along with purification fold 112.7. In this study, role of glyoxalase system in detoxification of MG was observed and subsequently, Gly I was purified from tolerant genotypes.

Keywords: Abiotic stress; glyoxalase system; methylglyoxal; salinity; water deficit.
Abbreviations: BSA_ Bovine serum albumin; EDTA_ Ethylenediaminetetraacetic acid; GSH_ Reduced glutathione; Gly I_ Glyoxalase II; Gly II_ Glyoxalase II; GST_ Glutathione S-transferase; MG_ Methylglyoxal; PAGE_ Polyacrylamide gel electrophoresis; ROS_ Reactive Oxygen Species; SDS_ Sodium Dodecyl Sulfate; SLG_ *S*-D -lactoylglutathione

Introduction

Crop plants being sessile expose to a number of adverse condition known as abiotic stress including salinity, water deficit, extremely high or low temperatures, toxic metals, waterlogging, elevated ozone, and ultraviolet radiation which adversely affect proper growth, metabolism and productivity of crop plants. Recent episodes of climate changes make the conditions worse. Abiotic stresses result in a series of physiological changes in plants that adversely affect the yield and even death. Reactive oxygen species (ROS) and methylglyoxal (MG) production in plant cell is the ultimate result under any kind of abiotic stresses (Singla-pareek et al., 2006; Hasanuzzaman et al., 2014; Nahar et al., 2016). Methylglyoxal is a highly toxic compound that produces under abiotic stress. It can inactivate the vital defense system causing metabolic disorder and cell death by reacting with proteins, lipids, and nucleic acid (Kaur et al., 2014; Nahar et al., 2015; Rahman et al., 2015).

During conversion of glyceraldehyde 3-phosphate (G3P) from dihydroxyacetone phosphate (DHAP) in glycolysis, MG is formed spontaneously in plants by non-enzymatic mechanisms under physiological conditions (Espartero et al., 1995; Yadav et al., 2005a). The rate of glycolysis increases under stress conditions, leading to an imbalance (in the initial and latter five reactions) in the pathway. Triose phosphates are very unstable metabolites, and removal of the phosphoryl group by β-elimination from 1, 2-enediolate of these trioses leads to the formation of MG (Yadav et al., 2005b). Therefore, MG production is an unavoidable consequence during stress. From G3P and DHAP, MG can also be formed enzymatically by triosephosphate isomerase that removes phosphate to yield MG (Silva et al., 2013).

The Glyoxalase system is ubiquitous in nature and consists of two enzymes: Glyoxalase I (Gly I, EC 4.4.1.5) and Glyoxalase II (Gly II, EC 3.1.2.6). These two enzymes catalyze 2-oxoaldehydes coordinately to convert into 2-hydroxyacids when reduced glutathione used as a cofactor (Silva et al., 2013). The reaction is catalyzed by Gly I and Gly II (Hoque et al., 2007; Nahar et al., 2016) (Fig 1). Methylglyoxal is a primary physiological substrate for Gly I (Kalapos, 1999; Hasanuzzaman et al., 2014). The Glyoxalase system has been found to be involved in protection against

MG induced cytotoxicity under various abiotic stresses and regulation of cell division and proliferation (Kaur et al., 2014). These properties of glyoxalase system make it an important tool for crop improvement against abiotic stresses. Understanding the importance of glyoxalase system in protection of plants under stress condition, this study was designed to investigate the role of glyoxalase system in contrast to plants of *Brassica* spp. under salinity and drought stresses. Glyoxalase system was studied in two *Brassica* spp. Finally, glyoxalase I enzyme was purified from BARI Sharisha16 which is a moderately saline tolerant genotype.

Results

Methylglyoxal level in Brassica plants under salinity stress

To check whether the upregulation of MG happened in plants in response to various stress duration, its level was measured in the seedlings under untreated control as well as various salinity stress condition. Salinity stress increased MG level sharply with the duration of salinity stress in both genotypes (Fig 2A). After 2-day of stress, MG level increased almost 103% in BARI Sharisha16 compared to control. At 4-day stress, MG level increased by 110% and 25% over control in BARI Sharisha16 and Tori7, respectively, while at 6- day, the increments were 136% and 61% over control in BARI Sharisha16 and Tori7, respectively.

MG level under drought stress

Methylglyoxal level increased with the duration of drought stress. Under control condition, MG level was higher in Tori7 than BARI Sharisha16. Unlike saline stress, MG contents were higher in Tori7 as compared to BARI Sharisha16 under drought stress and the contents were significantly higher in Tori7 than BARI Sharisha16 at 4 and 6- day (Fig 2B).

Gly I activity under salinity stress

The activities of Gly I in BARI Sharisha16 and Tori7 significantly increased under salinity stress at various durations (Fig 3A). After 2-day of stress, Gly I activity increased only in BARI Sharisha16 which was 41% higher than control. The activity further increased in time dependent manners. However, the activity was always higher in BARI Sharisha16 than Tori7 (Fig 3A).

Gly I activity under drought stress

Drought stress significantly increased the Gly I activities in BARI Sharisha16 and Tori7 (Fig 3B). After 2-day of drought stress, Gly I activities did not change significantly in BARI Sharisha16 and Tori7, compared to their respective control. At 4-day of drought stress, Gly I activity increased 76% and 32% over control in BARI Sharisha16 and Tori7, respectively. At 4-day and 6-day of drought stress, Gly I activities were higher in BARI Sharisha16 than Tori7.

Gly II activity under salinity stress

Gly II activities were increased under salinity stress both in BARI Sharisha16, and Tori7 (Fig 4A). Higher Gly II activity found in both stresses compared to control. At 2-day salinity stress, Gly II activity increased 145 and 52% in BARI Sharisha16 and Tori7 respectively. Noticeably, at 2, 4 and 6-day of salinity stress, higher Gly II activity found in BARI Sharisha16 than Tori7.

Gly II activity under drought stress

There was a significant increase in Gly II activity in response to drought stress (Fig 4B). A sharp increase of Gly II activity (191% and 93%) was observed in BARI Sharisha16 and Tori7 over respective control due to salinity stress within 2 days. At 4-day of drought stress, Gly II activities increased 189% and 86% in BARI Sharisha16 and Tori7, accordingly over control. Though Gly II activity slightly increased after 6 day of drought stress in both genotypes, the changes were not significant.

Purification of Gly I

Since Gly I was found to increase considerably under stress, an attempt was taken to purify the Gly I from BARI Sharisha16. The soluble protein fraction prepared from 50 g fresh seedlings was precipitated by 65% $(NH_4)_2SO_4$ and the dialysate was applied on DEAE-cellulose column chromatography (i.d. 1.7 × 20 cm) and eluted with a liner gradient of KCl (0-0.2 M) Fig 5. Total 140 fractions, each containing 5 ml, were collected and Gly I activity and absorbance at 280 nm of each fraction were measured. Gly I peak eluted at 76 mM of KCl (Fig 5). The fractions containing high Gly I activity was collected from the peak and pooled for measuring its activity. Among them, Gly I showed total activity of 4.31mmol min^{-1} with purification fold 9.23 and recovery of activity 3.73% (Table 1). The Gly I pools were applied on another DEAE column (DEAE-cellulose chromatography-2) again for further purification. Again, one Gly I peak eluted at around 76 mM of KCl (Fig 6). The Gly I activities of each fraction towards model substrate MG and absorbance at 280 nm were measured. The high active fractions were pooled for further purification. The pooled sample (15 ml) showed total activity of 2.3 mmol min^{-1} with purification fold 19.8 and recovery 2.06% (Table 1).

The active Gly I pools from DEAE chromatography-2 were applied on an affinity column chromatography of *S*-hexylglutathione-agarose to complete the purification. The Gly I sample was passed through the column following 10 ml 0.2 ml KCl for washing. The Gly I was eluted with 15 ml *S*-hexylglutathione. Gly I activity and absorbance A_{280} of affinity fractions were taken (Fig 7). The active fractions were pooled and dialyzed in B buffer overnight. The active fractions had specific activity 173.7 $\mu mol^{-1}mg^{-1}$ protein, total activity 0.44, purification fold 113 and recovery 0.38% (Table 1). The purities and molecular masses of the purified Gly I were examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The silver staining and CBB staining of the purified Gly I indicated that purified Gly I were highly purified and migrated as a single band on SDS-PAGE with an apparent molecular mass of 27 kDa (Fig 8A, B).

Discussion

It was observed that level of MG was higher under both stresses than control and the level increased with the increase in the duration of stresses (Fig 2A, B). Methylglyoxal is synthesized naturally by three enzymes: methylglyoxal synthase; cytochrome P450 IIE1 isozyme and amine oxidase participating in glycolytic bypass, acetone metabolism and amino acid breakdown, respectively. Removal of the phosphoryl group of triose phosphates produced during

Table 1. Summary of purification of Gly I from *Brassica*.

Purification steps	Total volume (ml)	Total protein (mg)	Total activity (mmol min^{-1})	Specific activity ($\mu mol^{-1}mg^{-1}$ protein)	Purification fold	Yield of activity (%)
Crude protein	192	375.36	115.68	1.54	1	100
$(NH_4)_2SO_4$ precipitate	35	200.07	97.27	2.43	1.57	84.08
DEAE-cellulose-1	23	6.06	4.31	14.22	9.23	3.73
DEAE cellulose-2	15	1.56	2.38	30.51	19.80	2.06
S-hexylglutathione-agarose	5	0.051	0.44	173.74	112.7	0.38

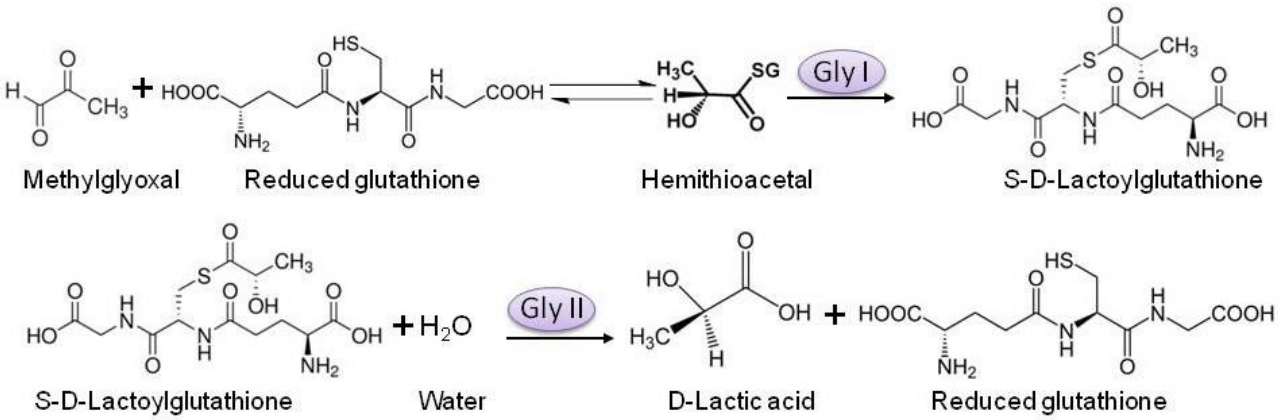

Fig 1. Mechanism of glyoxalase reaction to detoxify MG in living system.

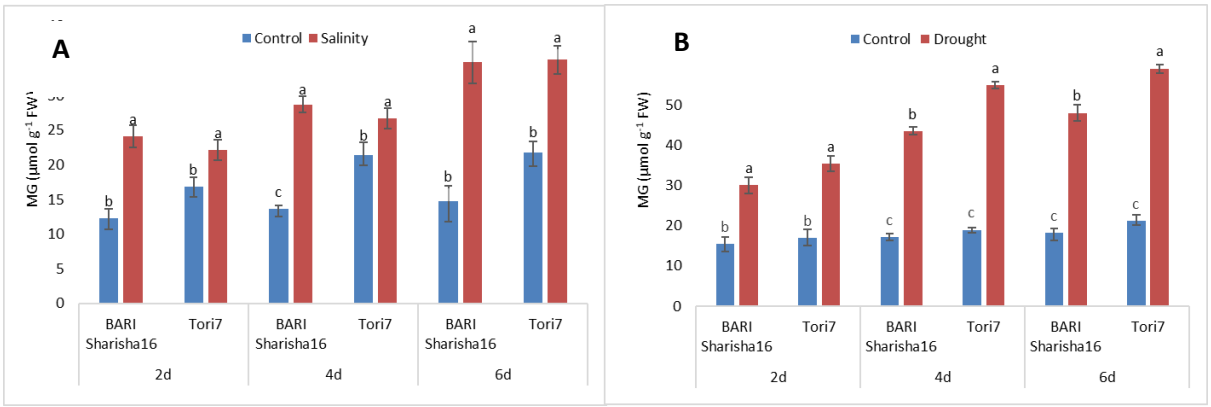

Fig 2. Changes in MG level under salinity (A) and drought (B) stress at 2, 4 and 6 days. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at P≤0.05 applying DMRT.

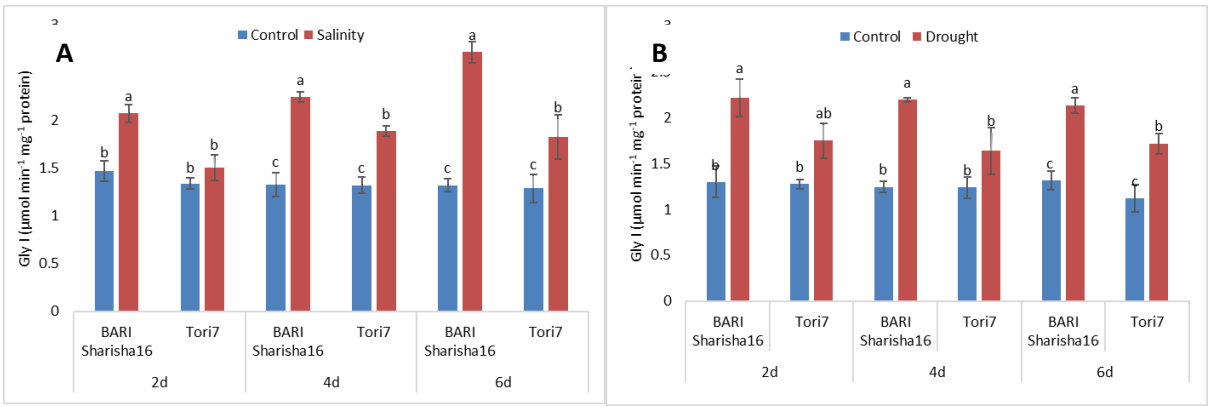

Fig 3. Changes in activity of Gly I under salinity (A) and drought (B) stress. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at P≤0.05 applying DMRT.

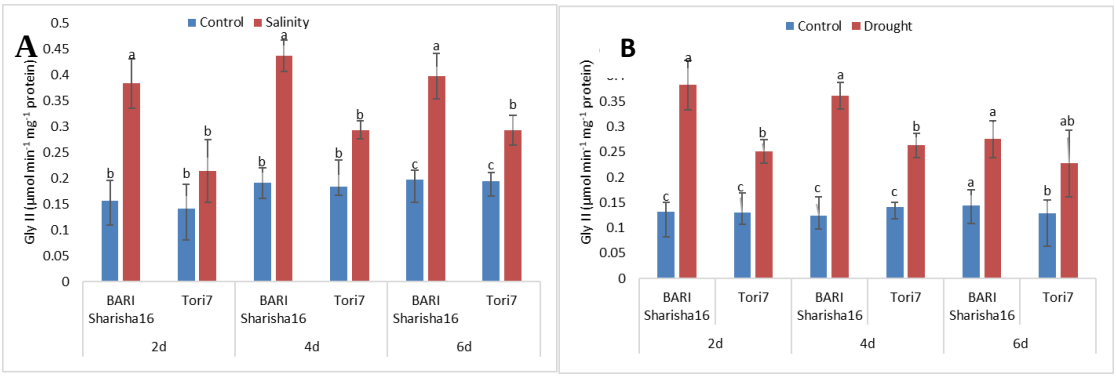

Fig 4. Changes in activity level of Gly II under salinity (A) and drought (B) stress. Mean (±SE) was calculated from three replicates for each treatment. Bars with different letters are significantly different at P≤0.05 applying DMRT.

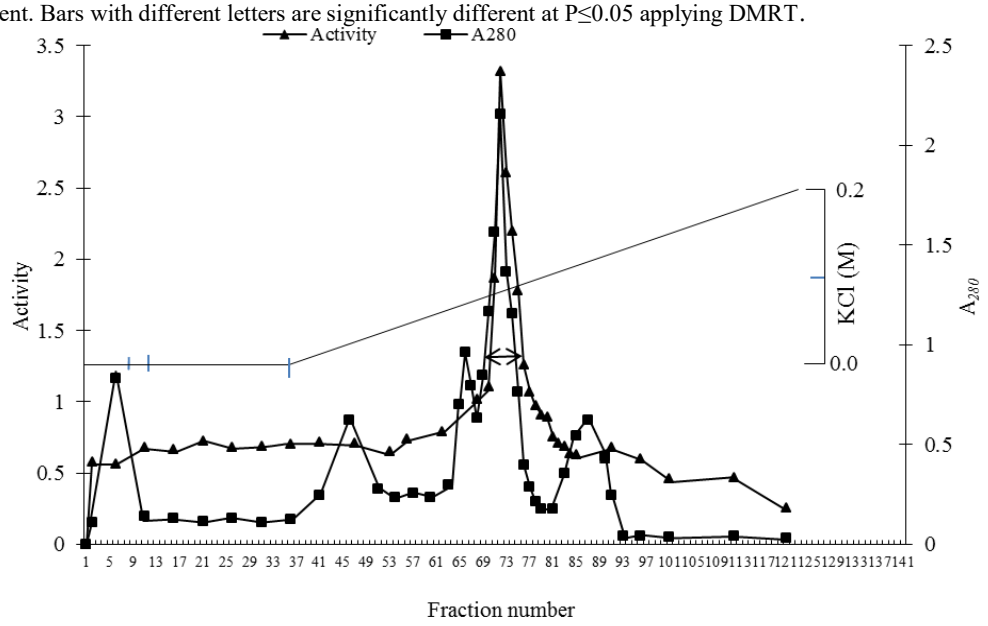

Fig 5. A typical DEAE-cellulose column chromatography of soluble crude protein prepared from 50 g fresh leaf of seedlings of BARI Sharisha16. For each fraction, absorbance at 280 nm (■) and Gly I activity (▲)were determined. Activity is expressed as µmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of Gly I. The fractions under the bar of Gly I, peak was pooled for subsequent purification. The curve shows the concentration of KCl (0-0.2 M).

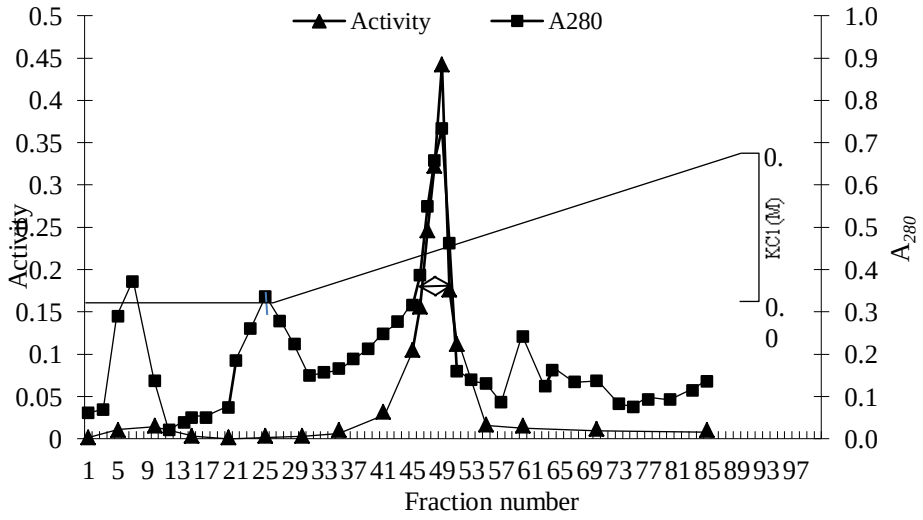

Fig 6. DEAE column-2 of Gly I pool from DEAE column-1. For each fraction, absorbance at 280 nm (●) and Gly I activity were determined. Activity is expressed as µmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of Gly I. The fractions under the bar of Gly I, peak was pooled for subsequent purification. The curve shows the concentration of KCl (0-0.2 M).

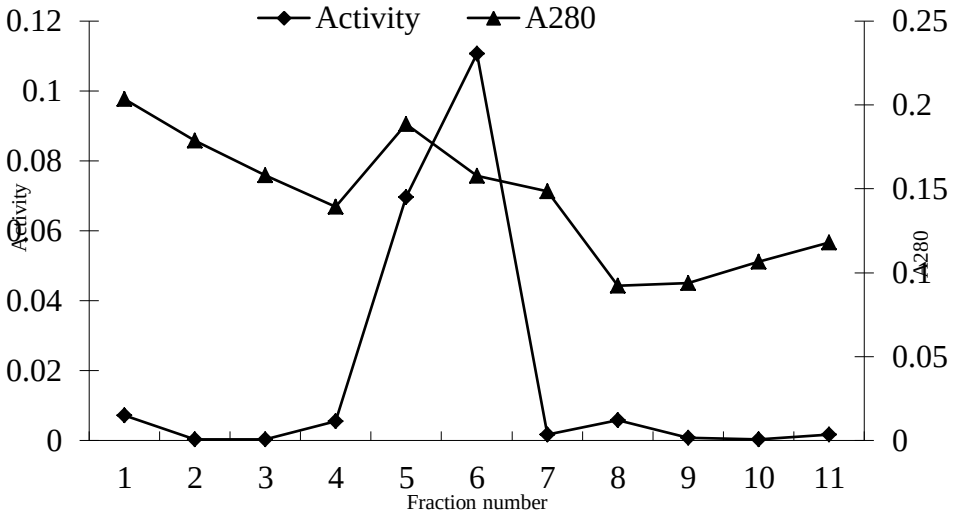

Fig 7. Elution profile of Gly I from affinity column chromatography (*S*-hexylglutathione-agarose). The fractions were eluted by *S*-hexylglutathione. For each fraction, absorbance at 280 nm (▲) and Gly I activity (♦) were determined. Activity is expressed as µmol min^{-1} ml^{-1}.

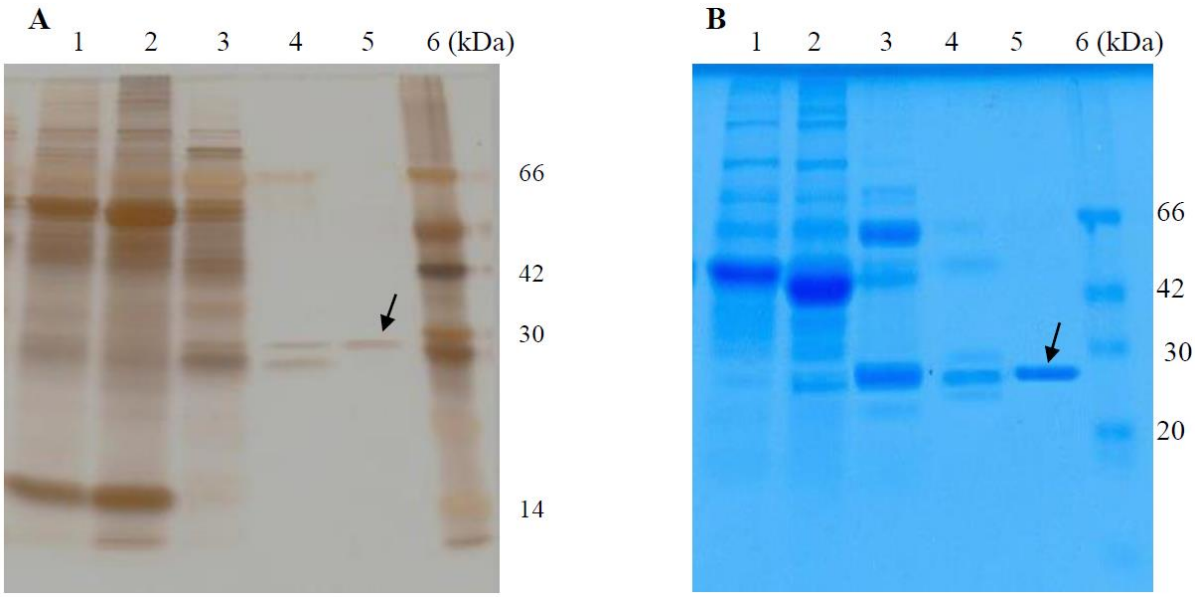

Fig 8. Silver staining (A) and CBB staining (B) of different fraction of Gly I purification from Brassica seedlings. Lane 1, Homogenous; Lane 2, $(NH_4)_2SO_4$ ppt; Lane 3, DEAE fraction-1; Lane 4, DEAE fraction-2; Lane 5, Purified Gly I; and Lane 6, Molecular weight.

glycolysis could generate MG (Kalapos, 1999; Vander et al., 2001; Distler et al., 2012). When plant exposed to stress conditions, enzymes of glycolysis and TCA cycles cells showed increased activity due to rapid functioning of cell (Umeda et al., 1994; Espartero et al., 1995; Sommer et al., 2001) and as a result increased triose phosphates are converted to MG instead of pyruvate only. So higher amount of methylglyoxal production under drought and salinity stresses confirmed by Hossain et al. (2009), Alam et al. (2014) and Nahar et al. (2015).

Various studies suggested a possible role of glyoxalase system in abiotic stress tolerance and also beneficial role in animal (Thornalley, 1993; 2003; Yadav et al., 2005b; El-Shabrawi et al., 2010). Various exogenous factors affected the activity of Gly I (Deswal et al., 1993). Previously, Gly I from tomato was shown to be upregulated under salt and water stresses (Espartero et al., 1995; Hasanuzzaman et al., 2011a). In this study, Gly I activities were higher in BARI Sharisha16 than control and Tori7. Gly I activity increased 41%, 69% and 105% in BARI Sharisha16 whereas 12%, 43% and 41% in Tori7 under salinity condition (Fig 3A). These results suggested that the higher Gly I activity in BARI Sharisha16 is involved in tolerance through detoxification of high level of MG. This also suggested that the upregulation of Gly I may be a general effect in response to abiotic stresses to survive. Methylglyoxal, substrate of Gly I, is produced from triosephosphates, increased activity of Gly I might be required whenever the rates of glycolysis or photosynthesis are enhanced. Therefore, beside MG detoxification, the higher Gly I activities in BARI Sharisha16 and Tori7 might involve in different physiological process. Several research groups have reported that the activity of Gly I was affected by various abiotic stress treatments including salt, water and heavy metal stresses (Alam et al., 2014; Nahar

et al., 2015; Rahman et al., 2015). The results of this study also addressed the situation.

Glyoxalase II converts the intermediate product produced by Gly I to D-lactate and release GSH to glutathione pool (Singla-Pareek et al. 2006, 2008). Therefore, in stress environment it plays important role in MG detoxification and GSH maintenance as well. In response to salinity stress and water stress, Gly II activity increased in BARI sharisha16 and Tori7 at 2, 4 and 6- day stress (Fig 4A, B). Previously salt sensitive BRRI dhan49 was reported to show slight increase in the activity of Gly II and conversely, in salt tolerant BRRI dhan54 treatment with 150 and 300 mM NaCl showed significant increase in Gly II activity as compared to control (Hasanuzzaman et al., 2014; Alam et al., 2013; Nahar et al., 2015). In this study, Gly II activity was found higher in BARI Sharisha16 than that in Tori7 suggesting that the MG detoxification ability in higher in BARI Sharisha16. On the other hand, GSH recycling ability might also be in higher BARI Sharisha16.

The specific activity of homogenous was 1.54 $\mu mol^{-1}mg^{-1}$protein and purified sample was 174 $\mu mol^{-1}mg^{-1}$protein. Total activity of homogenous was 115.68 mmol min^{-1} protein whereas 0.44 mmol min^{-1} protein in purified sample as in Table 1. As the sample passed through the columns specific activity increased, total activity decreased as the different proteins were removed through successive use of column chromatography Fig. 8A, B. The purified Gly I on SDS-PAGE appeared to be 27 kDa protein. In purification, Gly I were found to be highly purified with purification fold 113 and recovery 0.38%. Brassica Gly I had no isoforms (Veena et al., 1999). The size of the protein was in onion is 25 kDa confirmed after SDS-PAGE (Hossain and Fujita., 2009). Gly I purified from soybean cell suspension showed 1000-fold with a yield of 3.5% (Paulus et al., 1993). The Gly I purified was lined with in the sizes reported above.

Materials and Methods

Plant material

All plant materials, BARI sharisha16 and Tori7 were collected from the field laboratory of oil seed research centre, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh.

Stress treatments

Seeds of BARI Sharisha16 (tolerant) and Tori7 (susceptible) were grown in pot under greenhouse conditions (25 $^{\circ}$C and 12h photoperiod). Five-day-old seedlings were treated with NaCl solution to induce saline stress. The salinity level 16 dSm^{-1} was arisen by adding saline water prepared by NaCl, and the concentration of salinity in soil was measured by electrical conductivity meter (Hanna, Hungary). For drought stress, 10-day seedlings were stopped watering to induce drought stress until wilting. For both cases, one set of untreated control seedlings were maintained in normal growing condition for both tolerant and susceptible genotypes. Data were taken after 2, 4 and 6 days of stress implementation. For purification of Gly I, seven-day old seedlings of BARI Sharisha16 were used.

MG determination

Methylglyoxal was estimated according to Rohman et al. (2016) using N-acetyl-L-cysteine.

Extraction of enzyme

Fresh leaves (0.5 g) from seedlings of different treatments were homogenized in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10 % (w/v) glycerol with mortar and pestle. The homogenates squeezed in a nylon cloth and was centrifuged at 11,500×g for 10 min and the supernatant was used as soluble protein solution.

Glyoxalase I assay

Glyoxalase I assay was carried out according to the method of Islam et al. (2015) with slight modification. Briefly, the assay mixture contained 100mM sodium phosphate buffer (pH 7.5), 15 mM magnesium sulphate, 1.7 mM glutathione and 3.5 mM MG. The reaction was started by the addition of enzyme solution. The formation of thioester was measured by observing absorption at 240 nm for 1min. The activity was calculated using the extinction coefficient of 2.37 $mM^{-1}cm^{-1}$.

Glyoxalase II assay

Glyoxalase II activity was determined according to the method of Hasanuzzaman et al. (2011b) by monitoring the formation of reduced glutathione (GSH) at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB and 1 mM *S*-D-lactoylglutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG and the activity was calculated using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$.

Purification of Gly I

Extraction of crude protein

Fifty grams of leaves of 8 day old seedlings of tolerant genotypes BARI sharisha 16 was homogenized in equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10 % (w/v) glycerol with a waring blender. The homogenates squeezed in a nylon cloth and was centrifuged at 11,500×g for 10 min and the supernatant was used as crude protein solution**.**

Precipitation of soluble protein

Protein in the soluble protein solution was precipitated with ammonium sulfate at 65% saturation. The proteins were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) that contained 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA (buffer A) overnight. The dialyzate was diluted to 35 ml with same buffer.

Anion exchange chromatography

The dialyzate prepared from precipitation solution was applied to a column (1.77cm i.d. × 20 cm) of DEAE-cellulose (DE-52; Whatman, Kent, UK) that had been equilibrated with buffer A. This was termed as DEAE-cellulose chromatography-1. The column was washed with buffer A, and eluted with a linear gradient of 0 to 0.2 M KCl in 800 ml of buffer A. High active fractions of 5.0 ml were collected. The activity and absorbance (A_{280}) of each fraction were measured spectrophotometrically.

The fractions corresponding to the high Gly I active peaks were combined as the Gly I pool for further purification. The pooled Gly I sample was applied on another DEAE-cellulose

chromatography (termed as DEAE-cellulose chromate-graphy-2) and eluded with same gradient solution (600 ml). High actine Gly I fractions, each containing 5 ml, were collected and subjected to measure activity and absobance (A_{280}). High Gly I fractions were pooled for further purification by affinity chromatography to complete the purification.

Affinity chromatography

The high active Gly I peak of anion exchange chromatography-2 were applied to a column (0.76 cm i.d. ×4.0 cm) of *S*-hexylglutathione-agarose (Sigma, St. Louis, MO) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.01% (v/v) β-mercaptoethanol (buffer B). The column was washed with buffer B containing 0.2 M KCl and eluted with buffer B containing 1.2 mM *S*-hexylglutathione. Fractions of 2.5 ml were collected. Protein fractions eluted with Gly I were combined and dialyzed against buffer B. The dialysate was used as the purified Gly I solution.

Protein quantification

The protein concentration of each sample was determined by the method of Bradford (1976) using BSA as protein standard.

SDS-PAGE and Silver Staining

To check the homogeneity of the purified enzyme and to estimate its molecular mass SDS-PAGE was done in 12.5%(w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970) followed by silver staining.

CBB Staining

To visualize enzyme, the gel was shacked in concentrated CBB solution overnight. On next day, the gel was destained and scanned.

Measurement of molecular weight

The Molecular weight was measure by gel documentation system (Alpha-Inotech)

Data analysis

Data generated from this study were analyzed by STATISTIX 10 software where need. Data were analyzed following CRD design with three replications. Means were separated by Dancan's Multiple Range Test (DMRT) test and $P \leq 0.05$ was considered as significance level. The graphs were prepared in MS Excel, 2010. Mean values ± standard error (SE) were presented in graphs from at least three independent experiments, each containing three replications.

Conclusion

Glyoxalase system is known to play a very important role in abiotic stress tolerance. This two-step pathway detoxifies ubiquitously present cytotoxic metabolite MG, which otherwise increases to lethal concentrations under various stress conditions. In this study, role of glyoxalase system in detoxification of MG was observed and subsequent purification of a high active Gly I enzyme from tolerance plants. Further research will be conducted with this purified Gly I to examine its accumulation in plants under abiotic stress.

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