Purification and characterization of thermostable monomeric chloroplastic Cu/Zn superoxide dismutase from *Chenopodium murale*

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ABSTRACT

Superoxide dismutase is the first line of defense against oxidative stress and thus helps in maintaining the cellular integrity. *Chenopodium murale*, a weed species adapted to widely varying climatic conditions faces extremes of temperatures ranging from 4 °C to 45 °C (T_{max}) during growth and development. From this plant, we have purified a thermostable chloroplastic Cu/Zn superoxide dismutase (Chl Cu/Zn SOD) to homogeneity using minimal steps. Incubation of lysed chloroplasts at 70 °C for 1h reduced the interference of cytosolic SOD isoforms and reduced the protein content by 75 %. Chloroplastic SOD was purified from the heat stable fraction by gel filtration chromatography. The purified enzyme had a native molecular weight of 24 kDa, a half-life of 47.9 min at 80 °C and showed a single band at 24 kDa on SDS-PAGE. The N-terminus contained the conserved amino acids of chl Cu-Zn SOD. The Chl Cu/Zn SOD protein and its activity were enhanced under very high temperatures, high light intensities and in water stress/recovered *C. murale* plants under controlled environment conditions. Chl Cu/Zn SOD was also one of the predominant isoforms throughout growing period in field grown plants and declined during senescence. The Chl Cu/Zn SOD activity increased with the increase in ambient temperature and peaked in April with a 45 °C T_{max}. These results clearly indicate that the chloroplastic Cu/Zn SOD is stably expressed at extreme environmental conditions. The presence of stable monomeric chloroplastic Cu/Zn SOD might help the plants to maintain the cellular homeostasis against adverse environmental conditions. [Physiol. Mol. Biol. Plants 2009; 15(3) : 199-209] E-mail : renu_wtc@rediffmail.com

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INTRODUCTION

Abiotic stresses, especially salinity, drought, temperature, and oxidative stress, are primary causes of loss in crop productivity world wide (Vincour and Altman, 2005). Identification of stable protein and cloning of stress responsive genes from the adaptable plants to various environmental conditions is one of the important goal of the researcher in the field of stress biology. This will help in the genetic engineering of crop plants for enhanced tolerance to abiotic stress

(Cushman and Bohnert, 2000). *Chenopodium* species are weed species having wide adaptation to different climatic regimes and often experience abiotic stress during their life cycle. Abiotic stresses are known to induce oxidative stress in plants. It is likely that these weeds are endowed with an efficient defense mechanism, which enables them to maintain cellular redox homeostasis. *Chenopodium* is used as a model system to study the biochemical basis of thermotolerance in plants and several heat shock proteins have been isolated and characterized in this species (Downs *et al.*, 1999). We used *C. murale*, a weed as a model to understand biochemical basis of adaptation to high temperature stress in plants as it faces extreme temperatures ranging from 4 °C to 45 °C during growth

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and development in north India (Sabarinath, 2005). Similarly *C. album* ecotypes growing in habitats ranging from cool to warm helped in establishing the role of chloroplastic Hsps in imparting thermotolerance to photosynthetic e^- transport (Barua *et al.*, 2003).

Abiotic stresses like drought, salinity, cold, freezing, high temperature, anoxia, high light intensity and nutrient imbalances etc are known to induce oxidative stress in plants (Suzuki and Mittler, 2006; Schützendübel and Polle, 2002). Oxidative stress is caused by reactive oxygen species. These reactive oxygen species are scavenged by enzymatic (SOD, CAT, APX and GR) and non-enzymatic (glutathione, ascorbate and tocopherol) antioxidants (Levine, 1999). Superoxide dismutase [EC 1:15:1:1, SOD] is the first line of defense against ROS as it catalyses the dismutation of two superoxide radicals into hydrogen peroxide and oxygen. All SODs, are multimeric metallo-proteins that efficiently scavenge the superoxide radicals. In plants three classes of SODs have been reported based on the metal co-factor, which could be either a dinuclear Cu/Zn SOD, mononuclear Fe SOD and Mn SOD (Fridovich, 1997; Sabarinath *et al.*, 2005). SOD activity determines the concentration of the Haber-Weiss reaction substrates, O_2^- and H_2O_2 . Different SODs have been genetically engineered into plants and over expressed to assess their potential capacities for enhancing oxidative stress tolerance against abiotic stresses (Holmberg and Bülow, 1998). These findings confirm that SODs play a significant role in preventing cellular damage by oxygen free radicals and protect the plants from oxidative stress. SOD has also been applied in gene therapy especially in the prevention of oncogenesis (Mates and Sanches-Jimenez, 2000). Thermostable SODs are potentially useful in pharmaceutical and cosmetic industries because of their high stability (Kumar *et al.*, 2006).

We have earlier shown the occurrence of heat stable chloroplastic Cu/Zn SOD in *C. murale*. (Khanna Chopra and Sabarinath, 2004). *C. murale* plants face a broad range of temperatures during growth and development and have the ability to survive and reproduce in heat stress environments. It is likely that the expression of heat stable chl Cu/Zn SOD is modulated by the environment and the developmental stages and thus contributes to the heat stability and adaptability of the species. In this paper we report the expression of chloroplastic Cu/Zn SOD to abiotic stress treatments in *C. murale* plants grown in controlled environment and during growth and development in field grown plants. The enzyme was purified to homogeneity from chloroplasts by heat treatment and gel filtration

chromatography. The purified enzyme was characterized biochemically including N terminal analysis.

MATERIALS AND METHODS

Plant material, growth conditions and abiotic stress treatments

Chenopodium murale grows in the fields of Water Technology Centre, Indian Agricultural Research Institute, and New Delhi, India (77° 12' E, 28° 40' N, 228.6 m a.s.l) during November to May. Average maximum and minimum temperatures during the Rabi season, November 15th to May is shown in Fig. 1. The temperatures ranged from 4 °C minimum to 45 °C maximum during growth and development. The plants were marked at the seedling stage (in November) and sampling was done every month from the seedling stage upto maturity (May).

The plants were also grown in plant growth chambers (E-24 model, 3000 series Conviron, Canada) with a 16-h photoperiod, day night temperature 24 °C/15 °C, light intensity 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 70 % relative humidity. Five plants were maintained per pot. Three weeks old plants were used for studying the response of chloroplastic Cu/Zn SOD to abiotic stress treatments. The above conditions constituted the control. For high light intensity treatment (HL) three pots were shifted to a plant growth chamber with a light intensity of 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 2h, 4h and 24 h respectively. High temperature treatment (HT) was given to the plants by exposing to 32 °C (2h, 4h), 45 °C (2h) either with or without prior acclimation for 2 days at 35 °C and at 50 °C (2h). The plants were given water stress treatment by withholding water supply till leaf wilting was

Fig. 1. Average temperature maximum and minimum during the Rabi season (November to May).

observed (WS). Then plants were rewatered for recovery and sampling was done after 48hrs (WSR). All treatments were done at least in duplicate and the experiments were repeated twice.

Purification of chloroplastic Cu/Zn SOD

Young fully expanded leaves from field grown plants were plucked in March and used for isolating chloroplasts. Chloroplasts were isolated using Percoll step gradient method of Mullet and Chua (1983). Fresh leaves were homogenized in a pre-chilled blender for 2-seconds in 100 mL of ice cold grinding buffer (GR) contained 0.33 M sorbitol, 2 mM sodium-EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes-KOH (pH 7.5). Sodium ascorbate (0.1 g/100 mL) was added at the time of grinding. The homogenate was filtered through four layers of cheese clothes and centrifuged at 3000 g for 2 min. The pellet was re-suspended in 4 mL of GR buffer and was further purified by loading it onto a Percoll gradient (40-80 % step gradient) followed by centrifugation at 9,500 g for 6 min. After centrifugation, the lower band was transferred to a clean corex tube. The pellet was washed using ice-cold GR buffer several times to remove the Percoll. Then final chloroplast pellet was re-suspended in 4 mL GR buffer and the suspension was held in ice. The intactness of purified chloroplasts was evaluated by ferricyanide reduction test (Lilley *et al.*, 1975) using clark type O_2 electrode (Hansatech Instruments Ltd., England). Only chloroplast preparations with intactness higher than 90 % as estimated by the ferricyanide test were used. Chloroplasts were lysed by osmotic shock and the lysed chloroplasts were incubated at 70 °C for one hr in a sealed container in a shaking water bath. After incubation, the sample was centrifuged at 10,000 g for 10 min and the supernatant was used for ammonium sulfate fractionation, with the pH maintained at 7.8 by addition of ammonium hydroxide. The supernatant was slowly brought to 20 % saturation with $(NH_4)_2SO_4$ (114 g/litre) and then to 60 % saturation with $(NH_4)_2SO_4$ (262 g/litre). The 20-60 % $(NH_4)_2SO_4$ fraction was dissolved in, and dialyzed against 10 mM sodium phosphate buffer and 0.1 mM EDTA. The dialysate was further concentrated by Amicon stirred cell concentrator using PM-10 membrane (Amicon stirred ultra filtration cell, 8200 model, Millipore, Bedford, USA). The concentrated enzyme was applied to a Sephadex G-75 column (2 cm X 100 cm), equilibrated with 600 ml of 10 mM sodium phosphate buffer (pH 7.8) and 0.1 mM EDTA. Elution was done by using the same equilibration buffer. Fractions (3 ml) were collected at a flow rate of 30 ml h^{-1} cm^{-2} .

Enzyme extraction

Fresh leaf tissue (200 mg) was frozen in liquid nitrogen and homogenized in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.8) containing 2 mM EDTA and 5 mM β -mercapto- ethanol. During the homogenizing process 4 % PVP-40 was added (Donahue *et al.*, 1997). The homogenate was centrifuged at 30,000 g for 30 min at 4 °C and the supernatant was dispensed in aliquots for further analysis.

Enzyme and Protein Assays

Superoxide dismutase (EC: 1.15.1.1) activity was determined spectrophotometrically based on inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The reaction mixture contained 50 mM sodium phosphate buffer, pH 7.8, 58 mM NBT, 2.4 mM riboflavin, 9.9 mM methionine, and 0.025 % (v/v) Triton X-100. The assay mixture contained 50-80 mg protein of enzyme extract and its volume was made upto 0.1 mL using 50 mM sodium phosphate buffer, pH 7.8 and one mL of reaction mixture. Mixed gently and 2.4 mM riboflavin was added. This was exposed to light source (6 x 60W bulb) for 8 min. The reaction was stopped by switching off the light source and absorbance was measured at 560 nm. One unit of SOD is defined as the amount of enzyme required to inhibit the reduction of NBT by 50 % in 1 ml reaction volume. Total soluble protein was determined following the method of Lowry *et al.*, (1951). BSA (fraction V) was used as a standard.

Native-PAGE and in gel SOD assay

An in-gel SOD assay was performed as described in Beauchamp and Fridovich (1971). SOD isoforms were separated on a 10 % non-denaturing polyacrylamide gels at 4 °C using Mini Protean electrophoresis unit (Bio-Rad, USA). Gels were soaked in 50 mM sodium phosphate buffer, pH 7.8 containing 0.24 mM NBT and 28 mM riboflavin for 20 min in the dark at 25 °C, followed by immersing in 50 mM sodium phosphate buffer, pH 7.8 containing 28 mM TEMED, and then exposed under light source (60 Watts, 6 lamps) at room temperature.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) and proteins were visualized with Coomassie Brilliant Blue or Silver staining.

Thermal stability

The purified protein was incubated at 70 °C and 80 °C

in a sealed container in a continuous shaking water bath. Aliquots were withdrawn at 15 min interval and SOD activity was measured to obtain the thermal inactivation profile for the purified protein. The rate constant k_d for first-order inactivation was determined by Lin *et al.* (2001) from the slope of the inactivation time courses according to the equation $\ln (E_t/E_o) = -k_d t$. [E_t = enzyme activity after heat treatment, E_o = enzyme activity before heat treatment, control]

pH dependence and pH stability

The enzyme extract was incubated with three different buffer solutions and SOD activity was measured to see the effect of pH on the purified protein. sodium acetate buffer (0.2 M) for pH 3.0, 4.0 and 5.0, 0.2 M sodium phosphate buffer for pH 6.0, 7.0, 8.0, bicine buffer (0.2 M) for pH 9.0, 10.0, 11.0. Each sample was incubated at 37 °C for one hour (Lin *et al.*, 1995).

Molecular mass determination

Purified protein was loaded on sephadex G-75 column pre-calibrated with aprotinin (6.5 kDa), Cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa) and eluted using 10mM sodium phosphate buffer, pH 7.8 containing 0.1 mM EDTA.

NH₂ terminal amino acid sequencing

Purified Chl Cu/Zn SOD was separated by SDS - PAGE and electrotransferred to ProBlott™ membrane using CAPS 3-[cyclohexylamino]-1-propanesulfonic acid buffer. The protein band was sliced and NH₂ terminal portion of the peptide was sequenced by the Edman degradation method (Model 477 A protein/peptide sequencer, Applied Biosystems, CA, USA).

2-D gel electrophoresis

2-D gel electrophoresis technique was done by sorting proteins according to two independent properties in two discrete steps (Dunn and Corbett, 1996). First dimension step was carried out using Ettan IPGphor isoelectric focussing system on a 7 cm ready made IPG strip, pH range 3-10 followed by second dimension SDS-PAGE, by placing the equilibrated IPG strip on 7 % SDS gel in the tris-glycine system by using Multiphor II flat bed system (Amersham Bioscience, Aylesbury, UK), according to the manufacturer's instructions.

Antibody production and western analysis

The purified SOD (250 mg) was emulsified in Freund's complete adjuvant and injected subcutaneously into a

rabbit. Three booster doses, each containing 125 mg of protein emulsified in Freund's incomplete adjuvant, were administered at three weekly intervals. Ten days after the last injection, blood was collected and serum was separated and stored at -20 °C. The titration of the antibody in the immune serum by the ELISA method indicated an antibody titer of about 1:15000.

Fresh sample (250 mg) was ground using zivy buffer (Zivy *et al.*, 1983). PVPP was added at the time of grinding (0.05 g/g FW of sample). The homogenate was passed through cheesecloth and centrifuged at 10,000 g for 20 min. To the supernatant, eight volume of ice-chilled acetone was added and incubated at 4 °C over night for protein precipitation. The samples were again centrifuged at 10,000 g for 20 min and to the pellet Laemmli buffer was added (Laemmli, 1970). The protein samples were processed in boiling water for 4 min and aliquots were made and stored in -80 °C. For western blot analysis, proteins were electro transferred at 4 °C onto nitrocellulose membrane (Millipore) at 50V for 1 h in a transblot unit (Bio-Rad, Richmond, CA, USA). Immunodetection was carried out with polyclonal antibodies raised against purified Chl Cu/Zn SOD. The blot was incubated with the primary antibody and immunoreactive protein bands were visualized using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antiserum with 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium substrate.

RESULTS

SOD isozyme pattern during growth and development

Total soluble protein content and SOD activity increased from seedling stage to flowering and maturity but declined at senescence stage (Fig. 2). The maximum protein content and SOD activity was observed during Feb to April sampling. The SOD isoform pattern was regulated in relation to growth and environmental conditions. Among the two SODs, SOD V and SOD VI were the predominant isoforms up to the beginning of senescence stage (Fig. 2), SOD V is a chloroplastic Cu/Zn SOD showing stability even after boiling the leaf extract for 10 min while SOD VI is a cytosolic form (Khanna-Chopra and Sabarinath, 2004). During senescence SOD VI activity was higher than SOD V.

Purification of chloroplastic Cu/Zn SOD

Purified chloroplasts were used as the source of chloroplastic Cu/Zn SOD in order to reduce the interference of cytosolic heat tolerant SOD isoform VII

Fig. 2. SOD activity across developmental stages in *C. murale*, where it faces extreme temperatures from 5 °C to 45 °C. [A] SOD activity and total soluble protein [B] Native PAGE showing SOD activity. [C] Relative SOD activity as determined by scanning the gels using gel documentation system, area expressed as relative units. Vertical bars indicate SE (n=3).

which made its appearance in the heat treated leaf protein extract of *C. murale* at 70 °C (Khanna Chopra and Sabarinath, 2004). Heat treatment (70 °C, 1h) of lysed chloroplast fraction further helped to remove the heat sensitive chloroplastic proteins and thus minimize the purification steps as outlined in Table 1. The enzyme was purified to apparent homogeneity by (NH₄)₂SO₄ precipitation followed by gel filtration chromatography on Sephadex G-75. There was a 90 % reduction in the total protein, with a 4-fold enhancement in the total activity of chloroplastic Cu/Zn SOD after ammonium sulphate fractionation. The concentrated sample was loaded onto a Sephadex G-75 column and SOD activity was eluted as a single peak. The active homogenous fractions were pooled and concentrated using Centriplus 20 (10 kDa cut off; Millipore, Bedford, USA). The homogenous protein obtained after Sephadex G-75 showed total activity of 6,936 units as per the Beauchamp and Fridovich assay (1971). The purified protein showed 98-fold purification with a 36 % recovery of the initial SOD activity (Table 1). The band of SOD activity in isolated chloroplasts corresponded to the purified SOD protein, indicating that no modification occurred during purification process (Lane I & II, Fig. 3A). The preparation of purified Cu/Zn SOD was homogenous as judged by the results of native PAGE stained for protein and for SOD activity (Fig. 3A&B). The apparent molecular mass of the purified protein was found to be 24kDa by gel filtration on Sephadex G-75 column pre-calibrated with the known molecular mass protein (Fig. 3C). The protein pattern of each purification step examined by SDS-PAGE is shown in Fig. 4.

Properties of the enzyme

A single spot appeared on the 2D-gel, which confirmed that purified chloroplastic Cu/Zn SOD was a monomeric enzyme with a pI of about 5.6, close to the pI of BSA. (Fig. 5A). The enzyme exhibited maximum activity at pH 7.8. The purified protein was very stable in the alkaline pH range 7-10 but the activity decreased by 70 % at pH less than 4 (data not shown)

The purified enzyme heated at 70 °C for 1h retained 75 % of the original activity, while at 80 °C up to 25 min it retained 75 % activity. The inactivation of monomer fitted the first order inactivation rate equation $\ln(E_t/E_0) = k_d t$, where E_0 , E_t , represent the original activity and enzyme activity after heat treatment for time t , respectively. The thermal inactivation rate constant k_d calculated for the monomer was 0.014 min⁻¹ and the half life for inactivation was about 47.9 min at 80 °C (Fig. 5B). Our previous results (Khanna Chopra and

Fig. 3. Gel showing the purity and homogeneity of the purified protein. Purified fraction was loaded on a 10 % native PAGE. [A] One-half of the gel was stained for SOD activity, and [B] the next half was stained for protein using Coomassie Brilliant Blue R250. C- Chloroplastic fraction, P- Purified fraction, M- Native PAGE molecular weight marker. [C] Determination of molecular weight of thermostable Chl Cu/Zn SOD on sephadex G-75. Two mL was applied to a column of sephadex G-75, which was calibrated with bovine serum albumin (a), 66 kDa; carbonic anhydrase (b), 29 kDa; cytochrome C (c), 12.4 kDa and aprotinin (d), 6.5 kDa.

Fig. 4. Silver stained, 15 % SDS-PAGE showing the protein profile of the purification steps. Lane I, isolated chloroplasts; lane II, after heat treatment (70 °C, 1h), lane III, after 20-60 % $(\text{NH}_4)_2\text{SO}_4$ cut, lane IV, purified Chl Cu/Zn SOD protein. M- SDS-protein marker ranging from 14.4 – 116 kDa (MBI fermentas, MI, USA).

Fig. 5. 2D polyacrylamide gel electrophoresis of purified SOD protein [A]. pI marker (Biorad, CA, USA) pI marker loaded on a separate gel (not shown) and overlapped. [B] Thermal inactivation rate of purified chloroplastic Cu/Zn SOD from *C. murale*.

Sabarinath, 2004) showed the presence of heat stable chloroplastic Cu/Zn SOD in the crude leaf extract. This data shows that the thermostability is an inherent property of the purified chloroplastic Cu/Zn SOD protein.

N-Terminal analysis

The sequence of the purified SOD as determined by N-terminal Edman degradation of the enzyme gave an uninterrupted stretch of 15 amino acids, which showed that it carried the conserved region of the Cu/Zn SOD. Comparison of 15 amino acid sequence of the purified SOD with that reported for Cu/Zn SOD from other plants showed 67 % homology with other chloroplastic Cu/Zn SODs, 85 % homology with bovine and human Cu/Zn SOD and 93 % homology with SOD from *Caretta caretta* (Fig. 6).

Chloroplastic Cu/Zn SOD expression under abiotic stresses

Polyclonal antibodies were raised against purified chloroplastic Cu/Zn SOD. The response of Chl Cu/Zn SOD enzyme and protein was examined under various abiotic stresses. The chloroplastic Cu/Zn SOD activity and protein was significantly higher than that of control under very high temperatures, high light intensity (24 h) and in water stress recovered plants but not under water stress (Fig. 7 B, C & D).

DISCUSSION

We have previously reported the presence of heat stable chloroplastic Cu/Zn SOD in *C. murale* which maintains high activity even at 70 °C (Khanna Chopra and Sabarinath, 2004). This enabled us to include the heat precipitation step in the purification process. The procedure for the purification of Chl Cu/Zn SOD protein from *C. murale* was considerably shortened in the present

study by using isolated chloroplast as the source and subjecting the lysed chloroplast to 70 °C for 1h, which reduced the total protein content by 75 % (Table 1). Heat precipitation step has also been used earlier in the purification of SOD protein (Carter and Thornburg, 2000). The homogeneity of the purified SOD was established by the results of SDS-PAGE (Fig. 4) and 2-D gel electrophoresis (Fig. 5A) which confirmed that the purified SOD was a monomer with a Mol. mass of 24 kDa and pI of about 5.6, close to the pI of the Cyt and Cp-SODs, which are detected at pI 5.5 (Schinkel *et al.*, 1998). Chloroplastic Cu/Zn SODs so far isolated are homo-dimers or homo-tetramers having molecular mass (kDa) ranging from 30-33 kDa with an isoelectric point of 5.2 - 5.6 (Bordo *et al.*, 1994; Alscher *et al.*, 2002). In higher plants, so far only a cytosolic monomeric Cu/Zn SOD has been isolated from rice with the kDa of 17.5 kDa (Kanesmatsu and Asada, 1989). Here we report for the first time a monomeric chloroplast Cu/Zn SOD in *C. murale* with a mol mass of 24 kDa.

The thermostability of the Cu/Zn SOD has been well documented in animals and prokaryotes (Parege *et al.*, 1992; Battistoni and Rotilio, 1995) whereas in plants only few reports are available about thermal denaturation of Cu/Zn SOD (Steinman 1982; Kwiatowski and Kaniuga 1986; Walker *et al.*, 1991). In *Lens esculanta* and in brussel sprouts, 50 % inactivation of the purified SOD enzyme was observed at 50 °C and 60 °C respectively (Federico *et al.*, 1985; Walker *et al.*, 1991). In rice, the monomeric cytosolic form was stable at 70 °C for 50 min, whereas the dimeric chloroplastic Cu/Zn SOD lost 30 % activity after 5-min incubation at 70 °C (Kanematsu and Asada, 1989). The purified chloroplastic Cu/Zn SOD from *C. murale* has half-life value of 47.9 min at 80 °C (Fig. 5B), which is higher than that reported for monomeric cytosolic SOD from sweet potato, the value being 28 min at 85 °C (Lin *et al.*, 1995).

Table 1. Summary of the purification procedure of chloroplastic Cu/Zn superoxide dismutase (SOD) from isolated chloroplasts of *C. murale*.

Sample	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Fold purification
Isolated chloroplasts	1026.4	18988	18.5	100	1
Heat treatment at 70 °C, 1h	238.4	10513.4	44.1	55.36	2.38
20-60 % NH ₄ (SO ₄) cut	98.24	7230.5	73.6	38.1	3.97
Sephadex G-75	3.8	6935.98	1825.26	36.52	98.66

The reasons for the thermostability of SODs, well documented from hyperthermophiles, could be the presence of a large number of intrasubunit ion-pairs (Ursby *et al.*, 1998) and the close packing of the hydrophobic interface between the two halves of the β -barrel core resulting in the compactness of the β -barrel fold and the metal ions present in it (Bordo *et al.*, 1994; Battistoni *et al.*, 1998; Rodriguez *et al.*, 2002).

Comparison of the NH₂-terminal sequence of the purified SOD with that reported for Cu/Zn SOD from other plants showed 67 % and 43 % homology with the N-terminal region of the Cp and Cyt- Cu/Zn SODs respectively reported so far in plants (Fig. 6). However, it has 85 % homology with bovine and human Cu/Zn SOD and 93 % homology with *Caretta caretta* (marine turtle) Cu/Zn SOD. Schinina *et al.* (1993) showed that purified *Caretta* Cu/Zn SOD retained 80 % of its activity after 90 min at 70 °C and *Bovine* Cu/Zn SOD retained 87 % activity after 60 min at 70 °C (McRee *et al.*, 1990).

In the native habitat *C.murale* experiences increase in light irradiance and temperature as it grows from seedling stage to maturity. The temperature increases from 4 °C min. to 45 °C maximum and may be coupled with low water availability since rainfall is very minimal during this period. The abiotic conditions where in light irradiance is combined with chilling or high temperatures induce photo oxidative stress (Bowler *et al.*, 1992; Mittler *et al.*, 2004). Efficient anti oxidative defense plays an important role in enhancing survivability in stressful environments (Prashanth *et al.*, 2008). SODs constitute the first line of defense against highly toxic superoxide radicals by converting superoxide to H₂O₂ and molecular oxygen. SOD activity has been reported to increase in response to stress conditions such as high irradiance, low temperature (Mauro *et al.*, 2005) drought and high temperature (Lee *et al.*, 1999; Rhizhsky *et al.*, 2002; Selote and Khanna-Chopra, 2006). In our studies also chloroplastic Cu/Zn SOD protein content and activity increased in response to high temperature, high light and in water stress recovered plants (Fig. 7). However, the relative SOD activity typically did not show the response similar to that of relative Chl Cu/Zn SOD protein content. The lack of correlation between the Chl SOD activity versus the Chl SOD protein content might be because of the post translational modifications in the Cu/Zn SOD protein (Furukawa and O'Halloran, 2006). In field grown plants also both cytosolic and chloroplastic SOD activity increased from seedling stage to maturity indicating an increase in reactive oxygen species in response to changing environmental conditions (Fig. 2). Chloroplast

is a particularly rich source of ROS, especially under stress conditions (Foyer *et al.*, 1994; Asada, 1996). Efficient removal of ROS from chloroplasts is critical because very low concentrations of ROS can inhibit photosynthesis by oxidizing the thiol-modulated enzymes in the photosynthetic carbon reduction cycle (Kaiser, 1979). Chloroplastic Cu/Zn SOD plays an important role in ROS detoxification in chloroplasts. Novel Chl Cu/Zn isoforms have been shown to contribute to better fitness in photo-oxidative stress conditions in *A. thaliana* ecotype Cvi (Abarca *et al.*, 2001) and chilling responsive Chl Cu/Zn SOD isoforms have been identified in maize (Mauro *et al.*, 2005).

In conclusion we here report for the first time a monomeric chloroplastic Cu/Zn SOD in *C. murale* which is highly thermostable. The NH₂ - terminal sequence of the enzyme has the AT motif which characterizes the chloroplastic SODs of most species. It shows more homology with highly thermotolerant Cu/Zn SODs than chloroplastic SODs. The increase in enzyme activity in response to high light, high temperature and water stress/recovery emphasizes its importance in detoxifying ROS produced under multiple stresses in the field and thus contributes to the adaptation of *C. murale* to extreme environments. Hence, over expression of the *C. murale* chloroplastic Cu/Zn SOD in crop plants may help in improving abiotic stress tolerance through genetic engineering.

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