Cloning, Expression, and Purification of a Cu/Zn Superoxide Dismutase from *Jatropha curcas*

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We report cDNA cloning, expression, purification, and characterization of a novel Cu/Zn superoxide dismutase (SOD) from *Jatropha curcas* leaves. The full-length cDNA of this SOD contained a 496-bp open-reading frame (ORF) encoding 162 amino acid residues. The recombinant plasmid containing the SOD coding sequence was introduced into *Escherichia coli*, and the SOD was expressed as a fusion protein. The recombinant SOD was purified from a high-density fed-batch culture using a combination of immobilized metal ion affinity chromatography (IMAC) and Sephadex G25 desalting chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis indicated that the recombinant SOD was a monomeric protein with a molecular mass of approximately 16.4 kDa. Isoelectric focusing showed that this SOD was a basic protein with p*I* values of 7.04, 7.33, 8.62, and 8.77. The activity of the SOD was stable at 70 °C for 10 min, and in a broad pH range from 4 to 9. The presence of urea (up to 8 M), guanidinium chloride (up to 6 M), and 2-mercaptoethanol (up to 8 mM) had little effect on the activity. The activity decreased gradually with increasing concentrations of imidazole, hydrogen peroxide, and ethylenediaminetetraacetic acid (EDTA). Atomic absorption spectrometry showed the presence of 0.239 copper and 0.258 zinc atoms, respectively, in the SOD polypeptide.

Key words: Superoxide Dismutase, *Jatropha curcas*

Introduction

Superoxide dismutase (SOD), a family of metalloenzymes, catalyzes the disproportionation of superoxide to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). SOD is one of the most important enzymes in the plant defence system against oxidative stress, and it occurs ubiquitously in every cell of all types of plants (Mittler, 2002). SOD is multimeric metalloproteins. Based on the metal species present at their active sites, there are three distinct types of SOD in plant cells: copper/zinc (Cu/Zn-SOD) in the cytosol and chloroplasts, manganese (Mn-SOD) in the mitochondria, and iron (Fe-SOD) isozymes in the plastids (Alscher *et al*., 2002).

Reactive oxygen species (ROS) have the potential to interact with many cellular components, causing significant damage to membranes and other cellular structures. However, an elaborate and highly redundant plant ROS network, composed of antioxidant enzymes and antioxidants, is responsible for maintaining the levels of ROS under tight control (Mittler, 2002; Pitzschke *et al*., 2006). SOD is one of several important antioxidant enzymes with the ability to reduce oxidative damage caused by ROS. Increased levels of SOD can protect a plant against physical stress (chilling, drought, salinity, and high light intensity) and chemical stress (O_3, metal ions, $O_2^{\cdot-}$-generating herbicides), and improve biomass production (Alscher *et al*., 2002). SOD overexpression in some transgenic plants has been reported to protect against stress (Lee *et al*., 2007; Tseng *et al*., 2007). Thus, SOD is considered a key enzyme in the regulation of intracellular levels of ROS and in the maintenance of normal physiological conditions under oxidative stress (Mittler, 2002).

Jatropha curcas L., commonly known as physic nut, belongs to the family Euphorbiaceae and is today recognized for its conversion to biodiesel fuel. Its cultivation can also help in the reclamation of wastelands, degraded lands, and mine waste-contaminated lands. Various parts of the plant have been widely used in traditional folk medicine in many countries (Makkar and Becker, 2009). *J. curcas* is often grown on riversides and in mining areas where soils are contaminated by heavy metals. Our previous results indicated that *J. curcas* can adapt to lead and mercury stress by an effective antioxidant defence mechanism (Gao *et al*., 2009, 2010). SOD has not yet been characterized from *J. curcas*. We report here on cDNA cloning, heterologous expression, and determination of its biochemical characteristics of a Cu/Zn-SOD of *J. curcas* (JcCu/Zn-SOD). JcCu/Zn-SOD is one of the thermally stable enzymes among the published SODs.

Material and Methods

Plant and chemicals

Mature *J. curcas* seeds were collected in August 2010 from more than 10 individual wild trees in Panzhihua, Sichuan Province, China. Full seeds were selected, oven-dried at 30 °C, and stored in a plastic box (labelled No. 20100828) at 4 °C until processing. Nitroblue tetrazolium (NBT) and isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO, USA). Ni-Sepharose high-performance (Ni-Sepharose HP), Sephadex G25, and Superdex G75 resins were purchased from Pharmacia (Uppsala, Sweden). Restriction enzymes, T4 DNA ligase, and recombinant *Taq* DNA polymerase were purchased from TaKaRa (Dalian, China). All other reagents were of the highest purity available.

RNA isolation and cDNA cloning

J. curcas seeds were germinated, and seedlings were grown in the greenhouse with an even light supply at 30 °C. About 30 d after germination, the young leaves (0.1 g) were harvested, frozen in liquid nitrogen, and ground to a powder in a ceramic mortar. Total RNA was prepared using the RNAprep pure plant kit (Tiangen Biotech, Beijing, China). Total RNA was used as template for single-strand cDNA synthesis with a M-MLV kit (TaKaRa). In the present study, we amplified a 650-bp fragment using single-strand cDNA of *J. curcas* as a template and two primers [JcCu/Zn 3–1, 5′-(A/G)CCTGGTC(T/C)-(A/C/T)CATGGTTTC-3′, and JcCu/Zn 3–2, 5′-GACACCAC(C/T)AA(C/T)GGTTGCA-3′] based on the conserved sequences of SOD from *Arabidopsis thaliana* (NCBI Ref. Seq NM. 100757.3), *Thellungiella halophila* (GenBank accession no. EF405867.1), *Brassica juncea* (GenBank accession no. AF540558.1), *Brassica napus* (GenBank accession no. AY970822.1), *Zea mays* (GenBank accession no. DQ245740.1), *Brassica rapa* ssp. *Pekinensis* (GenBank accession no. AF071112.1), and *Raphanus sativus* (GenBank accession no. AF009735.1). On the basis of this DNA sequence, three primers were synthesized as follows: JcCu/Zn 5–1, 5′-TGCCA-CATCCAACTCTTGCTCCTG-3′; JcCu/Zn 5–2, 5′-ATTCCATCTGGCCCAACA-3′; and JcCu/Zn 5–3, 5′-CCCGCATGACGTTCTTTATC-3′. The single-strand cDNA was synthesized using the primer JcCu/Zn 5–1. Then two polymerase chain reactions (PCRs) were carried out. The primer pairs in each reaction were JcCu/Zn 5–2 and AP [5′-GCTGTCAACGATACGCTACGTAACG-GCATGACAGTG $(T)_{18}$–3′], as well as JcCu/Zn 5–3 and AP1 (5′-GTCAACGATACGCTACG-TAACG-3′). Thus, a 400-bp DNA fragment was amplified. Based on the above results, the primer pairs were designed to amplify the full length of the Cu/Zn-SOD coding sequence gene. The parameters for amplification were as follows: denaturation at 94 °C for 5 min for one cycle, followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. A final extension at 72 °C for 8 min concluded the reaction. All amplified PCR products were analysed on an 1% agarose gel, and all DNA fragments were subcloned into the pMD19-T cloning vector using *Escherichia coli* Top 10 as a host. The nucleotide sequences of these inserts were determined in both strands.

Construction of expression vector pET32-SOD

The cDNA region homologous to other Cu/Zn-SODs covering an open-reading frame (ORF) was amplified using two gene-specific primers. The 5′ upstream primer contains the ATG and Kpn I and enterokinase recognition sites (5′-GGGG-TACCGACGACGACGACAAGATGGCAG-GAACGG CAACC-3′), and the 3′ downstream primer contains the Hind III recognition site

(5′-CCCAAGCTTCTAAACAGATGAGTGAAG-3′). By use of 0.1 μg single-strand cDNA as a template and 10 pmol of each 5′ upstream and 3′ downstream primers, a 500-bp fragment was amplified by PCR. The fragment was ligated into pMD19-T and transformed into *Escherichia coli* Top 10. A positive clone was selected, and plasmid DNA was isolated from the clone and doubly digested using Kpn I and Hind III. The digestion products were separated, purified on an 1% agarose gel, and subcloned into a pET-32a(+) expression vector (pretreated with Kpn I and Hind III) (Novagen, Madison, WI, USA). The recombinant SOD protein was expressed in *E. coli* BL21(DE3), and the functional protein was identified by an activity assay as described below.

Overexpression and purification of JcCu/Zn-SOD

The transformed *E. coli* BL21(DE) was grown at 32 °C in 250 ml of Luria Bertani medium containing 50 μg/ml ampicillin until A_{600} reached 0.6–0.8. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM. The culture was incubated for an additional 8 h at 200 rpm, and then the bacterial cells were harvested by centrifugation. Cells (4 g fresh weight) were suspended in 80 ml buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) and ruptured by sonication. The mixture was centrifuged at 20,000 × *g* for 30 min at 4 °C. The final crude extract (about 80 ml) was adjusted to pH 8.0 and loaded onto a Ni-Sepharose HP column (5 ml, bed volume pre-equilibrated with buffer A). After washing with buffer A containing 50 mM imidazole, a batch elution with buffer A containing 200 mM imidazole was carried out, and active fractions were collected and pooled. Samples were then desalted to buffer B (50 mM NaH_2PO_4, 100 mM NaCl, pH 8.0) using Sephadex G25 resin. To cleave the tag from the fusion protein, the desalted samples were digested with enterokinase. The digested samples were loaded onto the same Ni-Sepharose HP column, which was pre-equilibrated with buffer B. After washing away unbound proteins, the absorbed proteins were eluted with buffer C (50 mM NaH_2PO_4, 150 mM NaCl, 35 mM imidazole, pH 8.0) and buffer D (50 mM NaH_2PO_4, 200 mM NaCl, 250 mM imidazole, pH 8.0), respectively. Active fractions were collected and pooled. The purified enzyme was either used directly for analysis or stored at –20 °C until use. The protein concentration was measured according to Bradford (1976) using bovine serum albumin as standard.

Determination of molecular mass and isoelectric focusing

The purified enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol (2-ME, 4% v/v), using a mini-Protein II apparatus (Bio-Rad, Hercules, CA, USA). The protein was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Isoelectric focusing was performed in a model 111 MINI IEF CELL apparatus (Bio-Rad). The p*I* standards (pH 4–10) were from Bio-Rad.

Determination of metal content

The metal content of the purified SOD was determined by inductively coupled plasma-mass spectrophotometry (ICP-MS), using a PQ ExCell instrument (VG Elemental, Franklin, MA, USA) after the enzyme had been dialyzed extensively against 10 mM phosphate buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA), followed by buffer lacking EDTA. Standard solutions of metal ions were used for quantitation.

Assay of SOD activity and activity staining

The SOD assay was performed according to Beauchamp and Fridovich (1971) with some slight modifications. The 3-ml reaction mixture contained 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, and 50 μl enzyme extract. Absorbance was read at 560 nm using a UV/Vis spectrophotometer (TU-1901; Purkinje General, Beijing, China). The amount of enzyme causing 50% inhibition of the reaction (one unit) was calculated. The activity was expressed as enzyme units per mg protein (U/mg protein).

The SOD activity in gels was determined by the Beauchamp and Fridovich (1971) method with some modifications. After completion of electrophoresis, gels were incubated in 50 mM phosphate buffer (pH 7.5) containing 28 μM riboflavin and 28 mM *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED) for 30 min in the dark, followed by

washing in distilled water for 1 min and incubation in the same buffer containing 2.45 mM NBT for 20 to 30 min under exposure to a fluorescent lamp at room temperature. Isoenzymes appeared as colourless bands on a purple background.

Thermostability, pH stability, and effects of additives on SOD activity

The effect of temperature on the enzyme stability was checked by incubating the enzyme in buffer B at various temperatures (30, 37, 40, 50, 60, 70 °C) for 10 – 60 min, and measuring the relative activity under standard assay conditions as described above. To investigate the pH stability, the protein samples were incubated with buffers of various pH values as follows: 0.2 M citrate buffer (pH 4, 5, and 6), 0.2 M phosphate buffer (pH 7), 0.2 M Tris-HCl buffer (pH 8), and 0.2 M glycine/NaOH buffer (pH 9 and 10). After 1 h incubation at 25 °C, the SOD activity was determined by the above-mentioned method.

To investigate the effects of denaturing reagents and other agents on the SOD activity, urea (1 – 8 M), guanidinium chloride (Gdn-HCl) (0.75 – 6 M), imidazole (0.125 – 1 M), H_2O_2 (2 – 16 mM), 2-ME (2 – 16 mM), and EDTA (2 – 16 mM) were added to the enzyme samples. After incubation of the mixtures for 1 h at 25 °C, the residual SOD activity was determined.

Statistical analysis

All treatments were arranged in a completely randomized design with three replicates. All data were expressed as means ± SD. Statistical significance was evaluated with a Student's t-test, and differences were considered significant if P values were ≤ 0.05.

Results and Discussion

Cloning and characterization of Cu/Zn-SOD cDNA

With the primers designed on the basis of the conserved amino acid sequences of known Cu/Zn-SODs, a product with the expected size of 650 bp was obtained by using rapid amplification of cDNA ends (RACE) PCR. Complementary to this fragment, three specific primers were designed. With these, a fragment of 400 bp was amplified. Sequence analysis revealed that combination of the 3′- and 5′-RACE fragments covered an ORF which encoded a SOD protein of 162 amino acid residues (GenBank accession no. JF509741). The sequence had high similarity with the sequences of a number of plant Cu/Zn-SODs (Fig. 1) and was designated JcCu/Zn-SOD. The amino acid sequence of JcCu/Zn-SOD (GenBank accession no. AFD34188.1) shared 84.0% identity and 88.9% similarity to hybrid aspen (GenBank accession no. CAC33847.1), 82.7% identity and 87.7% similarity to *Gossypium hirsutum* (GenBank accession no. ACC93639.1), 66.9% identity and 78.3% similarity to *Arabidopsis thaliana* (GenBank accession no. AAC24833.1), and 65.4% identity and 75.9% similarity to *Bambusa oldhamii* (GenBank accession no. ACX94084.1). Phylogenetic tree analysis of JcCu/Zn-SOD against the three types of plant SODs –Cu/Zn-SOD, Mn-SOD, and Fe-SOD– revealed that JcCu/Zn-SOD is indeed closest to the Cu/Zn-SOD family (Fig. 2). The

Fig. 1. Amino acid sequence alignment between Cu/Zn-SODs from several plant species using the software DNAMAN6.0. The GenBank accession number and the name for these sequences are as follows: JcCu/Zn-SOD, *J. curcas* Cu/Zn-SOD (AFD34188.1); HaCu/Zn-SOD, hybrid aspen Cu/Zn-SOD (CAC33847.1); GhCu/Zn-SOD, *Gossypium hirsutum* Cu/Zn-SOD (ACC93639.1); AtCu/Zn-SOD, *Arabidopsis thaliana* Cu/Zn-SOD (AAC24833.1); BoCu/Zn-SOD, *Bambusa oldhamii* Cu/Zn-SOD (ACX94084.1).

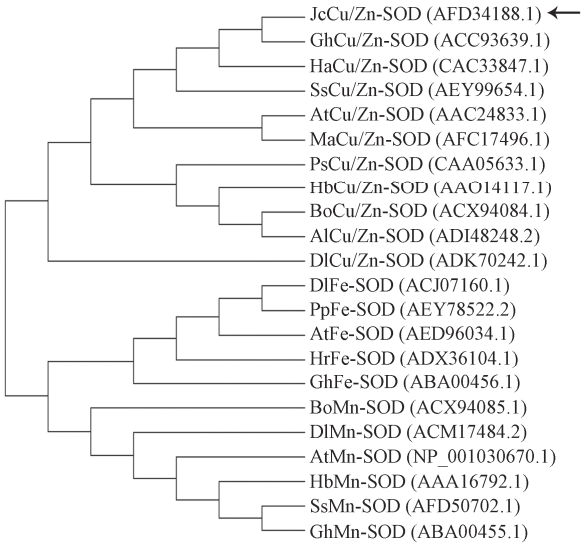

Fig. 2. Phylogenetic tree showing relationships between JcCu/Zn-SOD and diverse SOD proteins from other plant species. The sequences for the three types of plant SOD, *viz.* Cu/Zn-SOD, Mn-SOD, and Fe-SOD, were obtained from GenBank and aligned with that of JcCu/Zn-SOD (marked with arrowhead). The GenBank accession numbers are given in brackets. The tree was constructed using the MEGA5 software. Jc, *J. curcas*; Gh, *Gossypium hirsutum*; Ha, hybrid aspen; Ss, *Suaeda salsa*; At, *Arabidopsis thaliana*; Ma, *Musa acuminata*; Ps, *Pinus sylvestris*; Hb, *Hevea brasiliensis*; Bo, *Bambusa oldhamii*; Al, *Aeluropus littoralis;* Dl, *Dimocarpus longan*; Pp, *Prunus persica*; Hr, *Haberlea rhodopensis*.

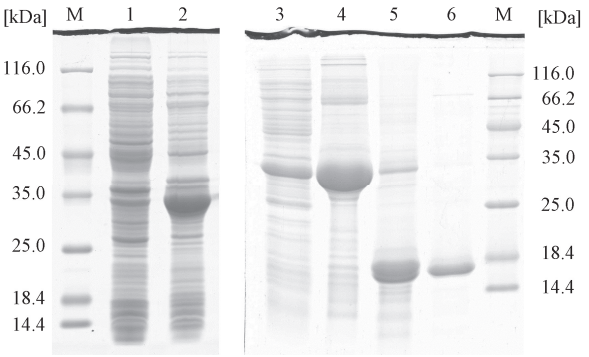

Fig. 3. SDS-PAGE (15%, 2-ME) of expressed proteins and purification of recombinant JcCu/Zn-SOD. About 15 – 20 μg protein were loaded per sample, and the gel was stained with Coomassie blue. Lane M, marker proteins; lane 1, no IPTG induction; lane 2, IPTG induction; lane 3, supernatant of crude extract; lane 4, extract after Ni-Sepharose HP column chromatography; lane 5, purified expressed protein after enterokinase treatment; lane 6, purified protein after re-chromatography on a Ni-Sepharose HP column.

localization of JcCu/Zn-SOD was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/), ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), and TargetP (http://www.cbs.dtu.dk/services/TargetP/). The results of SignalP and ChloroP showed that this protein does not have a signal peptide, but the prediction of TargetP indicated that JcCu/Zn-SOD is localized in the chloroplast. In order to determine the localization, further research must be done, such as immunohistochemistry.

Expression and purification of JcCu/Zn-SOD

The 489-bp JcCu/Zn-SOD ORF was amplified from the *J. curcas* cDNA and subcloned into the expression vector pET-32a(+) which was introduced into *E. coli*. Expression of

the recombinant SOD protein was induced with IPTG, and total cellular proteins were analysed by SDS-PAGE and Coomassie blue staining (Fig. 3, lane 2). JcCu/Zn-SOD was expressed as soluble fusion protein with a 6 His-tag and a Trx-tag (Fig. 3, lane 3). Thus, the SOD protein could be purified by Ni-Sepharose HP column and Sephadex G25 desalting chromatography. The crude cell extract was first applied onto a Ni-Sepharose HP column (Fig. 4A), which effectively bound the His-tagged protein which was then eluted with imidazole (peak P2) and found to have SOD activity. The P2 sample was passed through Sephadex G25 to remove excessive imidazole and NaCl (pattern not shown), and was then treated with enterokinase for 36 – 48 h at 4 °C to remove the tags and liberate a protein containing the amino acid sequence of native JcCu/Zn-SOD. The digested sample was loaded onto the same Ni-Sepharose HP column, and the first eluted peak (P1) showing enzyme activity was collected (Fig. 4B). Approximately 3.88 mg of the recombinant protein was obtained from 4 g cells. A summary of the purification steps is shown in Table I.

Determination of molecular mass and isoelectric point

The purified JcCu/Zn-SOD gave a single band with an apparent mass of 16.4 kDa using SDS-PAGE in the presence of 2-ME (Fig. 3, lane 6), which was close to the value (16,393 Da) calculated for the apoprotein, *i.e.* SOD without

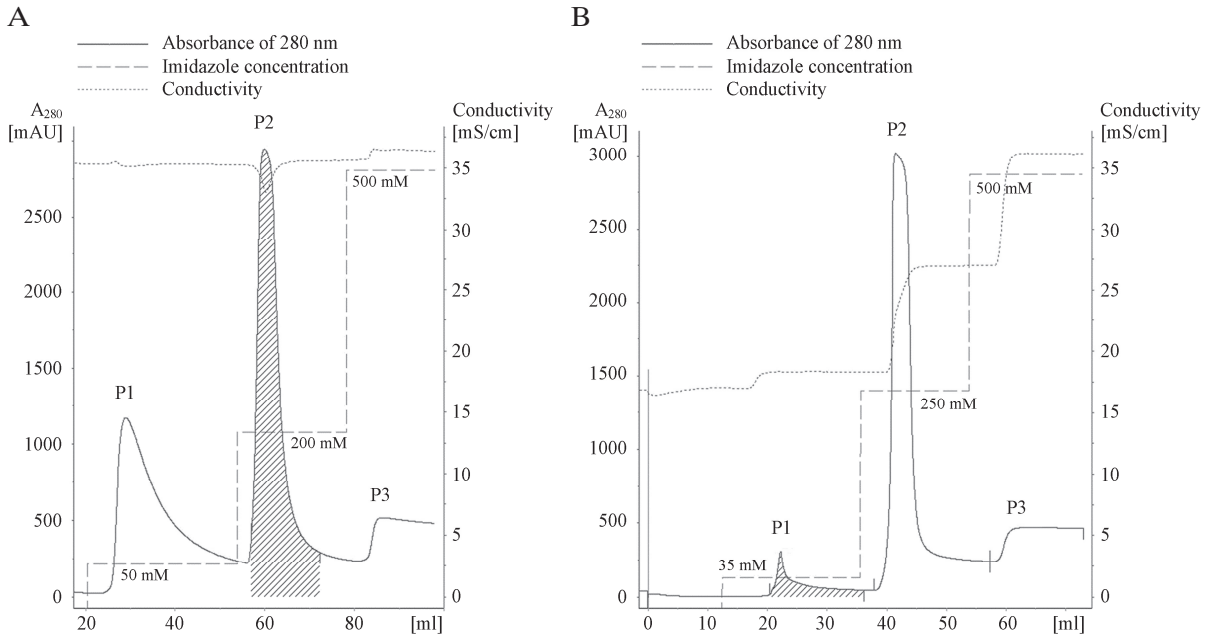

Fig. 4. Purification of JcCu/Zn-SOD by IMAC. (A) The column was equilibrated with buffer A and then was eluted with buffer A containing 50 and 200 mM imidazole, respectively. Only the peak labelled P2 contained the fusion protein and exhibited SOD activity. (B) The column was equilibrated with buffer B and then was eluted with buffers C and D, respectively. Only the peak labelled P1 contained the purified protein and exhibited SOD activity.

the metal ions, from the amino acid sequence. During gel filtration on a calibrated Superdex G75 column, the JcCu/Zn-SOD migrated as a single, nearly symmetrical peak of an apparent mass of 16.5 kDa (pattern not shown). MALDI-TOF MS of JcCu/Zn-SOD gave a major peak at *m/z* 16,385.4 (Fig. 5) and another smaller peak at *m/z* 16,454.0 in the irregular flank of the major peak. This is likely due to the heterogeneous status of the number of metal atoms bound to the protein (see below). The results of SDS-PAGE, gel filtration, and MS analysis indicated that the recombinant JcCu/Zn-SOD is a monomeric protein of 16,385.4 Da. This was different from other Cu/Zn-SODs. The molecular mass of many homodimeric plant Cu/Zn-SODs is in the range of 28 – 34 kDa (Haddad and Yuan, 2005; Madanala *et al.*, 2011). Recently, a few monomeric Cu/Zn-SODs have also been purified from *E. coli* (Battistoni *et al.*, 1996) as well as from the plant kingdom (Schinkel *et al.*, 2001; Sundaram *et al.*, 2009).

Plants have multiple genes coding Cu/Zn-SOD, and different Cu/Zn-SOD isoenzymes are specifically targeted to chloroplasts (Sundaram *et al.*, 2009), glyoxysomes (Bueno and Luis, 1992), peroxisomes (Bueno *et al.*, 1995), cytosol (Ogawa *et al.*, 1996), and the nucleus (Ogawa *et al.*, 1996). The presence of charge isomers in preparations of various eukaryotic Cu/Zn-SODs has also been frequently reported (Kajihara *et al.*, 1988; Liu *et al.*, 2002). For example, three charge isomers were discovered in *A. thaliana* (Kliebenstein *et al.*, 1998). Isoelectric focusing revealed four isoelectric variants in the recombinant JcCu/Zn-SOD, with p*I* values of 7.04, 7.33, 8.62, and 8.77 (Fig. 6). This was distinct from the p*I* of 7.22 calculated for the apoprotein. One possible explanation for the isoelectric variants is that the calculated value only considers the composition of amino acids, while the actual p*I* is related to protein conformation and post-translational modifications, especially binding of metals. In our SOD preparation, the metals bound to JcCu/Zn-SOD were less than 1.0 copper and 1.0 zinc atom equivalent per mol of apoprotein (data shown below), indicating that not all protein chains contained copper and/or zinc atoms. The different p*I* values of the four isoelectric variants were probably due to metal

Table I. Purification of the recombinant JcCu/Zn-SOD.

Step	Volume [ml]	Protein concentration [mg/ml]	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Yield (%)	Purification (fold)
Cell extract	320	1.79	574.16	7142.01	12.44	100	1.00
Affinity 1	15.5	8.69	134.75	6784.89	50.35	95	4.05
Enzyme treatment	25	5.31	132.70	5676.49	42.78	79.48	3.44
Affinity 2	16	1.67	26.68	4053.99	151.94	56.76	12.22

Specific activity, enzyme volume causing 50% inhibition of the reaction (one unit) expressed as enzyme units per mg protein. Total activity, total protein · specific activity. Data represent mean values ± SD (n = 3).

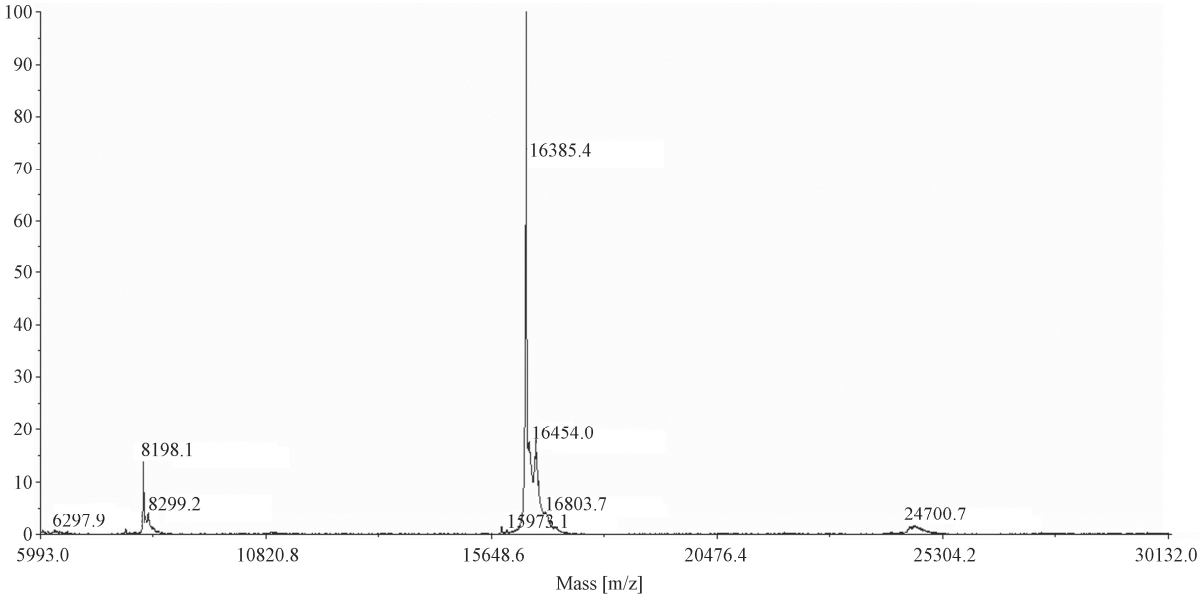

Fig. 5. MALDI-TOF MS of purified JcCu/Zn-SOD.

content variation or structural alterations around cysteine residues, as has been shown for human and avian Cu/Zn-SOD (Kajihara *et al.*, 1988; Liu *et al.*, 2002).

Determination of metal content

The metal content determination indicated that the sample contained 1.54 mg/l copper and 1.71 mg/l zinc atoms. According to the protein concentration of 1.67 mg/ml (Table I) and molecular weight of 16,385.4 Da, it could be calculated that 1 mol JcCu/Zn-SOD subunit contain 0.239 mol copper and 0.258 mol zinc atoms, respectively.

This result differed from most other Cu/Zn-SODs, which contain 2.0 copper and 2.0 zinc atoms per molecule (Bannister *et al.*, 1991; Yao *et al.*, 2007). Earlier results suggested that Cu atoms

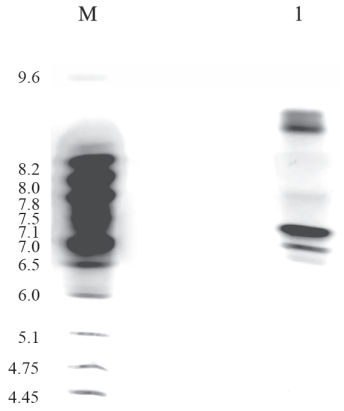

Fig. 6. Isoelectric focusing of *Jatropha curcas* SOD on 5% polyacrylamide gel using carrier ampholine in the pH range 4.0 – 10.0. Lane M, p*I* marker proteins; lane 1, purified JcCu/Zn-SOD.

are essential for SOD catalysis, while Zn atoms are wholly buried within the protein structure and are thought to play a role in protein stability. According to a generally accepted theory, the negative charge elsewhere on the protein surface reinforces the attraction by the positively charged channel around the copper atom, the catalyzed dismutation by Cu/Zn-SOD is believed to proceed by subsequent reduction and oxidation of a Cu ion acting as an electron carrier (Öztürk-Ürek and Tarhan, 2001; Sheng *et al.*, 2004; Branco *et al.*, 2005).

Thermostability, pH stability, and effects of various reagents on JcCu/Zn-SOD activity

When the enzyme was pre-incubated for 10 – 60 min at different temperatures from 30 – 70 °C, its subsequently determined activity decreased gradually within an hour to 74 – 79% at all temperatures up to 60 °C. However, at 70 °C, a significant loss of activity occurred, with approximately 34% activity remaining after 60 min (Fig. 7). Cu/Zn-SODs are generally known to have a rather high thermal stability. Hydrophobic regions of the protein are thought to play an important role in its thermal stability (Shaw and Bott, 1996). The present findings indicated that JcCu/Zn-SOD hads a relatively higher thermal stability than Cu/Zn-SOD from some other plants, such as garlic (He *et al.*, 2008) and *Radix lethospermi* (Haddad and Yuan, 2005). The activity of SOD was unaffected when the samples were incubated at various pH values from 4 to 9 for 1 h at 25 °C, suggesting this protein hads a broad pH tolerance. However, about 49% of the activity were lost when the pH was above 9, which was similar to the other reported Cu/Zn-SODs in plant species (Sheng *et al.*, 2004; He *et al.*, 2008). This pH tolerance range of JcCu/Zn-SOD was much broader than that of Cu/Zn-SOD from some other species (Madanala *et al.*, 2011). As shown in Fig. 8, urea, GdnHCl, and 2-ME had limited inhibitory effects on the activities of JcCu/Zn-SOD in this study. With increasing urea (8 M), GdnHCl (6 M) and 2-ME (up to 16 mM) concentrations, the activity of SOD decreased insignificantly compared to the control. The influence of various additives on the SOD activities indicated that JcCu/Zn-SOD may be inhibited gradually by imidazole, H_2O_2, and EDTA treatments, and the maximum remained activities were 69%, 57%, and 60% after incubation with 1 M imidazole, 16 mM H_2O_2, and 16 mM EDTA compared to the control, respectively.

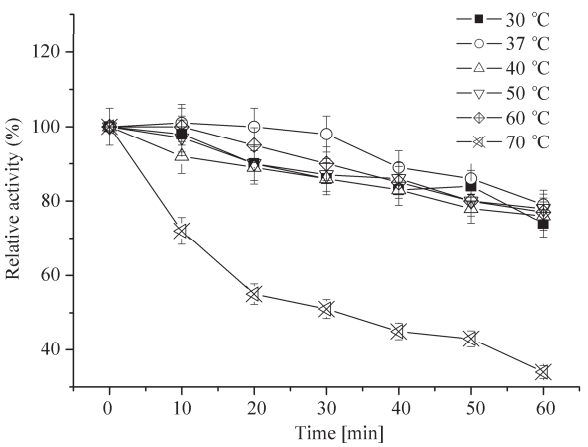

Fig. 7. Effect of temperature on the activity of JcCu/Zn-SOD. Data represent the average of three replicates. Full (100%) activity corresponded to 50 U of the purified enzyme.

In conclusion, we report the cloning, expression, purification, and characterization of a Cu/Zn-SOD from *J. curcas*. The recombinant enzyme appeared to be more stable than those from other plant species in a broad pH range, at higher temperature, and in the presence of denaturing agents and inhibitors. These properties provide a great potential for the commercial production and wide application as functional food supplement or as a therapeutic agent for treating inflammation. Investigation of the detailed structure and properties of JcCu/Zn-SOD would require fluorescence quenching studies, circular dichroism spectroscopy, and surface plasmon resonance analysis. The proposed purification procedure in this work has practical value for the large scale production of JcCu/Zn-SOD.

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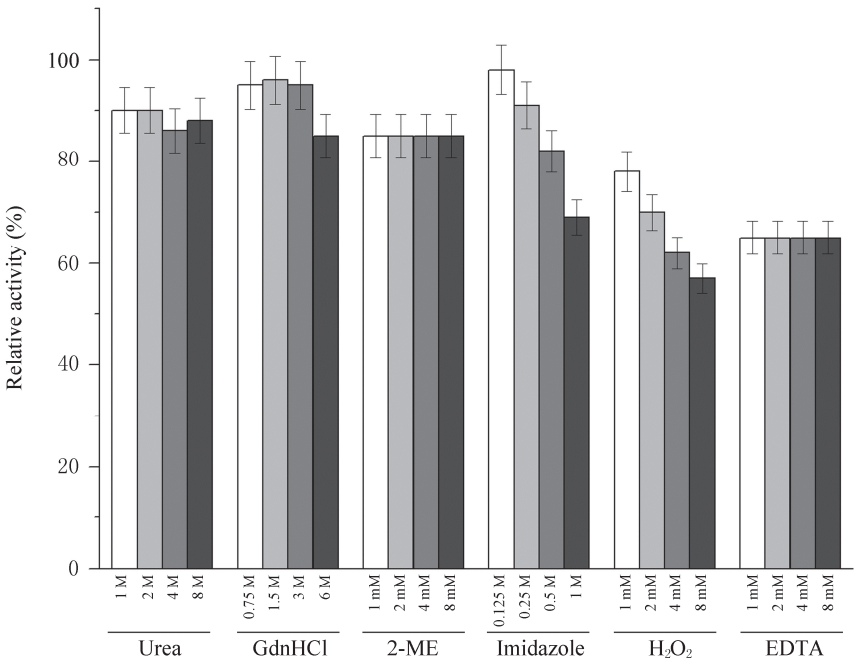

Fig. 8. Effects of denaturing reagents and additives on the activity of JcCu/Zn-SOD. Data represent the average of three replicates. Activity of the enzyme without treatment was set 100%.

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