Comprehensive characterization and molecular insights into the salt tolerance of a Cu, Zn-superoxide dismutase from an Indian Mangrove, Avicennia marina

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Superoxide dismutases are important group of antioxidant metallozyme and play important role in ROS homeostasis in salinity stress. The present study reports the biochemical properties of a salt-tolerant Cu, Zn-superoxide from Avicennia marina (Am_SOD). Am_SOD was purified from the leaf and identified by mass-spectrometry. Recombinant Am_SOD cDNA was bacterially expressed as a homodimeric protein. Enzyme kinetics revealed a high substrate affinity and specific activity of Am_SOD as compared to many earlier reported SODs. An electronic transition in 360–400 nm spectra of Am_SOD is indicative of Cu2+-binding. Am_SOD activity was potentially inhibited by diethyldithiocarbamate and H2O2, a characteristic of Cu, Zn-SOD. Am_SOD exhibited conformational and functional stability at high NaCl concentration as well in alkaline pH. Introgression of Am_SOD in E. coli conferred tolerance to oxidative stress under highly saline condition. Am_SOD was moderately thermostable and retained functional activity at ~ 60 °C. In-silico analyses revealed 5 solvent-accessible N-terminal residues of Am_SOD that were less hydrophobic than those at similar positions of non-halophilic SODs. Substituting these 5 residues with non-halophilic counterparts resulted in > 50% reduction in salt-tolerance of Am_SOD. This indicates a cumulative role of these residues in maintaining low surface hydrophobicity of Am_SOD and consequently high salt tolerance. The molecular information on antioxidant activity and salt-tolerance of Am_SOD may have potential application in biotechnology research. To our knowledge, this is the first report on salt-tolerant SOD from mangrove.

Oxidative stress in aerobic organisms refers to the production of oxygen byproducts and is triggered by various environmental factors. The most immediate response to these environmental stresses is the enhanced level of free radicals that include singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂), superoxide radical (O₂⁻), hydroxyl radical (OH), hydroperoxyl/perhydroxyl radical (HO₂), alkoxy radical (RO), peroxy radical (ROO⁻) and excited carbonyl (RO'). These reactive oxygen species (ROS) can lead to potential damage at cellular as well as genetic level leading to detrimental effects such as cell death and DNA mutation. In living cells, the antioxidant system is crucial for combating cellular oxidative stress. Superoxide dismutase (SOD; EC 1.15.1.1) is an essential component of this antioxidant system to provide first-line enzymatic defense by catalyzing the dismutation of superoxide radicals into O₂ and H₂O₂ at a diffusion-limited catalytic rate. Depending on the enzyme-bound metal co-factors, four different forms of SOD exist such as Cu, Zn-SOD and Fe-SOD (chloroplasts, cytosol, mitochondria, peroxyzome), Mn-SOD (mitochondria, peroxyzome), and Ni-SOD (prokaryotic cytosol). Such specific subcellular location of each isoform is thought to be important for compartmentalized redox signaling. Among all SOD enzymes, Cu, Zn-SOD is the most abundant type and is mostly localized in the cytosol, chloroplast, peroxyzome, and sometimes in extracellular spaces. Cu, Zn-SOD mostly exists in homo-dimeric form with non-covalently attached Cu and Zn ions in each subunit. While the zinc ion was found to be responsible for

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forming the enzyme assay as well as the impact of variation in environmental factors during sample collection. For each species, leaf tissues were collected from 6 different populations grown from each gram of leaf tissue of these 3 species is shown in Figure 1b in which of the SOD activity of the leaf. The comparative SOD activity in the crude extract (in unit per minute) prepared of Am_SOD was done. We also present here a deeper insight into salt-tolerant features of cal characterization of Am_SOD was done. The studies were a preliminary report that revealed a noticeably high halo-tolerance of this enzyme as well as some biochemical assays. However, a detailed understanding of the molecular basis of the high antioxidant activity of this enzyme in presence of high salt concentration is not yet available.

Results  
*Avicennia marina* showed the highest superoxide dismutase activity. The present study started with screening out the particular *Avicennia* species with maximum SOD activity. The free radical scavenging activity of 3 different species of *Avicennia* from the Indian mangrove forest (Figure 1a) was compared in terms of the SOD activity of the leaf. The comparative SOD activity in the crude extract (in unit per minute) prepared from each gram of leaf tissue of these 3 species is shown in Figure 1b in which *A. marina* displayed the highest activity among all the 3 species. For each species, leaf tissues were collected from 6 different populations grown at 6 different locations of the mangrove forest. The intra-species variation in SOD activity was very insignificant as evident from the standard deviation of the data that ruled out the possibilities of experimental error while performing the enzyme assay as well as the impact of variation in environmental factors during sample collection.

A 16 kDa protein of *A. marina* displayed SOD activity. Next, by employing a three-step purification strategy, the native SOD enzyme was purified from *A. marina* leaf into partial homogeneity. In step-1, proteins present in the pellet fraction after 60% ammonium sulfate cut were obtained and were subjected to strong anion exchange chromatography in step-2. Five eluted fractions obtained from step-2 (Figure 2a) were screened by SOD enzyme assay and fraction 5 displayed the highest SOD activity (Figure 2b). In the SDS-PAGE profile of this fraction, a 16 kDa protein was found to have been enriched along with some other proteins (Figure 2c). Hence, fraction 5 was re-fractionated in a gel filtration column in step-3. In gel-filtration, fraction 5 was separated into 3 sub-fractions (Fr 5A to 5C in Figure 2d). Fraction 5B was found to have the highest SOD activity (Figure 2e) and contain a 16 kDa protein with >90% purity in SDS-PAGE (Figure 2f). This protein band was excised from the gel, trypsin-digested, and analyzed by LC-MS/MS. As shown in supplementary Table S1, a total of 6 unique peptides were identified from this 16 kDa protein and all of them showed a significant match with a Cu, Zn-superoxide dismutase of *A. marina* in the UniProt database (Acc. no. Q9AXH2). Together, these 6 peptides account for about 48% sequence coverage to the intact protein. This identified protein is designated as Am_SOD throughout the entire study.

Recombinant Am_SOD was homodimeric. The mass-spectrometry deduced sequence of the purified protein with SOD activity was used to identify the gene from *A. marina* genome (NCBI genome ID: 16351)25. tBLASTn search with Am_SOD amino acid sequence revealed a 768 bp long mRNA transcript (GeneBank Acc. AF328859.1). The transcript was found to contain a 459 bp long ORF coding for the full-length Am_SOD protein. The full-length mRNA transcript and the ORF were separately PCR-amplified from the first-strand cDNA prepared from the total RNA of *A. marina* leaf. The full-length Am_SOD gene (with exons and introns) was also PCR amplified from the genomic DNA. The 768 bp long mRNA transcript and the full-length gene were separately cloned in the pCR<sup>T</sup>®2.1 vector by the TA-cloning method. The ORF was cloned in Nde1 and Xho1 sites of the pET22b+ vector under the control of the T7 promoter. Sequence analysis and comparison of these 3 cloned inserts revealed that the 2027 bp long gene consists of 7 exons with 6 introns. Upon splicing, a 768 bp long mRNA transcript is generated which consists of 459 bp long ORF with a 46 bp long 5' UTR and a 263 bp long 3' UTR. The domain architecture and nucleotide sequence of the full-length Am_SOD gene are shown in supplementary Fig. S1a, b. A positive clone with the Am_SOD cDNA insert positioned in an accurate reading frame (supplementary Fig. S2) was selected for recombinant expression. The expression of N-terminal 6xHis tagged recombinant Am_SOD was induced in *E. coli* cells with IPTG and the recombinant protein was found to be in a soluble form. The recombinant Am_SOD was then purified using Ni-NTA affinity column under native condition (Figure 3a) followed by the second round of purification in size exclusion column to remove undesired aggregates and non-specific *E. coli* proteins. The yield of recombinant Am_SOD was ~8–10 mg L<sup>-1</sup> of culture. The oligomerization status of the purified Am_SOD was checked in SDS-PAGE shown in Figure 3b. In non-

stabilizing the SOD dimer, the copper ion, via an alternate oxidation-reduction mechanism, helps this enzyme to catalyze a two-step superoxide dismutation reaction. Certain Cu, Zn-SODs were also found to exhibit unaltered catalytic activity even in the presence of ionic detergents, chaotropic agents, extreme pH, and high temperatures. Because of its cellular abundance, diverse organelar distribution, high kinetic stability, and oxidative stress-tolerance property, Cu, Zn-SOD has become a good candidate for biochemical studies and subsequent biotechnological applications. Some highly stable SOD enzymes have been reported from a wide range of extremophilic organisms like archaea, extremophilic bacteria, xerophytes, and halophytes. Halophytic adaptations are commonly found in mangrove plants which are continuously challenged with salinity stress. The ROS homeostasis of these mangroves is performed by robust antioxidant system including components such as SOD. Certain stress-combating enzymes from halophilic organisms have also been found functionally stable in highly saline microenvironments. Three major species under the genus *Avicennia* have been reported to be predominant constituents of Indian mangrove flora. Some studies have been performed to understand the mechanism of combating oxidative stress under highly saline conditions. A transgenic experiment was done in which introgression of Cu, Zn-SOD gene from *Avicennia marina* into rice resulted in enhanced salt tolerance. A recent study was performed on a Cu, Zn-SOD isolated from *Avicennia marina* growing in the mangrove forest of the Middle East coast. The study was a preliminary report that revealed a noticeably high halo-tolerance of this enzyme as well as some biochemical assays. However, a detailed understanding of the molecular basis of the high antioxidant activity of this enzyme in presence of high salt concentration is not yet available. In this communication, we report a full-length Cu, Zn-SOD enzyme (*Am_SOD*) isolated from the *Avicennia marina* of Indian Sundarban. A recombinant expression followed by a comprehensive biochemical, and biophysical characterization of Am_SOD was done. We also present here a deeper insight into salt-tolerant features of this enzyme at residue level through a rational mutagenesis approach.
reducing SDS-PAGE, *Am_SOD* appeared at ~33 kDa region, which corresponds to the MW of a dimer. However, in presence of β-mercaptoethanol, only the monomeric form was visible on the gel.

**Am_SOD displayed characteristic Cu, Zn-superoxide dismutase activity.** Sequence analysis of *Am_SOD* revealed the presence of a conserved SOD catalytic domain with multiple copper and zinc ion binding sites. The spectral pattern of *Am_SOD* in the visible region (300–800 nm) showed the evidence of electronic transitions at a region between 380–400 nm indicative of Cu²⁺ interaction with the imidazole ring of Histidine-62 (Figure 3c). Therefore, the enzymatic activity of the purified *Am_SOD* was studied by performing an assay using the riboflavin-NBT system. The principle of this assay is based on the fact that illumination of riboflavin generates free superoxide radicals which can convert NBT into blue-colored formazan which is spectrophotometrically measured at 560 nm. However, in presence of SOD, these radicals are scavenged and hence, the NBT conversion is reduced. Therefore, the high the A₅₆₀ value the less the amount of SOD enzyme present in the system and vice versa. As shown in Figure 3d, *Am_SOD* followed a typical Michaelis–Menten kinetics with an increasing concentration of riboflavin as substrate. The kinetic data were then plotted in a double reciprocal Lineweaver–Burk plot shown in Figure 3e. The *Vₘ₉₉₉* and *Kₘ* of recombinant *Am_SOD* were obtained to be 1557.14 unit/mg and 0.15738 µM respectively in 50 mM Tris buffer at pH 7.5. In addition to spectrophotometric assay, an in-gel activity assay was also performed in native PAGE shown in Figure 3f in which the *Am_SOD*...
appeared as a hyaline zone while the rest of the gel turned blue due to the oxidation of NBT. The enzymatic activity of Cu, Zn-SOD is specifically inhibited by diethyldithiocarbamate (DDC) and H$_2$O$_2$. In this study, the activity of Am$_SOD$ was specifically inhibited in a dose-dependent manner by sodium diethyldithiocarbamate trihydrate, and the IC$_{50}$ was obtained at 1.5 mM (Figure 3g). As compared to DDC, H$_2$O$_2$ was found to have a less inhibitory effect on Am$_SOD$ as the IC$_{50}$ value was obtained at 8 mM (Figure 3h). A similar pattern of Am$_SOD$ inhibition was observed in zymography assay performed with DDC and H$_2$O$_2$ (Figure 3i, j). The activity of Am$_SOD$ was fully inhibited by both inhibitors at high concentrations.

Am$_SOD$ showed halotolerance. Having a mangrove origin, Am$_SOD$ was expected to be a salt-tolerant protein. The salt tolerance was investigated and established through a couple of experiments. First, the tyrosine (Tyr)-fluorescence spectra of Am$_SOD$ were investigated at various NaCl concentrations (Figure 4a). In absence of salt, the wavelength of maximum emission for Am$_SOD$ was obtained at 310 nm, which is typical of a tyrosine residue. No significant change in the Tyr-fluorescence of Am$_SOD$ was observed in presence of NaCl at a concentration as high as 700 mM. Next, to understand further the salt-tolerant feature of Am$_SOD$ we performed the Tyr-fluorescence quenching experiment using acrylamide and potassium iodide (KI) as 2 complementary sets of water-soluble quenchers. Acrylamide is a neutral quencher and can enter the interior of a protein. On the other hand, iodide is a negatively charged and bulky quencher that can quench the fluorescence of the surface residues. Am$_SOD$ has no tryptophan residue but only a single tyrosine residue in its sequence. Therefore, in this study, the quenching data were analyzed by Stern–Volmer plot considering a homogenous emission from a single tyrosine. The quenching constant of this single tyrosine is reported here as effective Stern–Volmer constant (K$_{SV}$)$_{eff}$. The acrylamide and KI quenching data of Am$_SOD$ under control and high NaCl stress are represented in Figure 4b, c respectively. The values of (K$_{SV}$)$_{eff}$ and $f_q$ (quenchable fraction) are displayed in the tables adjacent to each corresponding plot. Considering the presence of only one tyrosine in Am$_SOD$, 100% quenching of fluorophore was observed in both experiments. Hence, this tyrosine residue is presumably located on the surface of Am$_SOD$. In acrylamide and KI quenching, insignificant change in the (K$_{SV}$)$_{eff}$ of Am$_SOD$ was observed both in absence of NaCl as well as in presence of 500 mM NaCl. The data indicated that there was a marginal conformational change in Am$_SOD$ in presence of a high concentration of NaCl as compared to no salt control. The conformational behavior of Am$_SOD$ in presence of NaCl was further investigated by Bis-ANS
fluorescence assay (Figure 4d) that exploits the surface hydrophobicity of a protein. Bis-ANS is a conformation-sensitive hydrophobic probe with a low quantum yield. However, it becomes highly fluorescent when binds to the hydrophobic pockets exposed on the protein surface. Unlike salt-sensitive proteins where hydrophobic pockets get buried under salt stress, AmSOD displayed a significant increase (~50%) in surface hydrophobicity. Next, the salt-induced aggregation pattern of AmSOD was studied by a single light scattering experiment shown in Figure 5a-i. A previously reported salt-sensitive and allergenic profilin Sola m 1 (a gift from Dr. Swati Gupta Bhattacharya of Bose Institute, Kolkata, India) isolated from eggplant26 was used as a control to compare fluorescence assay (Figure 4d) that exploits the surface hydrophobicity of a protein. Bis-ANS is a conformation-sensitive hydrophobic probe with a low quantum yield. However, it becomes highly fluorescent when binds to the hydrophobic pockets exposed on the protein surface. Unlike salt-sensitive proteins where hydrophobic pockets get buried under salt stress, AmSOD displayed a significant increase (~50%) in surface hydrophobicity. Next, the salt-induced aggregation pattern of AmSOD was studied by a single light scattering experiment shown in Figure 5a-i. A previously reported salt-sensitive and allergenic profilin Sola m 1 (a gift from Dr. Swati Gupta Bhattacharya of Bose Institute, Kolkata, India) isolated from eggplant26 was used as a control to compare
the results. *Am_SOD* did not show any aggregation even in the presence of 500 mM NaCl as evident from very insignificant/no increase in the absorbance at 360 nm. On the contrary, Sola m 1 started forming aggregates in the presence of 400 mM NaCl (Figure 5a-ii). All the above experiments are focused on studying the salt-induced conformational and structural changes in *Am_SOD*. In addition to these, the impact of salt concentration on the catalytic activity of *Am_SOD* was investigated as shown in Figure 5b. *Am_SOD* exhibited catalytic activity in presence of a wide range of NaCl concentrations. Maximum activity was observed at 25 mM NaCl and a further increase in salt concentration resulted in a gradual decrease in the specific activity. However, the enzymatic activity of *Am_SOD* was not drastically altered (<25% reduction) in presence of NaCl as high as 250 mM as compared to no salt control. Altogether, it was found that the biological function of *Am_SOD* was not considerably affected by high salt stress.

*Am_SOD* displayed enzyme activity at alkaline pH. The effect of pH on recombinant *Am_SOD* activity was studied using buffer systems of 4 different pH values. As illustrated in Figure 5c, the SOD activity was almost diminished at acidic pH of 3.6. However, the protein displayed enzyme activity in mildly acidic pH and the pH optimum was obtained at pH 7, which is a physiological pH. Interestingly, considerable retention of enzyme activity of *Am_SOD* was observed at a strongly alkaline pH of 10.

**Functional complementation of salt tolerant *Am_SOD* in *E. coli***. For functional identification of *Am_SOD* gene, a genetic complementation test was performed in a double SOD deficient mutant strain (ΔsodA and ΔsodB) of *E. coli* named QC774. The cells were transformed with *Am_SOD* cloned in pET22b+ vector. For control, QC774 cells and wild type *E. coli* K12 strain transformed with pET22b+ vector without any insert were used. Transformed cells were first selected on LB-agar plates containing ampicillin. An individual transformed colony was then streaked on LB-agar plate supplemented with ampicillin, methyl viologen dichloride for induc-
ing oxidative stress, and 500 mM NaCl for inducing salt stress. For untreated control, LB-agar plate was used without NaCl but with ampicilin and methyl viologen. Protein expression was induced by adding IPTG in all the

**Figure 5.** Impact of salt and pH on Am_SOD activity. (a) Plot of single light scattering experiment showing resistance of Am_SOD (i) to NaCl-induced aggregation. No significant increase in absorbance of salt-treated versus untreated Am_SOD at 360 nm (in y-axis) was observed over time (in x-axis). A salt-sensitive profilin protein, Sola m 1 from eggplant (ii) was used as control. (b) and (c) Plots showing specific activity (in y-axis) of 0.005 mg/ml of Am_SOD in presence of increasing concentrations of NaCl and 4 different pH values (in x-axes) respectively. 1.17 µM riboflavin was used as substrate in all reactions. Each data point is a mean of triplicate measure and SD as error bars. (d) Functional complementation of Am_SOD in E. coli on LB-agar plates with 500 mM NaCl (salt treated) and with 0 mM salt (untreated control). Both the plates were supplemented with methyl viologen to induce oxidative stress, IPTG for protein induction, and ampicilin for selection. Appearance of growth was observed for sod double mutant strain QC774 transformed with Am_SOD construct (area 1) on both the plates suggesting the ability of Am_SOD to remain functionally active for combating oxidative stress under high saline condition. K12 strain with functional native sod genes harboring pET22b+ vector (area 2) grew only in zero salt plate (under oxidative stress only). QC774 strain harboring pET22b+ vector (area 3) failed to grow under oxidative as well as salinity stress.
plates. As shown in Fig. 5d, only QC774 cells harboring Am_SOD constructs were able to grow under oxidative stress as well as salinity stress. On contrary, K12 cells with functional sod genes were able to survive only under the oxidative stress but couldn't grow in presence of high NaCl concentration. QC774 cells harboring empty vector were unable to survive under oxidative as well as salinity stress. This observation suggests the salt-tolerant feature of Am_SOD in addition to its potential role in combating oxidative stress.

Am_SOD displayed a certain degree of heat tolerance.  Deconvolution of CD spectra of Am_SOD (Figure 6a) at 25 °C revealed a correctly folded protein with predominantly β-sheets as evident from the minimum obtained at 215 nm. Also, a characteristic shoulder at 222 nm indicated the presence of a certain degree of α-helices. In step-wise thermal scanning, Am_SOD did not exhibit temperature-dependent denaturation since an inconspicuous change in the CD signal was observed at 90 °C as compared to what was observed at 25 °C (Figure 6b). A melting curve of Am_SOD shown in Figure 6c represents the ratio between α-helical fraction and β-sheeted fractions present in this protein at various temperatures. No significant decline in this melting curve of Am_SOD was observed when the temperature was gradually raised from 25 to 90 °C indicating no heat-induced conformational change in the protein. For comparison, a previously reported heat-sensitive pectate lyase Hel a 6 (a gift from Dr. Nandini Ghosh of Vidyasagar University, West Bengal, India) was used as a control. Hel a 6 protein was reported to show reversible heat denaturation27. Hence, the Hel a 6 melting curve exhibited a sharp decline with increasing temperature (AS or ascending scan) and the native folds were gradually lost. However, Hel a 6 partially refolded from a fully denatured state when the CD-scanning temperature was set back to 25 °C. To substantiate this observation, the effect of temperature on the catalytic activity of Am_SOD was investigated as shown in Figure 6d. Unlike the conformation-dependent melting curve
in Figure 6c, the enzymatic activity of Am_SOD remarkably declined at temperatures as high as 70 °C and 80 °C. However, Am_SOD was able to retain up to 70% of its catalytic activity at 60 °C. Reduction in surface hydrophobicity is linked to halotolerance of Am_SOD. A rational mutagenesis approach was undertaken to understand the role of a few selected residues in conferring salt tolerance to Am_SOD. Previous studies have shown that increased salt tolerance of a halophilic protein is associated with a noticeable increase in surface-exposed charge residues (negatively charged in particular) and reduction in surface hydrophobicity28–30. In this study, a comparison of Am_SOD with 3 non-halophilic Cu, Zn-SODs (Pa_SOD from Potentilla, Nt_SOD from tobacco, and Sl_SOD from tomato) by multiple sequence alignment (Figure 7a) revealed the presence of 8 less-hydrophobic residues in the N-terminus as compared to more hydrophobic residues on the corresponding positions of non-halophilic SODs. However, no significant change in surface-exposed charged residues was observed between Am_SOD and non-halophilic SODs. Hence, we anticipated the involvement of these 8 residues in the salt tolerance of Am_SOD. Out of 8, 5 residues were found to be sufficiently surface exposed on the tertiary structural model of Am_SOD (Figure 7b) and were estimated to have high SASA values as listed in supplementary Table S2. Residues of non-halophilic SODs corresponding to these 5 residues were also found to be solvent accessible. Each of these 5 residues on Am_SOD was found to have the lowest hydropathy index value (i.e. lowest hydrophobicity) as compared to the corresponding residues on 3 non-halophilic SODs (Figure 7c). Here, we decided to replace each of these 5 residues on Am_SOD with the residue having the highest hydropathy index value on the corresponding position among the 3 non-halophilic SODs. The strategy of amino acid substitution is illustrated in supplementary Table S2. In this way, 5 single-point mutants were generated by site-directed mutagenesis. A sixth mutant carrying all the 5 substitutions in the same protein was also generated by gene synthesis. The recombinant versions of all these 6 mutants were expressed in soluble forms and were found to remain in dimer as shown in non-reducing SDS-PAGE (data not shown). Now, the superoxide dismutase activity of these mutants was compared to that of the WT Am_SOD in gradually increasing NaCl concentrations. As shown in Fig 8A, the SOD activity of all the 6 mutants was nearly similar to that of the WT enzyme when assayed in presence of 25, and 100 mM NaCl. However, a significant reduction (p<0.05) in SOD activity of the 6 mutants was noticed when the NaCl concentration was increased up to 500 mM. Among the 6 mutants, the multiple-point mutant displayed maximum reduction (>50%) in SOD activity indicating a cumulative impact of these 5 substitutions on increasing the surface hydrophobicity and subsequently perturbing the halotolerance of the protein. In another experiment, the single light scattering pattern of the multiple-point mutant was compared with that of the WT Am_SOD under high salt stress. As shown in Fig-
ure 8b, the multiple-point mutant exhibited salt-induced aggregation in presence of 500 mM NaCl as compared to WT Am_SOD (Figure 8c) that remained considerably soluble.

Discussion
The present study presents a comprehensive characterization of a novel SOD enzyme isolated from a mangrove species of Indian origin using biochemical and biophysical methods. Mangroves are adapted to survive in high salinity environments. The generation of free radicals in the form of reactive oxygen species is a major manifestation of salt stress. To combat this challenge, mangroves are equipped with strong antioxidant systems that can function in a highly saline microenvironment. SOD enzymes are crucial members of the enzymatic antioxidant system. In this study, a high SOD activity of *A. marina* among 3 different *Avicennia* species was found to be associated with a 16 kDa protein designated as Am_SOD. The purity level and yield of natural Am_SOD protein purified from *A. marina* leaf were found to be considerably low. Hence, the full-length gene coding for this protein was isolated and purified in recombinant form. The analysis of the Am_SOD sequence revealed the presence of a conserved domain along with 6 conserved histidine residues responsible for metal ion (Cu$^{2+}$ and Zn$^{2+}$) binding which are characteristic of a Cu, Zn-SOD. Any organelle-specific putative signal peptide was not found in Am_SOD and its sequence showed homology mostly with cytosolic SOD enzymes. Interestingly, Am_SOD was sensitive to H$_2$O$_2$, a potent inhibitor of various Cu, Zn-SODs$^{31}$. Usually, peroxisomal SODs have been reported to have less sensitivity for H$_2$O$_2$ as observed in a recently characterized Cu, Zn-SODs from rice$^{32}$. Hence, the possibility of the peroxisomal location of Am_SOD can be ruled out. A further in situ localization study is warranted to confirm the subcellular location of this protein. Recombinant Am_SOD was found to
be a functional enzyme since it retained all the native folds as well as the catalytic activity. The kinetic data of Am_SOD represents a high substrate affinity and strong superoxide dismutation activity as compared to many previously reported Cu, Zn-SODs of eukaryotic origin. Such a robust activity of Am_SOD is thought to be the key for homeostasis of the exceptionally high level of ROS resulting from salinity stress and thereby protecting the cellular components from oxidative damage. To perform the biological activity, Am_SOD is thought to remain functional in a stressful microenvironment with extreme physiological conditions like high osmolarity and ionic strength. Here, we established the halotolerant feature of Am_SOD in terms of conformational stability and resistance to aggregation under high salt stress. The conformational stability of Am_SOD as observed in its tyrosine fluorescence quenching pattern was similar to the tryptophan fluorescence quenching reported for a halophytic rice protein PINO34. In Bis-ANS spectrofluorometric assay, a considerable increase in surface-exposed hydrophobic pockets in presence of high NaCl concentration was also noticed in another salt-tolerant protein DNA Pol-λ from Arabidopsis34. This structural stability of Am_SOD can be linked to the retention of its catalytic activity under highly saline conditions. The ability of Am_SOD to exert antioxidant activity under highly saline microenvironment was further confirmed by a functional complementation test where introgression of Am_SOD within a sod double mutant of E. coli conferred tolerance to salt as well as oxidative stress. In addition to salt tolerance, Am_SOD also displayed a certain degree of heat resistance. The CD spectra-based melting curve of Am_SOD indicates retention of >85% of its native structural folds at 95 °C. However, in temperature-dependent enzyme assay, Am_SOD exhibited a sharp decline in functional activity at 70 °C and onwards. This can be interpreted as even a small fraction of heat-induced conformational change has somehow perturbed the catalytic domain of Am_SOD. Thermostable SOD enzymes are predominantly found in peroxisomes. Assuming cytosolic origin, Am_SOD is probably an exceptional non-peroxisomal SOD that is resistant to heat denaturation. Am_SOD was also found to well tolerate the alkaline pH, which is not very common among the Cu, Zn-SODs. Hence, Am_SOD is less resistant to pH-induced conformational changes and metal–ligand leaching. Similar to halophilic Am_SOD, some SOD enzymes tolerant to alkaline pH were reported from marine organisms living in a saline environment35,36. Altogether, Am_SOD can be claimed as a stress-tolerant enzyme with strong free radical scavenging properties. The remarkably high salt tolerance of Am_SOD intrigued us to investigate its molecular basis at the residue level. For this purpose, a combinatorial approach consisting of comparative in silico sequence analysis with non-halophilic SODs followed by a mutational study was undertaken. The non-halophilic SODs were selected based on the availability of atomic details of their crystal structures. Many previous reports on extremophilic enzymes claimed that enhanced salt tolerance of a protein is linked to increased accumulation of negatively charged residues (such as aspartate and glutamate) and a decrease in hydrophobic residues on the surface of the protein28–30. Such a surface pattern is supposed to facilitate increased hydration even in presence of high salt in the protein microenvironment. Here, we reported the role of 5 residues located in the N-terminal portion of Am_SOD that are critical for its halotolerance. These residues are typically located outside of the conserved catalytic domain and were relatively less hydrophobic as compared to their non-halophilic counterparts. This observation was further experimentally corroborated by mutational analysis. It was also noted that not a single residue but the cumulative effect of all the 5 residue substitutions resulted in a drastic fall in salt tolerance.

Methods

Protein extraction. Avicennia sp. Leaves were collected from Lothian Wildlife Sanctuary of Sundarban, District South 24 pgs, West Bengal, India. About 100 mg of leaves were homogenized in 2 ml of 200 mM potassium phosphate buffer pH 7.8 containing 0.1 mM EDTA and 1% glycerol for 30 min at 4 °C. The supernatant was filter-sterilized in 0.2 µm membranes (Merck-Millipore).

SOD assay from leaf extract. 1 ml of each reaction mixture was composed of 2.4 µM riboflavin, 0.01 mM methionine, 0.167 mM NBT, 50 mM Na2CO3, and 200 µl of either leaf extract or buffer (enzyme blank). Samples were illuminated for 15 min and the absorbance was taken at 560 nm against corresponding non-illuminated samples (in dark) set as autozero. SOD activity was calculated as described in37.

Protein purification. 50 gm of Avicennia marina leaves were homogenized in extraction buffer (0.5 M Tris, pH 7.8; 250 mM NaCl; 50 mM KCl and 0.5% Arginine) at 1:5 (w/v) for 4 hour at 4°C. The clear extract was subjected to 60% ammonium sulfate precipitation. Pellet fraction was reconstituted in 25 mM Bis-Tris buffer pH 5.2 containing 0.13 M NaCl and 1% glycerol. Gel filtration was performed in Superdex S75 column (GE Lifesciences) equilibrated in the same buffer. Protein concentration in each fraction was estimated by Quick Start™ Bradford assay (BIO-RAD). Screening of the fractions was done by riboflavin-NBT based SOD assay and SDS-PAGE.

Mass spectrometry. The desired band was gel-excision, trypsin-digested as described in38 and cleaned up in ZipTipTM (Merck-Millipore). Peptides were subjected to RPLC-MS/MS analysis in a Xevo® G2-XS QT (Waters
from 300 to 800 nm at 25 °C in a double beam Hitachi U-2900 spectrophotometer (Japan). To 180 min at 25 °C.

NaCl (0–500 mM) and the absorbance at 360 nm was recorded in a UV-Vis spectrophotometer starting from 20 minutes to 180 min at 25 °C.

Riboflavin-NBT method as described in39. Each 200 µl reaction mixture consisting of 50 mM Tris-Cl pH 7.5, 9.9 mM L-Methionine, 0.57 µM NBT, 1 µg Am_SOD, 0.025% Triton-X, and serially increasing concentration of riboflavin (0–0.75 µM) was prepared. Enzyme blanks and non-illuminated sets were prepared for each riboflavin concentration. Absorbance was taken at 560 nm. The specific activity of Am_SOD for each riboflavin concentration was calculated by considering 1 unit of SOD enzyme equivalent to a 50% reduction in NBT conversion.

UV–Vis spectrophotometry. The absorbance spectra of 0.8 mg/ml of Am_SOD were taken at wavelength from 300 to 800 nm at 25 °C in a double beam Hitachi U-2900 spectrophotometer (Japan).

Enzyme kinetics. The specific activity and kinetic parameters (Vmax and Km) of recombinant Am_SOD were determined by the riboflavin-NBT method as described in39. Each 200 µl reaction mixture consisting of 50 mM Tris-Cl pH 7.5, 9.9 mM L-Methionine, 0.57 µM NBT, 1 µg Am_SOD, 0.025% Triton-X, and serially increasing concentration of riboflavin (0–0.75 µM) was prepared. Enzyme blanks and non-illuminated sets were prepared for each riboflavin concentration. Absorbance was taken at 560 nm. The specific activity of Am_SOD for each riboflavin concentration was calculated by considering 1 unit of SOD enzyme equivalent to a 50% reduction in NBT conversion.

SOD inhibition assay. The reaction mixtures were prepared as described in ‘Enzyme kinetics’ but with increasing concentrations of either sodium diethyldithiocarbamate trihydrate (0–3 mM) or H2O2 (0–10 mM) for 30 min. The riboflavin concentration was kept constant at 1.17 µM and specific activity was calculated.

Zymography. Purified Am_SOD protein was run in 10% non-reducing native PAGE. The gel was incubated in 1.26 mM NBT with gentle shaking for 20 min in dark followed by riboflavin buffer (10 mM potassium phosphate pH 8, 126 µl TEMED, and 34 µM riboflavin) with continuous illumination.

SOD assay under various physicochemical parameters. The reaction mixtures were prepared as described in ‘Enzyme kinetics’ but either with buffers of various pH values or various NaCl concentrations or various temperatures. In each assay condition, the rest of all the physicochemical parameters were kept constant except only the variable one. Comparative enzyme assay with NaCl-treated mutants was performed following the same method for WT Am_SOD. The riboflavin concentration was kept constant at 1.17 µM and specific activity was calculated.

Static light scattering. 0.5 mg/ml of Am_SOD or the mutant was mixed with various concentrations of NaCl (0–500 mM) and the absorbance at 360 nm was recorded in a UV-Vis spectrophotometer starting from 20 to 180 min at 25 °C.

Functional complementation test. Escherichia coli strain QC774 was transformed with either pET22b-Am_SOD construct or empty pET22b+. For control, WT E. coli K12 strain with functional sod genes was transformed with used. Cells were spread on LB-agar plates supplemented with 100 µg/ml ampicillin. An individual colony from each plate was streaked on LB-agar plate supplemented with 100 µg/ml ampicillin, 0.025 mM methyl viologen dichloride, 0.5 mM IPTG, and either 500 mM NaCl or without salt.

Circular dichroism spectrometry. CD spectra of 5 µM of either Am_SOD or Hel a 6 protein were recorded at 25 °C and 5 °C min-1 scan speed in Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). The raw CD data was converted in molar ellipticity in CAPITO server34. In a step-scan, the CD spectra were recorded by gradually increasing the system’s temperature from 20 to 90 °C at an interval of 10 °C. In a descending scan, the system was cooled down to 20 °C and the spectra were recorded once again.

Fluorescence spectroscopy. 0.05 mg/ml of Am_SOD in 25 mM Tris-Cl pH 7.8 containing 5% glycerol was separately incubated with 0–700 mM NaCl for 2 h. 2 ml of each sample was taken in a quartz cuvette (4 × 4 mm) and tyrosine auto-fluorescence was recorded in Hitachi F-7100 spectrophotometer (Japan). The excitation
wavelength was set at 276 nm and, the emission was scanned from 290 to 400 nm at 30 nm/min speed with 5 nm slit lengths. An average of 3 scans was taken and corrected for control buffer spectra. The maximum emission wavelength was determined by the instrument software with an in-built derivative analysis.

**Fluorescence quenching assay.** *Am* SOD (0.05 mg/ml) was incubated either with or without 500 mM NaCl for 3 h. Excitation was set at 276 nm. Emission of each sample was scanned at 310 nm, first without quencher, and then freshly prepared 5 M of either KI or acrylamide was added in 2 µl increment 10 times. After each addition, the solution was gently pipetted and left for 2 min to attain equilibrium. Quencher concentrations were corrected for ‘dilution effect’. Correction of ‘inner filter effect’ was done using Eq. (1).

\[
F_{\text{corr}} = \frac{F - \text{antilog}(A_{\text{ex}} + A_{\text{em}})}{2}
\]  

where \(F\) and \(F_{\text{corr}}\) represent the uncorrected and corrected fluorescence respectively. \(A_{\text{ex}}\) and \(A_{\text{em}}\) indicate the absorbance at excitation and emission wavelengths, respectively. The quenching data were analyzed according to the modified Stern–Volmer Eq. (2),

\[
\frac{F_0}{\Delta F} = \frac{1}{f_\alpha} + \frac{1}{f_\alpha} [Q]
\]  

where \(F\) is the difference between \(F_0\) (I304 without quencher) and \(F\) (I304 with quencher); \([Q]\) indicates molar concentration of quencher; \(f_\alpha\) is accessible fraction of Tyrosine; effective Stern–Volmer quenching constants \((K_{SV})\) are equal to \(f_\alpha\). \((K_{SV})\) values were obtained from the slope and intercept of the linear plot.

**Bis-ANS fluorescence assay.** NaCl treated or untreated *Am* SOD (0.02 mg/ml) was taken in a 3 ml quartz cuvette. A freshly prepared aqueous solution of 300 µM Bis-ANS was added in a 2 µl increment 10 times. After each addition, the solution was gently pipetted and left for 2 min to attain equilibrium. Emission and excitation were set at 490 nm and 390 nm respectively.

**Bioinformatics studies.** tBLASTn against NCBI nr and nBLAST against the *A. marina* genome were performed to identify the transcript and the full-length gene respectively. SOD sequences of *Potentilla atrosanguinea* (UniProt, B2CP37), *Solanium lycopersicum* (UniProt, Q43779), and *Nicotiana tabacum* (UniProt, A0A1S3ZTX1) were retrieved. Multiple sequence alignments were done in ClastalOmega server. Homology modeling of *Am* SOD was performed in SWISS-MODEL server using PDB:2Q2L as template followed by stereochemical quality checking in PROCHECK server. The hydrophathy index values of selected amino acids were recorded from. The SASA value of each residue was calculated in GETAREA server.

**Generation of mutants.** Mutant constructs in pET22b+ vector were generated by outsourcing from Bio-Bharati LifeScience Pvt. Ltd. (Kolkata, India) as illustrated in supplementary Table S2 and sequenced from Xcelris™ Genomics Labs Ltd., India. The mutant proteins were purified following the same method described for wild-type *Am* SOD.

**Statistical analysis.** Comparison of SOD activity was performed by students t-test in GraphPad prism software V6.1 and significance value was set as \(p < 0.05\).

**Ethical statement.** All the experimental research done on plants complied with the relevant institutional, national, and international guidelines. Leaf samples from the Lothian Island of Sunderban Biosphere Reserve were collected after obtaining official permission from the Directorate of Forest, Government of West Bengal vide approval letter no. 9(4)/SBR/C-227/17 (Part-II) dated on 09th April 2018. Only a single leaf was collected without destroying or uprooting the plant in presence of forest officials. Plants were identified by corresponding author Kashinath Bhattacharya in consultation with Botanical Survey of India. Voucher specimens were deposited (specimen no. VBH/2019/0012) at the herbarium of department of Botany of Visva- Bharati university.

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Author contributions
R.K.S., M.B., M.B.S., and G.S. performed the experiments. G.S., and K.N.B. analyzed and validated the data. All authors wrote the manuscript. No human samples, experimental animals, and cell lines were used in this study.

Competing interests
The authors declare no competing interests.

Additional information
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