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Radical scavenging property of a novel peptide derived from C-terminal SOD domain of superoxide dismutase enzyme in *Arthrospira platensis*

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A B S T R A C T

Superoxide dismutase (SOD) is an evolutionary conserved detoxification enzyme and powerful antioxidant which defends against the elevated ROS that are induced by various stresses. *Arthrospira platensis* (*Ap*) is known for its antioxidant-mediated immunostimulant properties, but there is no report on the SOD dependent antioxidant mechanism. Therefore, in this study, we have analysed the effect of H_2O_2 on growth and pigment composition in spirulina. Results showed that spirulina exposed to 10 mM H_2O_2 showed elevated growth pattern as well as increase in chlorophyll pigment composition especially during early days of exposure. Gene expression results showed that the expression profile of *Ap*SOD during oxidative stress stimulated by 10 mM H_2O_2 at different time intervals (0, 5, 10, 15 and 20 days) with highest expression on day 10 post-exposure. Together, the results confirmed the antioxidant role of *Ap*SOD in spirulina during oxidative stress induced by H_2O_2. Based on the amino acid arrangement and composition, we have predicted a short peptide ^{160}LGLDVWEHAYYL^{171} (LL12) from the catalytic centre of C-terminal SOD domain; further the peptide was synthesized. Antioxidant assays showed that LL12 peptide critically involved in radical scavenging mechanism. Also, LL12 peptide reduced the intracellular ROS level in H_2O_2 exposed leucocytes at a concentration of 12.5 μM. Cytotoxicity assay was performed on human leucocytes which showed that LL12 did not exhibit any cytotoxic activity against any of the leucocytes population. Overall, the study highlights the radical scavenging property of a novel short peptide derived from the C-terminal domain of *Ap*SOD which have the potential to develop as a biopharmaceutical drug.

1. Introduction

During energy production and other metabolic process in living organisms, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (^-OH) are generated as by-products due to oxygen metabolism. ROS, especially H_2O_2, disrupts the physiological balance in tissues by degrading the cellular components such as lipids, proteins and nucleic acids [1]. H_2O_2 is generated as by-products of various metabolic processes such as photosynthesis and respiration in microalgae and plants [2] which are mainly involved in key signalling pathways in development and stress responses. In freshwater and marine environments, H_2O_2 is commonly found at high concentrations such as 10 *pM* and it varies between 0.5 and 200 nM in sea water and up to 800 nM in freshwater lakes [3]. In freshwater ecosystem, high concentration of H_2O_2 can either harm or damage the cells of aquatic animals or prompt acclimation at moderate levels. H_2O_2 also acts as an intracellular precursor for more reactive oxidants as it moves rapidly across the membranes by diffusion [4]. Although, higher concentration of H_2O_2 is toxic to many aquatic organisms such as fish and shellfish; microalgae such as spirulina can withstand and grow as they exhibit unique antioxidant mechanisms to sustain and detoxify the elevated free-radicals and thus protect their cellular parts from uncontrolled oxidative damages [5]. The primary ROS scavenging enzymatic defense mechanism encompasses superoxide dismutase (SOD), glutathione peroxidase, (GPX), catalase (CAT) and peroxiredoxin (PrxR) [6].

SOD is one of the crucial antioxidant enzymes which primarily involves in the conversion of superoxide radicals (O_2^-) into O_2 and H_2O_2,

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thus protecting the cells and tissues from oxidative damage [7]. SODs are highly conserved metallo-enzymes which are categorised into four evolutionarily divergent groups based on their metal cofactor such as iron SOD (FeSOD), manganese SOD (MnSOD), nickel SOD (NiSOD), and copper/zinc SOD (Cu/Zn SOD). FeSODs are predominantly found in many bacteria, microalgae and plants [49]; NiSODs are found in microscopic organisms particularly in *Streptomyces* sp. [8] and MnSODs and Cu/ZnSODs are found in wide range of organisms especially in aquatic organisms [9,10]. SOD in microalgae plays a key role in protecting the cells from oxidative stress induced by various methods including chemical and biological stresses, heavy metals and radiations. Among all SODs, FeSOD is commonly found in majority of the microalgae and diatoms. Ismaiel et al. [11] reported that iron containing SOD may play a role in the survival of spirulina under stress conditions. FeSOD potentially responds to the H_2O_2 stress and protects cyanobacteria cells against O_2^- formed within the cytosol along with MnSOD which protects against O_2^- formed in the thylakoid lumen.

Arthrospira platensis (*A. platensis*) is mainly considered as a suitable immune-stimulant and natural antioxidant to humans and animals [12]. Spirulina is one of the well-studied microalgae with an extensive nutritive and therapeutic properties such as anti-cancer [13], anti-virus [14], anti-inflammatory [15,16], immune-stimulant [17], fatty-liver-preventing [18], neuroprotective [19] and cardio protectant [20]. Abd El-Baky et al. [4] reported that the antioxidant mechanism of *A. platensis* protected the cells up to 8 mM H_2O_2. So far, few antioxidant peptides with notable radical scavenging properties have been purified and derived from various sources such as plants, invertebrates, birds and higher vertebrates [21–24]. Recently, Jie et al. [25] reported that the hydrolysate of *A. platensis* comprises of unique antioxidant peptides, which might act as good source for therapeutics. Generally, the antioxidant activity of such short peptides is strongly influenced by their molecular mass, amino acid compositions, arrangement, structure and hydrophobicities [26]. These peptides involve in antioxidant reactions by acting as inhibitors of lipid peroxidation, chelators of transition metal ions or scavengers of free radicals by donating hydrogen. Thus, peptides acting as hydrogen donors serve as an excellent antioxidant peptide involving in multiple free radical scavenging mechanisms.

In this study, a superoxide dismutase (*ApSOD*) sequence was identified from the cDNA library of *A. platensis* developed by Illumina NextSeq500 technology. The identified full length cDNA sequence was reconfirmed by sequencing and BLAST alignment. Further, the physicochemical parameters of the cDNA sequence and its derived protein sequence were analysed. The effect of different concentrations of H_2O_2 on the growth of spirulina cells was evaluated and the modulation of *Ap*SOD transcripts during H_2O_2 stress in spirulina live culture was analysed using qRT-PCR analysis. To understand the antioxidant efficacy of the SOD domain region, a short peptide (LL12) was identified from the C-terminal region of Fe/Mn SOD based on the molecular mass, amino acid compositions, structure, sequences, hydrophobicities and evolutionary conserved region. The peptide (LL12) was synthesized chemically and its antioxidant mechanism was determined by various activity assays. In addition, the non-toxic nature of the peptide was confirmed by exposing human blood leucocytes to the peptides. Moreover, determination of intracellular ROS level was performed to confirm the radical scavenging activity of LL12 peptide to human blood leucocytes by flow cytometry.

2. Materials and methods

2.1. Spirulina cultivation

Water sample collected from Potheri Lake, Chennai (12.825527°N 80.039606°E) was microscopically examined for specific spirulina filaments with clear spiral morphology and diluted in sterile 6-well plates containing Zarrouk's medium until there is only one filament in the well. Further, the single cell was cultivated in modified Zarrouk's medium under 12:12 (day/night) illumination conditions in a specialized culture hood [27]. We obtained permission from National Biodiversity Authority of India for carrying out research experiments using the isolated spirulina cells (NBA/Tech Appl/9/742/14/16–17/450). The cyanobacteria isolate was identified as *A. platensis* by 16S rRNA sequencing and the sequence was submitted to NCBI database (Accession No. KY393096) [28].

2.2. H_2O_2 stress, algal cells collection and pigment analysis

The isolated spirulina cells were acclimatized for 30 days prior to experimentation. For each challenge, a set of 3 culture flasks were tested along with control. H_2O_2 (30% w/w in H_2O, Sigma-Aldrich) stress was given to algal culture at 10 mM concentration. Control flasks were maintained without H_2O_2 stress. Then algal cells were collected from challenged and control spirulina culture flasks at 5 various time points including days 0, 5, 10, 15 and 20 of post-challenge. To understand the post-exposure symptoms of different concentrations of H_2O_2 in spirulina cells, we have carried out the experiment for up to 20 days. Spirulina cells were collected from each Erlenmeyer flask from culture grown at different concentrations of H_2O_2 at different time points. The kinetics study of *A. platensis* growth was carried out in order to examine the ability of cellular growth of microalgae in every alternate day by measuring the optical density at 560 nm. Photosynthetic pigment chlorophyll *a* (Chl a), were quantified in *A. platensis* at different time points during H_2O_2 stress. Briefly, 0.5 g of the cells was grinded using 5 ml of 100% acetone (Fisher Scientific) for complete extraction of the pigments. Further, extracted pigments were centrifuged at 500 $\times g$ for 5 min and the supernatant was collected and the concentrations were determined using UV spectrophotometer (Shimadzu, Japan). Following formula was used to calculate the pigment concentrations [29]: Chl a = 11.24 $A_{661.6}$–2.04 $A_{644.8}$ (μg/ml). All the spirulina cells were flash frozen in liquid nitrogen and stored at −80 °C until further use.

2.3. RNA isolation, cDNA synthesis and gene expression analysis

The spirulina cells were collected from the culture medium, filtered through a glass microfiber filter-GF/C (Whatman, Kent, UK) and total RNA was isolated using TRIzol solution (Life Technologies, Rockville, MD, U.S.A.) as per manufacturer's protocol. The quantity and purity of the isolated RNA was analysed using NanoDrop (Thermo Scientific, USA). Finally, the purified RNA was converted to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's method with oligo (dT) as primer. The concentration of cDNA is unified as 100 ng in all the samples prior to analysis. qRT-PCR analysis was performed to quantify the transcripts of *Ap*SOD in normal cells and H_2O_2 treated cells, using Fast SYBR® Green Master Mix (Roche Diagnostics GmbH, Germany) in Light Cycler 96 Real Time PCR system (Roche). Relative quantification of *Ap*SOD gene was performed using 16S rRNA as internal control gene and the change in the folds of expression between control and treated cells were recorded. The primers designed previously by Kumaresan et al. [28] were used to amplify *Ap*SOD mRNA: *Ap*SOD F1, GGC TAA TAT GTC CCT GGA AGA G (Sense) and *Ap*SOD R2, AGA CTT GGG CAG CGT TAT T (Anti sense) and 16S rRNA (GenBank Accession No. KY393096): 16S rRNAF3, CGTAAA CCT CTC CTCAGT TCA G (Sense) and 16S rRNA R4, GAACGGATT CAC CGC AGT AT (Anti sense). Quantitative real time PCR assay was performed using the following cycles: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C for 60 s by means of a 20 μl qRT-PCR reaction mixture containing 2 μl cDNA, 10 μl Fast SYBR® Green Master Mix, 0.5 μl of each PCR forward/reverse primers and 7 μl nuclease-free water. To verify the purity of the amplification product, melting curve analysis was performed. Finally, the gene expression results were analysed by $2^{-\Delta\Delta CT}$ method using the Light Cycler 96 software (Version1.1.0.1320). The Cq values were recorded for each reaction and all the reactions were performed in

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