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Full Length Article

Amplification and Posttranslational Modification Study of Manganese Superoxide Dismutase Gene in *Sordaria fimicola*

Jawaria Rana[1], Rabia Arif[1*], Muhammad Ishfaq[1], Siu Fai Lee[2] and Muhammad Saleem[1]
[1]*Molecular Genetics Research Laboratory, Department of Botany, University of the Punjab, Lahore, 54590, Pakistan*
[2]*Department of Biological Sciences, Macquarie University and CSIRO Black Mountain Laboratories, Building 101, Clunies Ross Street, Black Mountain, ACT 2601, Australia*
[*]For correspondence: phdgenetics@gmail.com

Abstract

Manganese Superoxide dismutase (MnSOD) is an important enzyme which is present in all organisms like plants, animals, bacteria and fungi. It protects the cells from oxidative damage. In this manuscript, we have amplified gene encoding MnSOD enzyme from four strains of *Sordaria fimicola* collected from the Evolution Canyon-1. Point mutations on 264 (G) and 330 (T) in S1 and S2 strains were observed. Post translational modifications (PTMs) of MnSOD were predicted and compared with the reference sequences of *Neurospora crassa* and *Sordaria macrospora* by using different PTMs predictor servers. Phosphorylation and glycosylation in *S. fimicola* as well as *S. macrospora* and *N. crassa* was calculated on Serine (S), Tyrosine (Y) and Threonine (T) residues by NetPhos and YinOYang. No acetylation was predicted in *S. fimicola* however, it was observed on different lysine residues of *S. macrospora* and *N. crassa* by PAIL.

Keywords: MnSOD; Amplification; Mutations; Phosphorylation; Glycosylation

Introduction

Post-translational modifications of proteins regulate many natural processes side by side by activating, inactivating, or gaining function of the proteins and these modifications also modifies proteins by modulation of their molecular interactions, stability and localization (Jensen, 2004; Leech and Brown, 2012). Current improvements in mass spectrometry have facilitated to analyze detailed structure of covalent modifications of proteins and also have shed light on the post-translational modification of superoxide dismutase. PTMs are common in eukaryotes but rarely present in prokaryotes like bacteria (Hermann *et al*., 2000). There are several types of PTMs. Till now about 300 different PTMs have been described for biologically important proteins (Lee, 2013). It is reported in the UniProt Knowledge (UniProtKB) that up to 2012, about 72320 PTMs has been experimentally done. Among these, 49062 are phosphorylations, 5164 acetylation and 5736 gylcosylations (Silva *et al*., 2013).

Superoxide dismutases are ubiquitous family of enzymes that work to efficiently catalyze the dismutation of superoxide anions (Fridovich, 1995; Xu, 2006). Fungal SODs are involved in various biological processes including stress response, cell differentiation and infection (Aguirre *et al*., 2005). SODs are useful in medical treatments, and as beauty enhancing agent (Neuilly-sur-Seine *et al*., 1978), nutrients (Kumar *et al*., 2006), farming (Zhang *et al*., 2011) and chemical industries.

MnSOD has been found in the cytosolic fractions of prokaryotes and in the mitochondrial matrix of eukaryotes (Ravindranath and Fridovich, 1975). So far 74 MnSODs have been recognized from different classes of fungi (Bannister *et al*., 1987). MnSOD is considered a key scavenger of detrimental reactive oxygen metabolites in the matrix of mitochondria, like Copper-Zinc SOD in the cytosol (Keele *et al*., 1970). The MnSOD hunts superoxide radicals formed inside the mitochondria and protects it from the destructive effects of ROS (Holley *et al*., 2011). MnSODs provide protections to aerobic fungi against certain environmental factors (Longo *et al*., 1999). The mutants of *Candida albicans* and *Schizosaccharomyces pombe* are more sensitive for many stresses than parental strains when Mn-SOD gene has been removed from the cell (Jeong *et al*., 2001). MnSOD is vital for the survival of many fungi when they are in stationary phase, because at this time the transcription rate is very high in compound media (Rhie *et al*., 1999). In *C. albicans*, both mitochondrial (SOD2) and cytosolic MnSOD (SOD3) play a role during the stationary phase with their expression increased, while *SOD1* expression decreased (Lamarre *et al*., 2001).

S. fimicola is a coprophilous fungus (Lamb *et al*., 1998) and usually self-fertile, homothallic and haploid pyrenomycete in which about 8 spores are produced as a result of cell division (Chambers and De Wet, 1987). The natural habitats of *S. fimicola* and other two species have

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been mainly defined in dung of herbivorous animals (Dickinson *et al.*, 1981). The fungus has decomposition effect on wood and plant wastes (Alma *et al.*, 2000).

To the best of our knowledge, no study on post translational modifications of SOD in *S. fimicola* is available. Applications of the recently established proteomic predictor software have brought us findings regarding the new post-translational modifications of SODs in *S. fimicola*. This study was conducted to predict some covalent modifications of superoxide dismutase protein such as phosphorylation and glycosylation in *S. fimicola.* Nitration has been the most extensively analyzed modification both *in vitro* and *in vivo*. Regulation of SOD activity by the post-translational modifications could represent a new field of interest regarding the cell signal process. In this study, we have, for the first time amplified superoxide dismutase gene in *Sordaria fimicola* collected from contrasting environments and studied the impact of environmental stress on posttranslational modifications of this gene.

Materials and Methods

Organism

Stock cultures of all *S. fimicola* strains used in the present study were provided by Molecular Genetics Research Laboratory, Department of Botany, University of the Punjab, Lahore. These cultures were originally collected from south facing slope (SFS) and north facing slope (NFS) of Evolution Canyon, Israel. All the stock cultures were sub-cultured under sterile conditions and maintained on Potato Dextrose Agar (PDA) at 17°C.

Genomic DNA Extraction

By using modified Pietro method (Pietro *et al*., 1995), DNA of given strains was extracted and subjected to agarose gel electrophoresis using 1% agarose with ethidium bromide as staining dye and ladder of about 1 Kb. The gel was photographed under gel documentation system. After genomic DNA extraction, general PCR was used for amplification of DNA in order to study the variations among different strains of *S. fimicola*, using Clustal Omega online alignment tool available at https://www.ebi.ac.uk/Tools/msa/clustalo/.

General PCR

MnSOD gene was amplified using these primers (F 5-GTGGCCGAGATTGAAAAGAC-3 and R 3-CGGGATCCTGATCCTTAGTG-5).

Amplification of DNA was programmed at following conditions: Initial denaturation was maintained at 94°C for 5 min followed by 40 cycles of denaturation. Then denaturation at 94°C for 1 min leading to final elongation step at 72°C for 5 min.

PCR mixture of about 50 μL was used which contained $MgCl_2$, PCR buffer, DNTPs, DdH_2O, primers, taq Polymerase and template DNA.

For amplification of manganese superoxide dismutase gene in different strains of *S. fimicola*, primers were designed. Resultant nucleotide sequences were analyzed after sequencing by using Chromas software. This software determines the identity, similarity and differences of the sequence in data.

Clustal Omega was used for multiple sequence alignment. It has the ability to deal with very large no. of DNA, RNA and protein sequences.

Post Translational Modification Tools

Different bioinformatics tools were used for post translational modification which are: YinOYang 1.2 Server (available at: http://www.cbs.dtu.dk/services/YinOYang/), NetPhos 3.1 Server (available at http://www.cbs.dtu.dk/services/ NetPhos/), NetAcet 1.0 Server available at: http://www.cbs.dtu.dk/services/NetAcet/), PAIL http://pail.biocuckoo.organd NetNES 1.1 Server (available at: http://www.cbs.dtu.dk/services/NetNES/) to calculate YinOYang sites (the interplay between glycosylation and phosphorylation), phosphorylation sites, and nuclear export signals (NES), respectively. The amino acid sequences of amplified genes were obtained from online tool "EMBOSS Transeq" available at https://www.ebi.ac.uk/Tools/st/emboss_transeq/ while the amino acid sequences of all the reference strains, i.e. *N. crassa*, *S. macrospora*, and *S. fimicola*, were retrieved from Uniprot available at: http://www.uniprot.org/proteomes.

Results

Genomic DNA which was extracted from different strains of *S. fimicola* was subjected to amplification of manganese superoxide dismutase gene. A final product of about 435 bp nucleotides was obtained for this gene after sequencing of PCR amplicons of four strains. The sequences were subjected to BLAST tool at NCBI (https://www.Ncbi.nlm.nih.gov/BLAST) to check homologous sequences to those found for *S. fimicola*.

All the sequences were aligned by pairwise multiple sequence alignment using Clustal *O* to observe nucleotide variations between the strains (Fig. 1).

O-Glycosylation, Phosphorylation and Nuclear Export Signals

All possible O-glycosylation, phosphorylation and NES predicted sites for MnSOD enzyme are given in Table 1 and Fig. 2, 3 and 4

Acetylation on Leucine Residues

Acetylation occurred on Leucine residues of *N. crassa* and *S. macrospora* while no acetylation occurred in strains of *S. fimicola*.

Table 1: Prediction of PTMs on different residues of *N. crassa*, *S. macrospora* and different strains of *S. fimicola* by using different bioinformatic tools

Software used to Predict PTMs	*N. crassa*	*S. macrospora*	S1	N6
NetPhos	Phosphorylation			
Serine	5,6,13,14,42,67,114,140,156,165,167, 174,220, 240, Total= 14	6,33,80,91,113,122,131,140,142, 146, 152,153,162,208, Total= 14	6,9,47,80,98,129,185,Total = 7	6,9,47,80,98,129, Total= 6
Threonine	49,130	6,152,153	113,124,129	113,124,129
Tyrosine	40,73,125,156,185,186,225, Total= 7	14,22,39,48,188, Total= 5	58,63,108, 119, 120, Total= 5	58,63,108,119,120, Total= 5
YinOYang	Glycosylation			
Serine	14,20,83,84,124,167	56,97,140,142	23, 64,108	23,64, 109
Threonine	33,68,118,121	41,91,94,141,152,153	8,58,61,108,119, 120	8,58,61,108,119, 120
NetNes	Leucine Rich signal			
	176	19	93, 95	93,95
PAIL	Acetylation on Leucine			
	61,125,242	34,217	Nil	Nil

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S1    TTTGACGGCTTCAAAGAGGCCCTGGCTGCTGCGCTCTTGGGCATCCAGGGCAGTGGTTGG    300
S2    TTTGACGGCTTCAAAGAGGCCCTGGCTGCTGCGCTCTTGGGCATCCAGGGCAGTGGTTGG    300
N6    TTTGACGGCTTCAAAGAGGCCCTCGCTGCTGCGCTCTTGGGCATCCAGGGCAGTGGTTGG    300
N7    TTTGACGGCTTCAAAGAGGCCCTCGCTGCTGCGCTCTTGGGCATCCAGGGCAGTGGTTGG    300
      *********************** ************************************

S1    GGCTGGCTCGTCAAGGAGTTTACTTCCGAGTACAGCAGCAGGCTGCGCATCGTCACCACT    360
S2    GGCTGGCTCGTCAAGGAGTTTACTTCCGAGTACAGCAGCAGGCTGCGCATCGTCACCACT    360
N6    GGCTGGCTCGTCAAGGAGTTTACTTCCGAGAACAGCAGCAGGCTGCGCATCGTCACCACT    360
N7    GGCTGGCTCGTCAAGGAGTTTACTTCCGAGAACAGCAGCAGGCTGCGCATCGTCACCACT    360
      ****************************** *****************************

S1    GAGCATGCATACTAC    435
S2    GAGCATGCATACTAC    435
N6    GAGCATGCATACTAC    435
N7    GAGCATGCATACTAC    435
      ***************
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Fig. 1: Multiple sequence Alignments of different strains of *S. fimicola* for MnSOD enzyme. Highlighted region shows mutation on that point

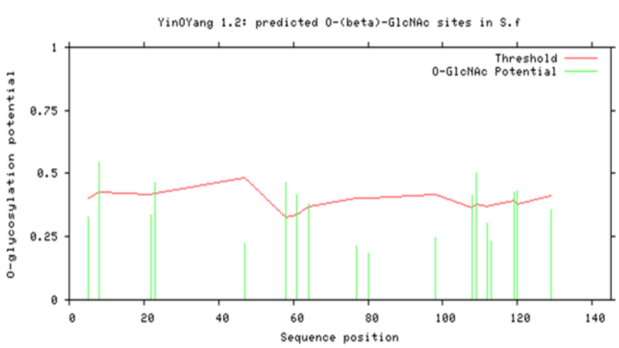

Fig. 2: Prediction of potential Glycosylation sites on Serine and Threonine residues of MnSOD protein in *S. fimicola* N6 using YinOYang 1.2 server

Discussion

Manganese superoxide dismutase is a nuclear-encoded and mitochondria-matrix localized oxidation-reduction (redox) enzyme that regulates cellular redox homeostasis. Sometimes, it is essential for proteins to obtain stable or transitory molecular structures for proper functions. In the current study MnSOD gene has been amplified in four strains of *S. fimicola* that were isolated from Evolution Canyon to measure genetic variations between the strains of two contrasting environments. The S2 and S3 strains under study were taken from the south slope of EC while N5 and N6 were isolated from the north slope of EC. Point mutations on two positions *i.e*., 264 (G) and 330 (T) in S1 and S2 strains was observed (Fig. 1) when compared with the reference sequence. No variation was observed in the strains that were isolated from the north slope of EC. This finding favors the hypothesis that more variations are expected in the strains that were isolated from the south slope of EC because the s-slope of EC has more UV lights, high solar radiations, temperature and pH (Saleem *et al*., 2001; Arif *et al*., 2017).

The next step after amplification was the prediction of PTMs in MnSOD protein in *S. fimicola* and comparison was made with the model fungi *N. crassa* and *S. macrospora*. Post-translational modifications (PTMs) mostly cause proteolytic cleavage or covalent modifications at particular amino acid. Proteolytic cleavage is permanent modification, whereas covalent modifications might be changeable, *e.g*., phosphorylation of proteins.

Phosphorylation in diverse subtypes of histones on projected Ser/Thr amino acids caused decondensation of chromatin network which is essential for the regulation of transcription and expression of certain genes, while the *O*-GlcNAc modification taking place on the similar Ser/Thr residues cause condensation of chromatin material as described by Butt *et al.* (2011).

We have predicted 14 phosphorylation sites on serine, 2 on threonine, and 7 on tyrosine residue on MnSOD protein of *N. crassa*, in case of *S. macrospora* 14 phosphorylation sites on serine, 3 on threonine and 5 on tyrosine while in case of strains S1 and N6 phosphorylation modifications were observed on different positions *i.e*., 7 phosphorylation modifications on serine, 3 on threonine and 5 on tyrosine residues out of 435 amino acid of MnSOD protein were predicted. By comparing the conserved region of this protein, it was found that phosphorylation on one amino acid, which is 6S, is conserved in *N. crassa*, *S. macrospora* and S1 and N6 strains of *S. fimicola*. Amongst these was the YinOYang prediction method, which predicted Yin Yang sites in proteins (sites, where *O*-glycosylation and phosphorylation may compete with each other). Moreover acetylation and methylation would also work together to regulate FOXO1 transcriptional activity. This study suggested that phosphorylation and acetylation deactivate FOXO1's transcriptional activity by disrupting binding between DNA and FOXO1, and promote its cytoplasmic localization and degradation of the FOXO1 transcription factor. Furthermore, glycosylation and methylation increased the DNA binding affinity and enhance nuclear accumulation of FOXO1 and promoted transcriptional activity. The interplay between phosphorylation and glycosylation regulated sub-cellular localization of FOXO1, affect processes such as apoptosis, gluconeogenesis and lipogenesis. Thus this *in silico*

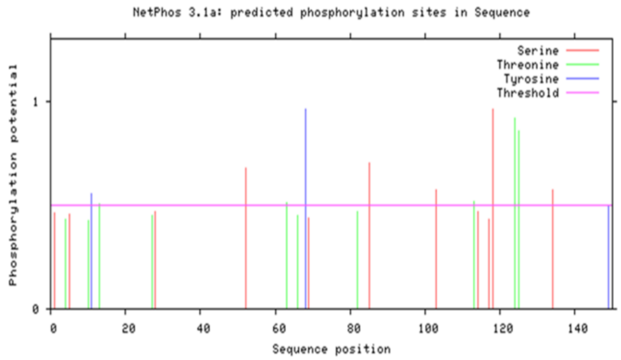

Fig. 3: Phosphorylation prediction sites on amino acid residues serine, tyrosine and threonine in *S. fimicola* in N6 strain

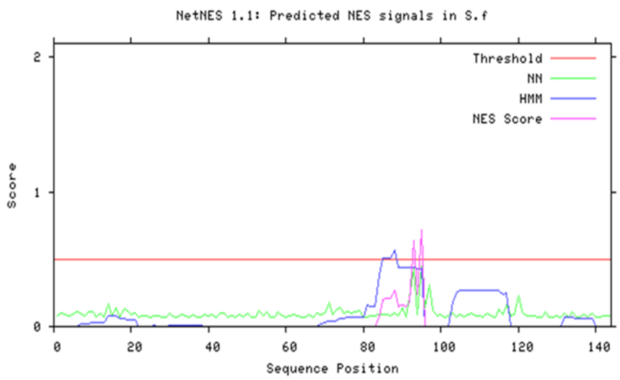

Fig. 4: Leucine rich nuclear export signals in *S. fimicola*

work suggested that different modifications played an important role in the regulation of FOXO1's transcriptional activity and its target genes. Acetylation occurred on K13, K41, K95 and K104 positions in different strains. Phosphorylation occurred on Y31, T57 and S1 (Ishfaq *et al*., 2016).

PTMs were common among all strains of *S. fimicola* except for the acetylation, which was predicted in N7 strain at K104 and K114 as described by Ishfaq *et al*. (2017). *S. fimicola* strains collected from diverse environment that were evaluated for their laccase enzyme activity while *Aspergillus niger* was used as control fungus. The post-translational modifications (PTMs) potential was predicted for laccase protein in *S. fimicola* by using different servers like LysAcet and PredMod for Acetylation, BPS for Methylation, DISPHOS and YinOYang for Phosphorylation and for Glycosylation NetNGlyc 1.0 and YinOYang were used. Molecular Evolutionary Genetics Analysis (MEGA 6.0.5) software was used for phylogenetic analysis (Tamura *et al*., 2013). The PTMs of the laccase proteins in *S. fimicola* strains from the opposite slopes of the EC 1 were compared with each other and these were found to be common among all the five strains of *S. fimicola* except for the acetylation by server PredMod.

The sod2 gene has 797-bp long ORF with three intronic regions and is projected to translate a polypeptide of 208 amino acids (Zelko *et al.*, 2002). Transcription of the sod2 gene in *C. graminicola* was strictly related with formation of semicircular conidia. SOD_2 is up-regulated in response to oxidative stress generated as part of the signaling pathway that regulates conidiogenesis.

Conclusion

In conclusion, superoxide dismutase enzyme is ubiquitous metallo-enzyme important for the existence of all aerobic organisms because it catalyzes the dismutation of the highly reactive superoxide radical anion O^{-} to O_2 and $H_2O_2$and therefore this study would be an important contribution to the existing knowledge about life's responses towards environmental stress.

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