WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Purification And Molecular Characterization Of Superoxide Dismutase From An Extreme Halophilic Strain Halomonas Utahensis SM1by Electrospray Ionization Mass Spectrometry

S.Murugan[1], Neyyoor[2], T.Subha[3]

[1] Assistant professor, Department of Biochemistry, Lakshmipuram college of Arts and Science,

[2] Kanyakumari district Tamil Nadu; E-mail:muruganlpc@gmail.com

[3] Assistant professor, Department of Allied Health Sciences, Noorul Islam Center for Higher Education,Kumarakoil ,Kanyakumari district, Tamil Nadu E-mail: subhabio86@gmail.com.

Corresponding Author: S. Murugan, E-mail: muruganlpc@gmail.com

ABSTRACT :

Superoxide dismutase from culture of Halomonas utahensis SM1, isolated from Puthalam salt pan located in the southern most peninsular coast of India, was purified by gel filtration chromatography. The apparent molecular mass of the enzyme was 42.8 KDa and was shown to be a dimer with similar subunits of molecular mass 21.4 KDa. MOLDI-TOF MS analysis of isolated bands (Band Id-1 and 2) Found to contain 265 and 23 ions respectively. PSMs analysis indicates that the found in fragment 1 were repeated 502 times within the sequence and was matched with 11 isoenzymes of superoxide dismutase. Peptides of fragment 2 were repeated 1059 times and were paired with 11 isoenzymes of superoxide dismutase compared with the superoxide dismutase of Pseudomonas aeroginosa VRFPO1.

Keywords: Superoxide dismutase, Isoenzyme, *Halomonas utahensis.*

1. INTRODUCTION :

Superoxide dismutases (SODs; EC 1.15.1.1.) are a class of metal proteins, catalyzing the dismutation of the superoxide radicals (O_2^-) to oxygen and hydrogen peroxide. In the recent years, due to the big practical interest in the microbial SODs the attention was focused on the thermostable ones produced by thermophilic microorganisms. The SOD of the anaerobe bacterial hyperthermophile *Aquifex pyrophilus* was with half-life 175 min at 95°C [Lim *et al*., 2001]. The most thermostable SOD, described recently in the literature was of archaebacterial origin. A strain of *Sulfolobus sulfataricus* produced Fe-Mn SOD with half-life of 2 hours at 100°C [Russo *et al*., 1997]. The SOD metalloenzymes can be separated into three classes based on the metal cofactors at their active sites: copper/zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD), and iron SOD (FeSOD) [Fridovich, 1995]. Recently, SODs have been used in gene therapy and therapeutic treatments for oxidative damage in the treatment of postischemic reperfusion injury, rheumatoid arthritis and osteoarthritis, brain trauma, influenza-induced lung pneumonitis, breast cancer, nervous system dysfunction, persistent pulmonary hypertension, and tissue damage. SODs are considered to be clinically useful for a wide variety of applications, including the prevention of oncogenesis, tumor promotion, and tumor invasiveness, and the reduction of the cytotoxic and cardiotoxic effects of anticancer drugs [Angelova *et al*.,2001, Farrow et al.,2008, Laurila *et al.,* 2009, Teoh *et al*.,2009,Zemlyak *et al.,* 2006]. A SOD biosensor has also been used to determine the antioxidant properties of acetylsalicylic-acid-based drugs and the antiradical activity of healthy and cancerous human brain tissues [Emregul, 2005]. Today, thermostable enzymes play very important role in industry because of their stability. Among these, thermostable SODs from thermotolerant or thermophilic microorganisms have received increasing attention [Li *et al*., 2005].

Protein identification by mass spectrometry (MS) can be performed using sequence-specific peptide fragmentation or peptide mass fingerprinting (PMF), also known as peptide mass mapping [Aebersold R and Mann, 2003]. The standard approach to identify proteins includes separation of proteins by gel electrophoresis or liquid chromatography. Mass spectrometry (MS) has been used for the analysis of proteins and peptides since 1989, when two new “soft” techniques for gas phase ionization of large, polar and highly charged molecules were introduced [Fenn *et al.*,1989].Different analyzers can be coupled with various sources, so that the number of possible instrumental configurations is large. In particular, two different mass spectrometer configurations were used to produce most of the proteomic data published thus far: ion traps coupled to ESI sources (ESI-IT) and TOFs coupled to MALDI sources (MALDI-TOF) [Schwartz *et al*., 2002]. Electrospray ionization mass spectrometry (ESI-MS) has become an important tool for the characterization of macromolecules in recent years. ESI-MS allows the ionization of various macromolecules with little or no fragmentation, enabling accurate molar mass determination by making the unfragmented structure amenable to mass separation. Combining ESI-MS to high resolution mass analyzers such as quadrupole-time-off light (Q-TOF) instrument provides exact molar masses for the polymers analyzed with mass accuracies in the

ppm region. Therefore, valuable information on the chemical constitution of the macromolecule can be derived. The interfacing of this soft ionization method with collision induced dissociation (CID) presents a powerful technique for the detailed structural characterization of polymers [Fenn, 2003]. Considering the above, in the present study, an attempt was made to identify the peptide sequence of SOD produced by *Halomona sutahensis SM1* strain by ESI-Q-TOF- MS.

2. Materials and Methods :

2.1. Collection of samples

The study site was Puthalam saltpan of Kanyakumari district.Water samples were collected from the saltpan at 3 different sites. The collected samples were transferred to sterile polythene bags to prevent direct contact with air and were transported to the laboratory in an ice box for further examination.

2.2. Screening of SOD activity

Culture of *Halomonas utahensis* SM1 was inoculated in tubes containing marine broth supplemented with 2% pyrogallol and incubated the tubes in an incubator at 37˚C for 12 days. Supernatant was obtained from the early stationary phase culture by centrifuging the culture broth at 3000 rpm for 10 minutes. The supernatant obtained was used as enzyme source. 2% pyrogallol in 0.05MTrisHCl buffer at pH 7.5 acts as the substrate. The SOD activity was assayed by using Marklund and Marklund method [Marklund and Schinner, 1974].

2.3. Media for SOD production

A synthetic medium used for increasing the production of SOD was prepared by adding 29% NaCl, 5% fructose, 9 % glucose, 5% sucrose, 6% galactose, 4% maltose, 4% lactose, starch 10% and 1.5 % of cysteine, 1% histidine, 0.8% arginine, 0.8% glycine and 2% tryptophan and tyrosine 5% and 20% $MgSO_4.7H_2O$, 15% $MnSO_4$, 10% $MgCl_2.6H_2O$ and 2% pyrogallol along with marine broth. The pH was adjusted to 8.8 by using Na_2CO_3. The ingredients were dissolved in 100ml of distilled water, which serve as a medium for SOD. The pH was adjusted to 8.8 using Na_2CO_3.

2.4. Preparation of enzyme source

A loop full of culture was inoculated into SOD nutrient broth medium. The inoculum prepared was then placed in a shaking incubator at 42˚C for 12 days. The broth culture was taken and centrifuged at 3000 rpm for 10minutes which was used as enzyme source for SOD. SOD activity was assayed by Marklund and Marklund method [Marklund and Schinner, 1974].

2.5. Estimation of protein

The protein concentration of the enzyme was estimated by Lowry *et al*., [Lowry *et al.*, 1951].

2.6. Purification

2.6.1. Precipitation by solvent

The cell free fermented media was collected and subjected to different steps of purification. The enzyme protein was partially purified using ice cold ethanol precipitation method [Sharma *et al.*,2000]. Solvent was added to 100 ml of culture filtrate to get 70% saturation and most proteins were precipitated out. The precipitation yield was increased by adding four volumes of ethanol per volume of sample [Petkarmedha *et al.*, 2013].

2.6.2. Desalting of protein

The precipitated protein was dissolved in 10mMTrisHCl buffer and the solution was taken in dialysis bag. The dialysis process was taken at 4ºC.

2.6.3. Purification by chromatography

All the purification steps were carried out at 4˚C or on ice. The dialyzed protein sample was loaded on SephadexG150column (7×1) of hydroxylapatite purchased from sigma (0.75 by 79cm), equilibrated with 4MNaCl in 10mM sodium phosphate (pH 7.0), 10 ml of 4MNaCl in 250mM sodium phosphate (pH 7.0) and 10 ml of 2MNaCl in 10mM sodium phosphate (pH 7.0). SOD was eluted by adding 2MNaCl-300mM sodium phosphate (pH 7.2). Fractions were collected throughout with a flow rate of 15ml/hour [Bardford, 1976]. The elute was then dialyzed against 4MNaCl-50mmTris hydrochloride (pH 7.2), concentrated to 1.5 ml by dialysis against solid polyethylene glycol 8000, chromatographed on Sephadex G-150 column (95 × 1.6 cm) in 4MNaCl-50mMTris hydrochloride (pH 7.2). Elution buffer routinely contained 0.5mMNaCl because of the salt requirement of the enzyme. Enzyme sensitivity was lost when using high concentration of salt during crude enzyme preparations. This is due to the presence of respiratory components in crude extracts which yield superoxide and whose activity is induced by high concentration of salt.

2.7. Sample preparation for SDS-PAGE analysis

The extracted protein samples were subjected to one dimensional SDS-PAGE analysis according to Laemmli [Laemmeli, 1970], by diluting the sample in 1X loading or cracking buffer containing 0.08MTrisHCl, pH-6.8, 2.7% SDS, 13.7% glycerol, 0.97M β-mercaptoethanol and 0.3% bromophenol blue, and kept at 100˚C for 3 minutes before loading.

2.7.1. SDS-PAGE Electrophoresis

Proteins were resolved by SDS PAGE as described by Ausubel and his group [Ausubel and Frederick, 1989]. 0.75 mm thick gels were cast in a gel apparatus using separating and stacking gels. The separating gel mixture was poured into a glass plate cassette assembly, overlaid with water saturated with isobutanol (1:1) and allowed to polymerize for 2 hours at room temperature. After polymerization water saturated isobutanol layer was removed and the separating gel was overlaid with 0.1% SDS and stored over night at 4˚C. After removing 0.1% SDS, the top of the gel was rinsed with water and the stacking gel was then poured on the top of the separating gel. Comb was then inserted into the stacking gel giving 1cm space between the bottom of the well and the top of separating gel. Then the stacking gel was allowed to polymerize for 45 minutes at room temperature. The comb was removed carefully and the wells were cleaned by rinsing with electrophoresis buffer. Protein samples were loaded on to the wells. 1X electrophoresis buffer was then added in the upper and lower chamber of the gel casting unit and the gel was run at a constant voltage of 50 volt until the proteins reach the separating gel. After that the voltage was raised to 150 volt.

2.8. Protein identification and mass spectroscopy

Protein bands were further subjected to Nano LC-MS/MS analysis for identification. Zip tip purified peptides were analyzed using Nano- RPLC (Thermoscientific, USA) coupled with an Orbitrap Elite Mass spectrometer (Thermoscientific, USA). The peptide mixtures were dissolved in 2 % CAN with 0.1% formic acid and loaded onto a guard column. Purified peptides were released into a C18 capillary column (100µM×10cm) and separated using a linear gradient solvent system (5 to 100% CAN) for 80 minutes at a flow rate of 300nl/minute. Peptides were ionized by positive mode electro spray with an ion range M/Z of 350 to 4000 Dal using X caliber software.

2.9. Gene ontology by DAVID software and Protein interaction by STRING

Analysis of functional enrichment of differentially regulated proteins was performed in DAVID 6.7 software [Dennis *et al*., 2003] http://david.abcc.ncifcrf.gov /according to standard protocol [Huang *et al.*, 2009]. The combined list of official gene symbols corresponding to the identified proteins was used for input.

3. RESULTS :

3.1. Enzyme production in optimized medium

The SOD activity was determined from control and optimized medium. The results were recorded and tabulated in table 1.

Table1: Activity of SOD in optimized medium

Particulars	Enzyme activity (U/ml)
Control	3.96
Optimized medium	12.98

The results indicate that the yield of SOD in optimized medium was found to be more (12.98 U/ml) than the control (3.96 U/ml).

3.2. Extraction and purification of SOD

SOD from isolated strain was extracted by centrifugation and concentrated by ice cold ethanol solvent followed by dialysis and Gel filtration chromatography, according to [May and Dennis,1987]. Number of fractions collected, enzyme activity, protein content and specific activity of SOD are calculated (Table 2).

Table 2: Activity of SOD in ice cold ethanol solvent fraction

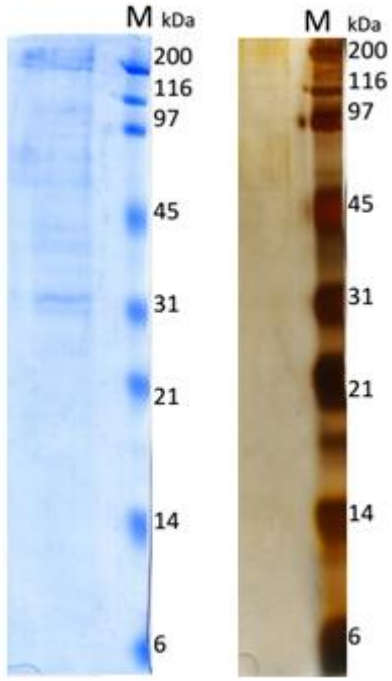
Solvent fraction	Enzyme Activity (U/ml)	Protein Content (mg/ml)	Specific activity (Units/mg)
I	35	6.3	5.55
II	42	7.0	6
III	68	7.8	8.71
IV	30	5.4	5.55
V	40	5.5	7.27

Table 3: Purification of SOD

Sample	Enzyme Activity (U/ml)	Protein Content (mg/ml)	Specific Activity (U/mg of Protein)
Culture Supernatant	39	5.27	7.4
Crude fraction	156	10	15.6
Sephadex G-150	230	0.6	383.333

The activity of SOD was found to be 39 U/ml and the specific activity was 7.4 U/mg of protein in culture supernatant, 156 U/ml and the specific activity was 15.6 U/mg of protein in crude fraction and 230 U/ml and the specific activity was 383.33 U/mg of protein in Sephadex G-150 purified sample as shown in table 3.

3.3. SDS-PAGE analysis of SOD



a **b**

Figure 1 a&b: Shows SDS-PAGE analysis of SOD

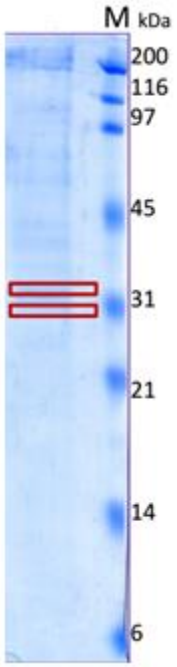


Figure 2: SDS-PAGE analysis of SOD indicating bands selected for Mass spectrum analysis

Table 4: Mass spectrum analysis of Chromatogram view Band ID-1 and 2

Mass Spec Analysis

Accession	Description	Score	Coverage	# proteins	# unique peptides	# Peptides	# PSMs	# AAs	MW [kDa]	Calc.pI
A0A086BWN4	Superoxide dismutase OS = *Pseudomonas aeruginosa* VRFPA01 GN = G039_0320565 PE = 3 SV = 1-[A0A086BWN4 PSEAI]	762.71	41.97	11	7	8	502	193	21.4	5.40

A0A086BWN4	Superoxide dismutase OS = *Pseudomonas aeruginosa* VRFPA01 GN = G039_0320565 PE = 3 SV = 1-[A0A086BWN4 PSEAI]	1566.47	41.97	11	7	8	1059	193	21.4	5.48

Mass Spectrum Analysis

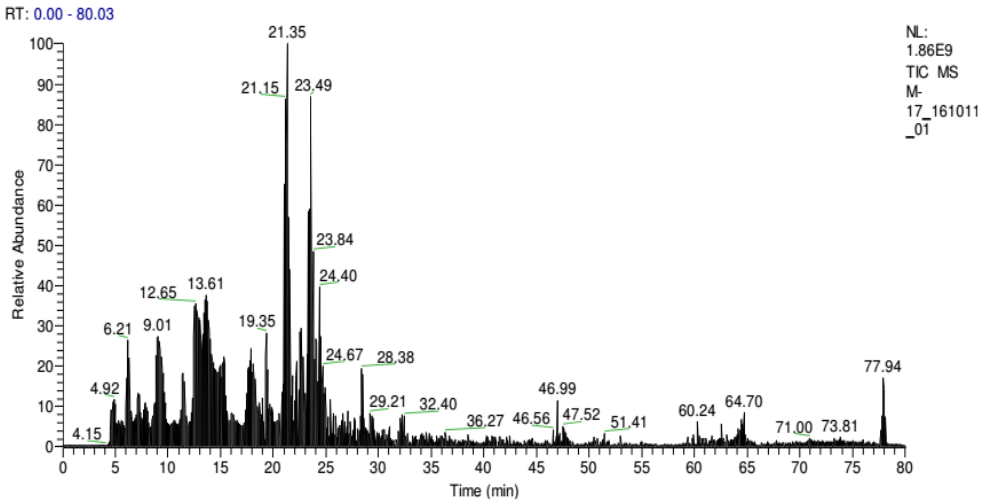


Figure 3: Chromatogram view – Band ID1

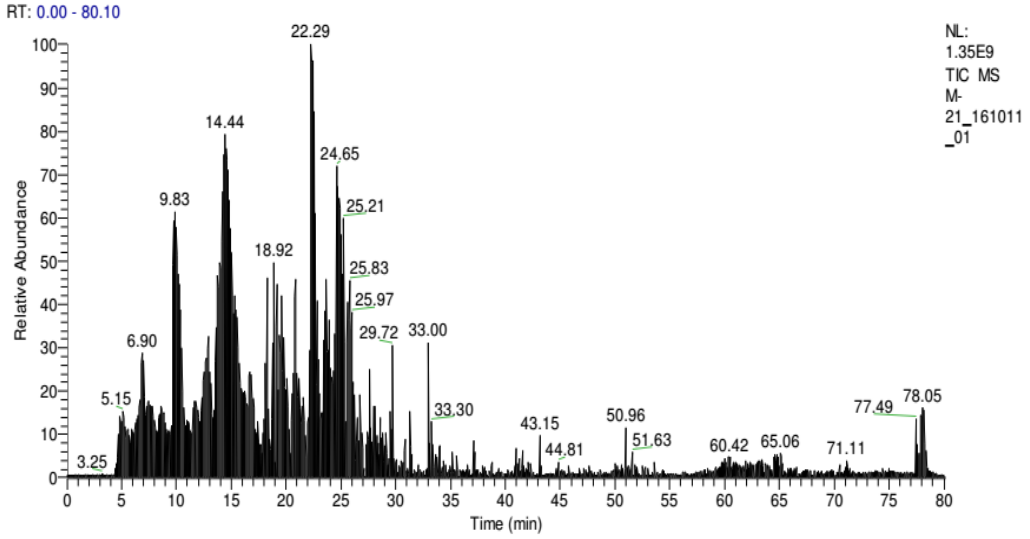


Figure 4: Chromatogram view - Band ID2

The proteins purified and isolated from the strain were studied using SDS-PAGE. The protein bands obtained from the gel were further subjected to trypsin digestion. The aminoacid sequence of SOD from the isolated strain *HalomonasutahensisSM1* showed closed homology with the SOD of type strain *Pseudomonas aeroginosa* VRFPAO1. The extracted gel bands were prepared for MALDI-TOF MS analysis by treating with trypsin. The peptide spectrum of fragment-1 (Band Id-1) and fragment 2 (Band Id-2) by trypsin digestion were found to contain 26 and 23 ions respectively as shown in fig (3 and 4). Height of the peak represents the intensity of the respective ions. The fragments and their finger print information of LC/MS spectrum of the peak were analysed. The score, intensity of coverage, molecular weight, number of aminoacids, number of peptides as well as isoelectric point (pI) value were calculated and shown in table (4). The results showed that the fragment 1 contains 8 peptides and 193 aminoacids.It has significant score

value of about 762.71% with a coverage of 41.97. It contains 7 unique peptides and 8 peptides. The Peptide Spectral Match (PSMs) analysis indicates that the peptides were found to be repeated 502 times within the sequence and was found to be matched with 11isoenzyme forms of SOD. The molecular weight was found to be 21.4 Kda and the number of aminoacids present was determined as 193 with a pI value of 5.48, compared with the SOD of *Pseudomonas aeroginosa* VRFPAO1. Similarly fragment 2 showed the score value of 1566.4%, coverage of 41.97 and was found to be matched with 11 isoenzymes of SOD. This fragment contains 8 peptides and 7 unique peptides containing 193 aminoacids. The peptides of fragment 2 were found to be repeated 1059 times in the whole protein sequence of SOD and were paired with11isoenzymes of SOD. The molecular weight and isoelectric point (pI) were calculated as 21.4 KDa and 5.48 respectively.

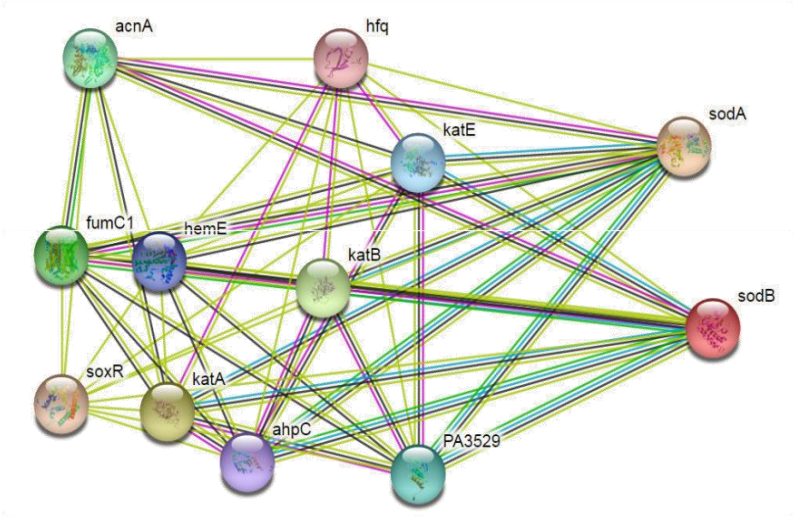


Figure 5: Protein – Protein interacting partners – soda

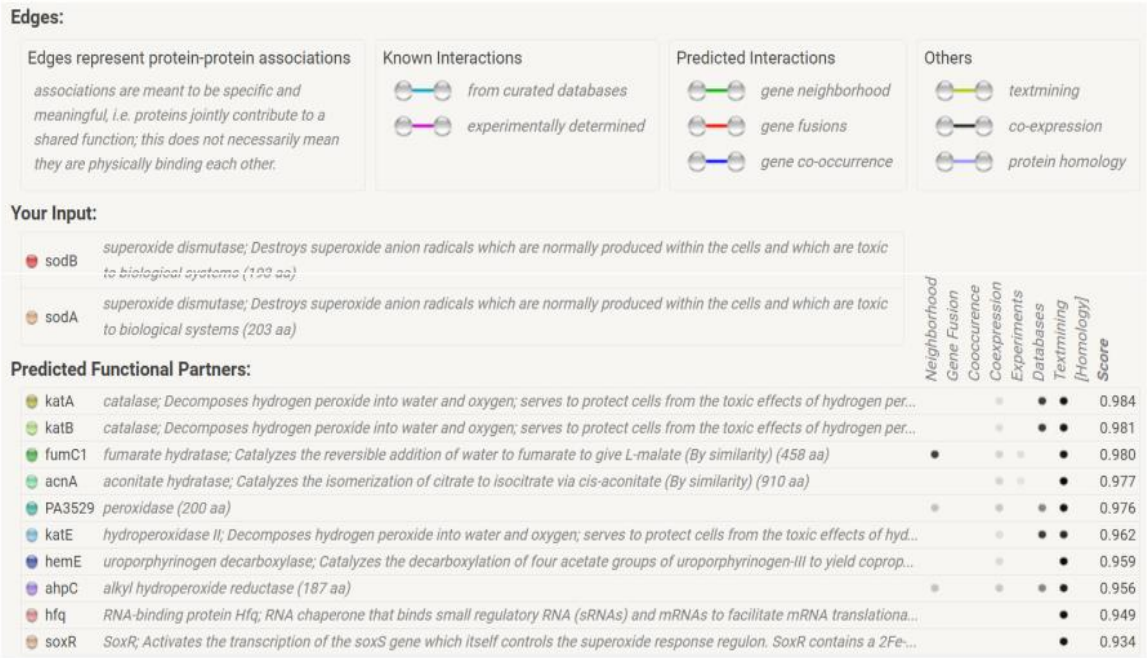


Figure 6: showing the interaction network list of proteins with sod A

The identified protein was subjected to STRING (v.9.05) analysis to reveal functional interactions between the deregulated proteins. Each node represents a protein and each edge represents an interaction. Ten additional interacting proteins were added to provide a more comprehensive view of the interactions. Different line colors represent the types of evidence for the association (Fig.6).The nodes of the network (the marbles in the figure) represent the proteins, while the edges of the network (the lines between the marbles) represent the predicted functional associations between the proteins. The color of each of the edges represents the type of evidence that exists for that interaction: a red line indicates the presence of fusion evidence, a green line indicates neighborhood evidence, a blue line indicates co-occurrence evidence, a magenta/purple line indicates experimental evidence, a yellow line indicates text-mining evidence, a light blue line indicates database evidence, and a black line indicates co-expression evidence. The data in figure 6 represents the interaction network lists of proteins, those found to interact with SOD A in the first column, with details of the

proteins' full names and sizes in the second column. The third through tenth columns indicate what type of evidence exists for the interaction of each of the proteins with SOD A. The last column gives the confidence score, as determined by the evidence, for the interaction of each protein with SOD A.

4. DISCUSSION :

In this study superoxide dismutase from *Halomonas utahensis* SM1 has been purified and characterized. This is the first report on the purification and molecular characterization of superoxide dismutase isolated from *Halomonas utahensis* SM1. According to Seatovic *et al.,* [Seatovic *et al.,2004*], the molecular weight of SOD from *Halobacterium halobium* by gel filtration column equilibrated with 0.05M phosphate buffer of pH 7.2 showed 37 KD. The Mn-SOD isolated from bacteria and mitochondria are either homodimers or heterotetramers with subunit molecular weight of about 20 KD [Cannio *et al*.,2000]. SDS-PAGE analysis of SOD in present investigation shows that the enzyme is a homodimer made up of 2 identical subunits of same molecular weight of about 21.4 kda. Each subunit is composed of 193 aminoacids and the isoelectric point was calculated as 5.48. The molecular characterization of the enzyme explains that SOD in the present analysis shows closed homology with the SOD of *Pseudomonas aeroginosa WRFPO1* and the molecular mass of its subunits is larger than the others. Further studies on this enzyme could reveal new insights into the structural basis for thermal adaptations of proteins at high temperatures and may provide a plausible explanation on how microorganisms can deal with extreme environments.

Acknowledgement

We would like to thank the management of Lakshmipuram College of Arts and Science, Neyyoor, for providing facilities to do this work.

REFERENCES :

1. Wang S , Yong-Bin Y , Dong Z. Contributions of the C-Terminal Helix to the Structural Stability of a Hyperthermophilic Fe-Superoxide Dismutase (TcSOD). Int J Mol Sci. 2009 ;10(12):5498–5512.
2. Russo, A. D., R. Ruilo, G. Nitti, M. Masuilo, V. Bochini, *Biochim.Biophys.Acta*, 1343 (1997), pp.23-30.
3. Fridovich, I, Superoxide radical and superoxide dismutases.*Annu. Rev. Biochem*., 64 (1995), pp.97–112.
4. Angelova, M, Dolashka-Angelova, P, Ivanova, E, Serkedjieva, J, Slokoska, L, Pashova, S, Toshkova, R, Vassilev, S, Simeonov, I, Hartmann, HJ, Stoeva, S, Weser, U &Voelter, W, A novel glycosylated Cu/Zn-containing superoxide dismutase: Production and potential therapeutic effect.*Microbiology*, 2001;147(6) :1641–1650.
5. Farrow, KN, Lakshminrusimha, S, Reda, WJ, Wedgwood, S, Czech, L, Gugino, SF, Davis, JM, Russell, JA &Steinhorn, RH, Superoxide dismutase restores eNOS expression and function in resistance pulmonary arteries from neonatal lambs with persistant pulmonary hypertension. *Am. J. Physiol. Lung Cell Mol. Physiol*., 2008:295(6): 979–L987.
6. Laurila, JP, Castellone, MD, Curcio, A, Laatikainen, LE, Haaparanta-Solin, M, Gronroos, TJ, Marjamaki, P, Martikainen, S, Santoro, M &Laukkanen, MO, Extracellular superoxide dismutase is a growth regulatory mediator of tissue injury recovery. *Mol. Ther*., 17 (2009), pp.448–454.
7. Teoh, M.L.; Fitzgerald, M.P.; Oberley, L.W.; Domann, F.E, Overexpression of extracellular superoxide dismutase attenuates heparanase expression and inhibits breast carcinoma cell growth and invasion. *Cancer Res*., 69 (2009), pp.6355–6363.
8. Zemlyak, I.; Nimon, V.; Brooke, S.; Moore, T.; McLaughlin, J.; Sapolsky, R, Gene therapy in the nervous system with superoxide dismutase.*Brain Res*, 1088 (2006), pp.12–18.
9. Emregul, E, Development of a new biosensor for superoxide radicals.*Anal.Bioanal.Chem.*, 383 (2005), pp. 947–954.
10. Li, DC, Gao, J, Li, YL & Lu, J, A thermostable manganese-containing superoxide dismutase from the thermophilic fungus *Thermomyceslanuginosus. Extremophiles*, 9 (2005), pp.1–6.
11. Aebersold, R& Mann, M,'Mass spectrometry based proteomics',Nature, 422 (2003), pp.198-207.
12. Fenn J.B, Mann M, CK Meng, SF Wong, CM Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules.*Science.* 246, (4926) (1989), pp. 64-71.
13. Schwartz JC, Scnko MW, Syka JE, A two dimentional quadruple ion trap mass spectrometer.*J AM S Oc Mass Spectrom.* 13 (6) (2002), pp.659-669.
14. Fenn JB, Electrospray wings for molecular elephants (Nobel lecture).AngewandteChemie International Edition, 42 (33) (2003), pp. 3871-3894.
15. Marklund, & Schinner, Involvement of superoxide iron in the auto oxidation of pyrogallol and a convenient assay for superoxide dismutase, *European J. Biochem*, 47 (1974), pp.469-474.
16. Lowry, OH, Rosebrough, NJ, Farr, A & Randall, RJ, Protein measurement with the folin's phenol reagent, *J. Biol. Chem*, 193 (1951), pp.265-275.
17. Sharma A., S.Khare, N. Gupta, Hydrolysis of ricehyll by crosslinked*A.niger, J. Chem*, 1 (2000), pp.1-3.
18. PetkarMedha B, PillaiMeena M, KulkarniAmarja A, BondreSushma H, Rao KRSS, Purification and characterization of Superoxide dismutase isolated from sewage E.Coli. *J MicrobBiochem Technol*.,5 (2013), pp.102-106.
19. Bradford, MM,'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding',Analytical Biochemistry, 72 (1976), pp.248-254.
20. Laemmeli, UK, Cleavage of structural proteins during the assembly of head bacteriophage T4, *Nature*, 227 (1970), pp.680-685.

21. Ausubel, Frederick M, Short protocols in molecular biology: a compendium of methods from current protocols in molecular biology. *Molecular biology methods laboratory manuals*, (1989), pp: 372-375.
22. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki Ra, DAVID: Databaase for Annotation, Visualization and Integrated Discovery. *Genome Biol*, 4 (5) (2003), pp.3.
23. Huang D, Kaluarachchi S, Van Dyk D, Friesen H, Sopko R, Ye W, Bastajin N, Dual regulation by pairs of cyclin dependent protein kinases and histone deacetylasescontrols G1 transcription in budding yeast. *PLOS Biol*. 7 (9) (2009), pp.100-188.
24. May, BP, Dennis, Superoxide dismutase from extremely halophilic archeabacteria, *J. Bacteriol*., 169 (1987), pp.1417-1422.
25. Seatovic, S.; Gligic, L.; Radulovic, Z.; Jankov, R.M, Purification and partial characterization of superoxide dismutase from the thermophilic bacteria *Thermothrix*sp. *J. Serb.Chem. Soc*, 69 (2004), pp.9–16.
26. Cannio, R, Fiorentino, G, Morana, A, Rossic, M &Bartiolucci, S, *Frontiers in Bioscience*, 5 (2000), pp.768.