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Original Article

STUDY ON THE PROPERTIES OF PURIFIED RECOMBINANT SUPEROXIDE DISMUTASE FROM *STAPHYLOCOCCUS EQUORUM*, A LOCAL ISOLATE FROM INDONESIA

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ABSTRACT

Objective: Superoxide dismutase (SOD) (E. C: 1.15.1.1) from *Staphylococcus equorum* which catalyzes the dismutation of the superoxide anion ($O_2^{\cdot-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), is one of the most important classes of antioxidant enzymes and are used in pharmaceutical or cosmetic applications. SOD of *S. equorum* was purified from total protein into *homogeneity* and characterized to determine the unit activity, ion metal cofactor, optimum temperature and pH, kinetic parameters, and effect of denaturing and reducing agents and UVC exposure on the rSOD activity.

Methods: The protein was purified in a single-step purification using Ni-NTA affinity column with various imidazole concentrations. SOD activity was analyzed by colorimetric and activity staining using nitroblue tetrazolium (NBT). The purified rSOD was exposed to different temperatures and pHs, different concentrations of denaturing agents, reducing agents, and to UVC exposure.

Results: SOD protein with high purity was obtained when imidazole concentrations of 100 mM, 200 mM and 250 mM were applied. The purified rSOD displayed specific activity of 1666.7 U $mg^{-1}$when measured at 30°C and pH 7.8. The presence of conserved manganese-binding sites (H28, H83, D171, H175) and the inhibition of rSOD activity by NaN_3 but not by H_2O_2 or KCN and indicated that rSOD was Mn-dependent. The optimum temperature and pH were determined to be 40°C and 6.0, respectively. The Michaelis constant (K_m), maximum velocity (V_{max}), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were found to be 371.2 µM, 1.738 μMS^{-1}, 1.358 s^{-1}, and 3.7x10^{-3} $S^{-1}\mu M^{-1}$, respectively. The rSOD activity was slightly affected in the presence of detergents (0.5% SDS, 0.5% Triton-X 100), denaturing agents (6 M GdnHCl and 6 M urea) and reducing agent (5 mM βME). After exposure of rSOD by UVC for 45 min, it retained half of its activity.

Conclusion: This is the first study to report the stability of the SOD of *S. equorum* against environmental factors. The SOD displays some thermostability, is active in wide pH, stable in the presence of denaturing and reducing agents, however it is relatively unstable to UVC exposure

Keywords: *Staphylococcus equorum*, Manganese superoxide dismutase, Expression, His-tag purification, Characterization.

INTRODUCTION

SODs are mettaloenzymes that functions as the body's first-line of defense, and is one of the important classes of antioxidant enzymes. SODs protect macromolecules such as DNA, proteins, and lipids from reactive oxygen species (ROS) responsible for DNA mutation, changes of protein structure and function, and membrane lipid peroxidation [1]. These enzymes, which are produced by both prokaryotic and eukaryotic cells, catalyze the dismutation of superoxide anions ($O_2^{\cdot-}$) to O_2 and H_2O_2 which are further reduced by catalase or peroxidase to water (H_2O) [2]. Based on the metal cofactors at their active sites, SODs are classified into four types: manganese SOD (MnSOD or SodA), iron SOD (FeSOD or SodB), copper/zinc SOD (Cu/ZnSOD or SodC) and nickel SOD (NiSOD or SodN). MnSODs is found as dimers in bacteria, except in *Thermus thermophilus* and *T. aquaticus* as tetramers [3] while SODs from eukaryotes usually are in the tetramer form. MnSOD and FeSOD are closely related in their amino acid sequence and show structural homology [4].

SODs play an important role in biological system, as they prevent adverse changes to the structure and function of macromolecules, including protein. The native structure of a protein is its three-dimensional conformation when placed in its natural environment. Alterations in the environment, such as the addition of denaturing or reducing agents, may cause denaturation of the protein and cause it to unfold fully or partially, depending on the denaturation mechanism, thus losing its native structure [5]. However, denaturing or reducing agents are routinely used in protein extraction and purification processes. Example of denaturing agents include Sodium dodecyl sulfate (SDS), urea, and guanidine hydrochloride (GdnHCl). SDS has been reported to decrease the activity of MnSOD from a new species *Tatumella ptyseos* ct [6]. Cu/Zn SOD of mungbean (*Vigna radiata*) was more stable in the presence of urea than GdnHCl, the denaturation by GdnHCl and urea were reversible when the enzyme was incubated in GdnHCl or urea at 30° C [7]. Catalytic activity of bovine SOD was unaffected by urea or by SDS. The histidine residues of the enzyme were resistant to carboxymethylation in neutral buffer and urea [8]. Urea, GdnHCl and βME had little effect on activity of Cu/Zn SOD *Jatropha curca* [9]. Bovine SOD retained full activity after 1 hour in 4% SDS at 25°C but rapidly lost activity in 1% SDS plus 0.1 M βME [10].

The presence of DTT (0.1, 0.5, or 1.0 mM), caused a significant decrease on SOD activity in rat liver cytosol [11]. UV radiation causes modification or destruction of amino acids and inactivation of proteins [12]. *Deinococcus radiophilus* is an obligate aerobic bacterium extraordinarily resistant to UV, oxidative stress, and ionizing radiations. The extreme resistance of SOD from *D. radiophilus* against UV would be associated with its morphological characteristics and efficient ROS scavenging systems [13]. *Effect of UV radiation on SOD activity has never been reported before.*

SODs are widely used in therapeutic and cosmetics applications as well as in the agricultural, food and chemical industries [14]. The therapeutic role of SODs is in preventing oxidative damage in the treatment of cardiovascular diseases, neurodegenerative disorders, aging, rheumatoid arthritis, diabetes, autoimmune diseases and cancer [15]. Food supplement comprising of α-lipoic acid and SOD can be used in the treatment of neuropathies. *S. equorum* is a gram-positive, aerobic, coccus, catalase-positive, coagulase negative, non-pathogenic, non-motil and no-spore forming bacterium with an optimum growth temperature of 30°C [16]. This bacterium was originally isolated from skin horse and contributes to the formation of flavor in fermented foods [17]. Preliminary results using a local bacterial isolate obtained from soil in Indonesia showed that *S. equorum* produced a single that has never previously reported [18]. The *sod* gene coding for SOD from *S. equorum* was chemically

synthetized and expressed as an active enzyme in *E. coli* BL21(DE3) through the use of the pJexpress414 expression vector. In this study, the SOD of *S. equorum* was purified from total protein into *homogeneity* and characterized to determine the ion metal cofactor, optimum temperature and pH, kinetic parameters, and effect of denaturing agents and reducing agents and UVC exposure on the rSOD activity.

MATERIALS AND METHODS

Materials

Isoprophyl thiogalactopyranoside (IPTG), nitro blue tetrazolium (NBT), Coomassie brilliant blue R-250, Bovine serum albumin (BSA), urea, guanidine hydrochloride (GdnHCl), imidazole, sodium dodecyl sulfate (SDS), Triton-X 100, beta-merchaptoethanol (ßME), dithiothreitol (DTT), potassium cyanide (KCN), sodium azide (NaN_3), hydrogen peroxide (H_2O_2), riboflavin were obtained from Sigma-Aldrich Chemical (St Louis, MO, USA), unstained protein ladder were obtained Amersham Bioscience (Buckinghamshire, UK). All chemicals were reagent grade.

Protein overproduction

E. coli BL21(DE3) carrying the *sod* gene coding for SOD (pJExpress414*sod*) was grown in Luria Bertani (LB) medium containing 100 µg/mL of ampicillin. A 4% overnight culture was transferred to 1L of fresh LB. The culture then incubated at 37°C with agitation of 150 rotation per minute (rpm) until an OD_{600} reading of 0.6-0.8 was reached. After IPTG induction with final concentration of 1 mM, culture was further incubated at 37°C for 4 hours. The cells were then harvested by centrifugation (5,000 x g; 20 min; 4°C) and washed with phosphate buffer saline (PBS) at pH 7.8. The cell pellet was then re suspended in (1:2) 1x Lysis Equlibration Wash (LEW) buffer (50 mM NaH_2PO_4. H_2O, 300 mM NaCl, pH 8.0). rSOD was produced intracellular. Hence cells were disrupted to release the protein. Cell lysis was performed on ice bath by sonication for 2 min, followed by centrifugation. The total protein in supernatant was then analyzed using SDS-PAGE.

Protein purification

All purification procedures were performed at pH 8.0 and 4°C under non denaturing condition. The protein was purified in a single-step purification procedure using Ni-NTA agarose as an affinity chromatography with gradient concentrations of imidazole for elution. The Ni-NTA column was first equilibrated with buffer (50 mM NaH_2PO_4. H_2O, 300 mM NaCl, 15 mM imidazole) to remove unspecific binding from the column. A 500 µL of supernatant was loaded in micro tube containing 500 µL Ni-NTA resin.

The micro tube was inverted 20 times, centrifuged at 1,000 x g for 2 min and the supernatant was discarded. Bound protein was then eluted twice with 1x LEW buffer followed by subsequent elutions with 1x LEW containing (15 mM, 50 mM, 100 mM, 200 mM, and 250 mM imidazole). Buffer-exchange was carried out with PBS using a *Nanosep® 10 kDa* filters *(Pall* Life Sciences, Germany*)* by centrifugation at 5,000 x g for 5 min. The rSOD was analyzed by 15% SDS-PAGE [19] and proteins concentration was determined by the Bradford method (1976) using BSA as the standard reference.

Enzyme assay and determination of unit activity

SOD activity was determined using NBT auto-oxidation method developed by Indrayati *et. al.* (2011). Briefly, after electrophoresis, gel was then soaked for 15 min at room in 100 mM potassium phosphate buffer at pH 7.4 containing 1.23 mM NBT rinsed with water, incubated in the dark for 15 min at room temperature in 100 mM potassium phosphate buffer containing 0.028 mM riboflavin and 28 mM TEMED and finally washed again with water. The gel was illuminated using a fluorescent lamp (23 Watt, with distance of 5 cm) for 10 min.

In the spectrophotometric assay, SOD activity was presented as the percentage of inhibition (% inhibition) calculated by comparing the absorbance (A) of samples containing SOD to those without SOD. Phosphate buffer pH 7.8; 66 µL, 0.1 M EDTA and 33 µL, 1.5 mM NBT was added to protein samples. The solution was then homogenized, incubated for 2 min in a light box, added 25 µL of 0.12 mM riboflavin further incubated in a light box for 4 min and the absorbance was determined in λ560 nm. Unit activity was performed according to [20] with modification, a series of rSOD with masses ranging from 75 to 19,200 ng was used and enzyme activity was determined previously. One unit activity (U) of SOD is defined as that amount of protein that inhibits NBT reduction by 50%. Specific activity is defined as the rSOD activity per milligram of total protein (U/mg).

Determination of SOD type

To predict the SOD type, the amino acid sequence of *S. equorum* rSOD were compared with those of known MnSODs from mesophilic bacteria deposited in the NCBI Gen-Bank database (http: //www. ncbi. nlm. nih. gov) and were then aligned using the Clustal Omega software (https: //www. ebi. ac. uk/Tools/msa/clustalo) to identify and conserved regions in the sequences. To determine the metal cofactor, the rSOD was pre-incubated in 67 mM phosphate buffer at pH 7.4 in the presence of each inhibitor i. e. 20 mM potassium cyanide (KCN), sodium azide (NaN_3), or hydrogen peroxide (H_2O_2) for 1 h in 37ºC and followed by an SOD assay as describe above.

Determination of optimum pH and temperature

The optimum pH and temperature for rSOD activity were tested by a modified procedure from Kumar *et. al.* (2014) [21]. The optimum pH was determined using different buffers of a wide pH range (pH 4-10). 0.1 M citrate buffer ($C_6H_8O_7$,$C_6H_5O_7Na_3.2H_2O$) for the pH range 4-6, 0.1 M phosphate buffer (Na_2HPO_4, NaH_2PO_4) for the pH range of 7-8 and 0.1 M Glycine-NaOH buffer for the pH range of 9-10. All pH values were adjusted at room temperature. The optimum temperature was determined at optimum pH at different temperatures ranging from 30 to 70° C.

Determination of kinetic parameters

Enzymes kinetic properties were determined on optimum pH and temperature using the modified procedure introduced by Areekit *et. al.* (2011) and the SOD activity were measured as described above. Kinetic parameters were calculated from Lineweaver-Burk plots with a stander curve for the formation as a function of the formazan concentration (mM) at absorbance of 560 nm. The kinetic parameters included Michaelis constant (K_m), maximum velocity (V_{max}), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m).

Effect of denaturing, reducing agents and UVC exposure on rSOD activity

A 0.5 units of rSOD was incubated for 1 h at 37ºC in 67 mM phosphate buffers pH 7.8, each containing four types of denaturing agents (0.5% SDS, 0.5% Triton X-100, 6 M GdnHCl, and 6 M urea) and two types of reducing agents (5 mM DTT and 5 mM βME). The effect of UVC exposure on the rSOD activity was performed at λ254 nm with a distance of 5 cm (Vilbert Lourmat, France, VL-6. LC 230 V 50/60 Hz, intensity 7 mW/cm^2). A 2.0 unit of rSOD were exposed with a time of exposure 15; 25; 35; 45; 55; 65 and 75 min. Enzyme activity was measured as describe above.

RESULTS

Protein overproduction and purification

The rSOD fraction showed several protein bands when eluted with imidazole at concentrations of 15 and 50 mM (Fig. 1a, lanes 5-8) and single protein band of rSOD of 23.45 kDa were obtained when imidazole concentrations of 100 mM, 200 mM and 250 mM were applied (Fig. 1a, lanes 11-16). The 23.45 kDa protein appeared to coincide with the molecular weight of monomer form of the rSOD. The final rSOD yield after affinity chromatography and specific activity was found to be 10 mg/L and 1,666.7 U mg^{-1} respectively (Fig. 1b).

Determination of SOD type

The SOD of *S. equorum* contained four residues, three histidines and one aspartate residues (H28, H83, D171, and H175) and amino sequence at the N-terminal region (LPNLPYWF) which is conserved in all reported MnSOD (Fig. 2a). The result of BLAST analysis also indicated that the amino acid sequence of the SOD was highly homologous to MnSODs of *S. xylosus* (92.35% identity and 7.65%

similarity) and *S. epidermidis* (97.46% identity and 2.54% similarity). The enzyme's activity was inhibited by NaN_3, but not by H_2O_2 and KCN, as shown by the significant difference in the achromatic zones in activity stained gel treatment with NaN_3 (Fig. 2b). The results from both data strongly indicated that *S. equorum* SOD was manganese type enzyme.

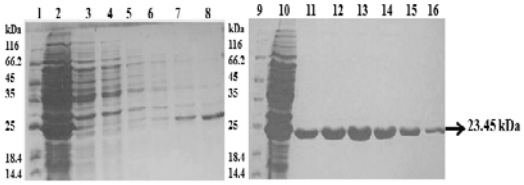

Fig. 1a: SDS–PAGE profile of rSOD visualized by Coomassie brilliant blue stain. Lanes 1 and 9, protein ladder; lanes 2 and 10, intracellular total proteins of E. coli BL21(DE3); lane 3, nickel column flow through; lanes 4, rsOD eluted with 1x LEW buffer; lanes 5-18 except line 10, rSOD eluted twice with 1x LEW buffer containing (15 mM, 50 mM, 100 mM, 200 mM, and 250 mM imidazole) respectively.

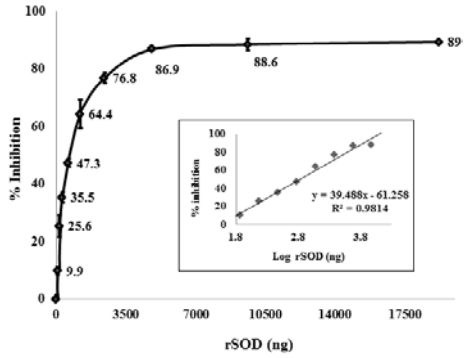

Fig. 1b: Inhibition by rSOD determined by NBT auto-oxidation method. The amount rSOD that inhibited NBT reduction by 50% was defined as 1 unit enzyme (600 ng). Insert: graph of percent inhibition vs log rSOD concentration

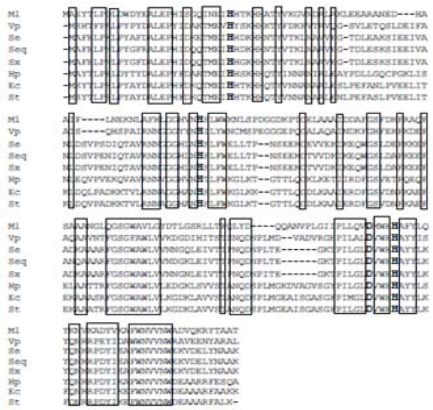

Fig. 2a: Multiple alignment of the amino acid sequences of *S. equorum* (Seq) with those of bacterial MnSODs from Gen-Bank NCBI data base. Alignment was performed using Clustal Omega program. The corresponding proteins are listed as follows: *Mycobacterium lepraemurium* (Ml, D13288), *Vibrio parahaemolyticus* (Vp, AB041845), *S. epidermidis* (Se, AF462457), *S. xylosus* strain DSM 20266 (Sx, AY571689), *Haemophilus parasuis* (Hp, AY195847), *E. coli* (Ec, X03951) and *Salmonella typhimurium* (St, U20645). Residues in bold are predicted to bind metal mangan cofactor. The boxes region designates positions where all sequences share the same amino acid residue and gaps are indicated by dashes to improve the alignment

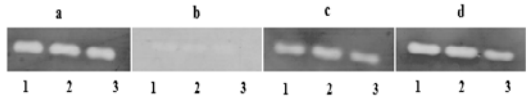

Fig. 2b: Activity staining, of rSOD in the presence of various inhibitors. The zymogram was performed with 15% native polyacrylamide gel electrophoresis (600 ng protein per lane) gel in triplicates. (A) without inhibitor, (B) with 20 mM NaN_3, (C) with 20 mM H_2O_2 and (D) with 20 mM KCN

Determination of optimum pH and temperature

The purified rSOD showed high enzyme activity between pH 4.0 and 7.0, with the highest activity at pH 6.0. The pH curve was characterized by a rapid decrease in enzyme activity above pH 9.0 and below pH 5.0 (Fig. 3a). *S. equorum* rSOD exhibited high thermal stability, the enzyme was relatively stable at temperature below 50˚C, with the optimum temperature for enzyme activity at 40˚C, but was inactivated rapidly at 70˚C (Fig. 3b).

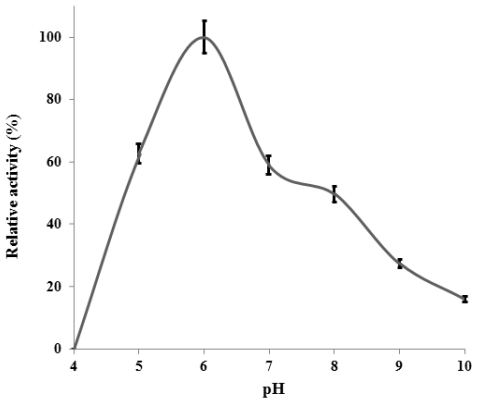

Fig. 3a: Optimum pH of rSOD from *S. equorum*. The purified rSOD was assayed at different buffer of a wide pH range (pH 4–10). The optimum pH for the SOD activity was 6.0. The experiment was conducted in triplicate

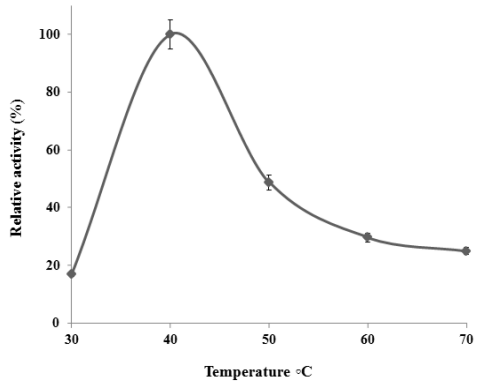

Fig. 3b: Optimum temperature of rSOD from *S. equorum*. The purified rSOD was assayed in 0.1 M citrate buffer pH 6.0 at different temperatures values. The optimum temperature for the rSOD activity to be found 40°C. The experiment was conducted in triplicate

Kinetic parameters of purified SOD

To obtain the enzymatic performance of rSOD, kinetic parameters were determined for the purified protein. Our results demonstrated for the first time that our SOD possessed Michaelis constant (K_m),

maximum velocity (V_{max}), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were found to be 371.2 μM, 1.738 μMS^{-1}, 1.358 s^{-1}, and 3.7x10^{-3} $S^{-1}\mu M^{-1}$respectively**.**

Effect of denaturing, reducing agents and UVC exposure on rSOD activity

In general, the rSOD activity slightly decreased in the presence of denaturing and reducing agents. The enzyme retained much of its activity in the presence of 6 M urea and GdnHCl (Fig. 4a), and around 70% of its activity after treatment with 0.5% Triton-X 100 and SDS (Fig. 4b). The addition of 5 mM DTT and *β*ME decreased the SOD activity to 50% and 80%, respectively (Fig. 4c). Exposure to UVC for 45 min decreased the rSOD activity by half, while UVC exposure for 65 and 75 min, reduced the rSOD activity to below 50% (Fig. 4d).

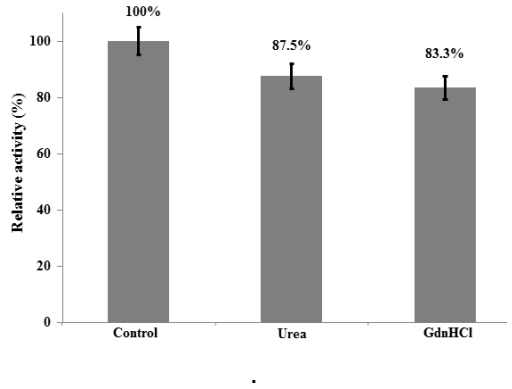

Fig. 4a: rSOD activity in the presence of 6 M urea and GdnHCl. (A). control, (B) with 6 M urea, (C) with 6 M GdnHCl. A 0.5 unit of rSOD containing urea and GdnHCl respectively was pre-incubated for 1 h at 37°C prior to SOD assay. The experiment was conducted in triplicate

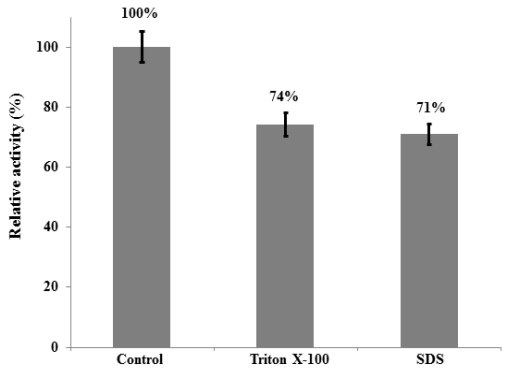

Fig. 4b: rSOD activity in the presence of 0.5 % Triton-X 100 and SDS (A). control, (B) with 0.5% Triton-X 100 (C) with 0.5%. SDS. A 0.5 unit of rSOD containing SDS and triton-X 100 respectively was pre-incubated for 1 h at 37°C prior to SOD assay. The experiment was conducted in triplicate

DISCUSSION

In pharmaceutical field, SOD can be used as the cosmetic active agent and for treatment of several degenerative diseases. This current work focused on the partial characterization of the SOD from *S. equorum* that has never been previously studied. An SOD of 23.45 kDa from *S. equorum* was expressed from a synthetic gene in *E. coli* BL21(DE3) and this study showed for the first time that it was a manganese-dependent. When measured at 30°C and pH 7.8, the specific activity of the enzyme was 1666.7 Unit/mg proteins. We also determined the optimum pH and temperature of the SOD were 6.0 and 40°C, respectively. Our results showed that the SOD was stable in the presence of denaturing and reducing agents and fairly stable against UVC exposure.

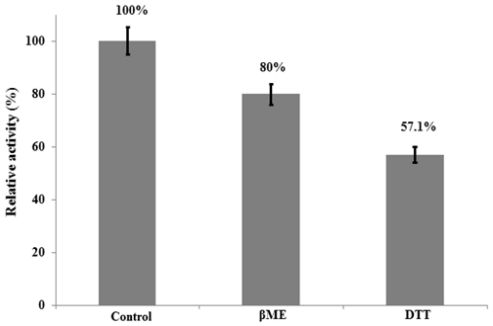

Fig. 4c: rSOD activity in the presence of 5 mM ßME and DTT. (A). control, (B) with 5 mM ßME, (C) with 5 mM DTT. A 0.5 unit of rSOD containing ßME and DTT respectively were pre-incubated for 1 h at 37°C prior to SOD assay. The experiment was conducted in triplicate

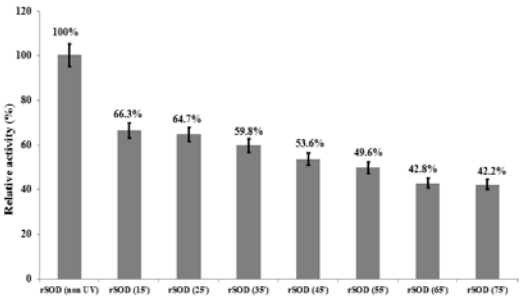

Fig. 4d: rSOD activity in the presence of UVC radiation. A 2.0 unit of rSOD were exposed to UVC (254 nm, distance of 5 cm) with time of exposure 15, 25, 35, 45, 55, 65, and 75 min

E. coli *is the most commonly used host for high yield expression of recombinant proteins. However, several proteins expressed in* E. coli *hosts have high affinity to nickel ions due to the presence of histidine residues exposed on the surface. Protein purification using low imidazole concentrations produced several contaminants, the main being the* Lac *protein repressor that recognize the* lac *operator and binds to it tightly with* dissociation constant of 10^{-}[11] to 10^{-}[13] M [24]. Purification using higher imidazole concentrations (100, 200, and 250 mM) resulted in a better elution of target protein, though these imidazole concentrations are higher than advised for most IMAC column washing procedures. The specific activity measured was 1,666.7 U $mg^{-1}$of our rSOD is lower than that of SOD reported from *Bacillus* sp. MHS47 [22], *Methylobacillus* sp. strain SK1 [25] and *Tatumella ptyseosct* [6] but similar to that of a thermostable SOD from *Thermus* sp. JM1 [23]. This indicates that SODs from *S. equorum* and *Thermus* sp. JM1 have the ability to convert its s substrate into products at the same rate. Enzyme with the needed properties such as higher activity and specificity in an industrial application can be obtained by improving process conditions and by protein engineering. Specific activities of *Bacillus* sp. Protease and keratinase were significantly increased when their genes were engineered by site-directed mutagenesis [26].

SODs are generally divided into four types based on their metal content at the active site and each type of SOD is sensitive to different inhibitors. Multiple alignment of rSOD *S. equorum* with other bacterial MnSOD using Clustal Omega program showed that an amino acid comparison of the active site and the conserved regions of *S. equorum* rSOD similar to those regions of other bacterial MnSODs [27]. The rSOD activity was inhibited by the azide group (N_3), which acts as a substrate analogue, and this suggests that the rSOD produced is an MnSOD. The binding of the azide to the active

site of rSOD blocks its substrate or other small molecules at the active site. Azide binding residues include the three His metal ligands (H28, H83, D171, and H175) and are affected by electrostatic interactions as well as steric interference by the mutational changes studied. This is the first report on MnSOD from *S. equorum*, whereas MnSOD from other bacteria was previously reported [22].

The optimum pH of *S. equorum* rSOD was 6.0 lower than that of *Bacillus* MHS47 rMnSOD with an optimum pH of 7.5. This rSOD was found to be more stable in an acidic environment, retaining 73.9% of enzyme activity under such conditions. This is particularly useful in cosmetic applications as for example skin cleansing preparations with a relatively low pH (about 6.0 or less) exhibit low irritation or anaphylaxis. The rSOD was more thermostable as compared to SOD from other mesophilic bacteria such as rMnSOD from *Bacillus* sp. MHS47 that has an optimum activity at 37°C [22]. An important requirement for SODs in industrial applications is their thermal stability as thermal denaturation is a common cause of enzyme inactivation. While the mechanisms responsible for protein thermostability are unclear. Possible reason such as increased charged and hydrophobic residues [28].

The total number of charged hydrophilic residues residues both positive (lysine, arginine, histidine), and negative (aspartate and glutamic) in rSOD is 53, higher than that of *Bacillus* sp. MHS47 (total 50), which explains the rSOD's thermostabilty. Michaelis-Menten kinetics parameters were determined for purified rSOD, and the results showed that the K_m of rSOD was lower than the SOD from the anaerobic bacterium *Propionibacterium shermanii* [29]. A low K_m value indicates that the rSOD binds efficiently with its substrate. The K_m value of both enzymes was 371.2 µM at pH 6.0 and 0.54 mmol/l at pH 9.0. The V_{max} value$_s$ of rSOD and *P. shermanii* SOD are 1.738 $\mu MS^{-1}$and 2.000 mol. s^{-1} respectively showing that rSOD is capable of a higher rate of catalysis.

The diverse applications of protein in the pharmaceutical and food industry as well as analytical biochemistry highlights the importance of studies on interaction between proteins with denaturing and reducing agents. Our results suggests in the presence of urea, rSOD was more stable compare to *Thalassiosira weissflogii* SOD shown by 14% inhibition of rSOD and 60% of *T. weissflogii* SOD activity [30]. In comparison with SOD from bovine, rSOD is remarkably stable in the presence of GdnHCl but less stable to SDS. A 6 M of GdnHCl caused 16% inhibition of rSOD whereas 0.2 M GdnHCl decreased 40% SOD activity from Bovine. Many proteins unfold fully in strong denaturants such as 6 M GdnHCl or 8 M urea [5]. rSOD retained 71% activity after 1 h in 0.5% SDS at 37°C, whereas SOD from bovine retained full activity after 1 h in 4% SDS at 25°C [5]. Only few SODs from bacteria are biochemically characterized for their kinetic behavior and stability. The mechanism responsible for rSOD stability against denaturing and reducing agents still remains *unclear*. It may utilize a mechanism similar to CuZnSOD from bovine which metal cofactor plays a structural role for stabilizing the native structure of the enzyme [10].

Our finding shows that UVC decreased the activity of rSOD enzyme, possible reasons for UVC effect on rSOD activity such as the presence of high number of aromatic amino acids in the protein and protein oxidation. *S. equorum* rSOD contains 21 aromatic amino acids (tyrosine, phenylalanine and tryptophan). The aromatic amino acids have strong absorption in the λ250-298 nm region. It effects not only the modification or destruction of amino acid residues, but leads to inactivation of entire proteins [31]. UVC generates ROS which oxidize amino acid residues, some amino acid residues including lysine, arginine, proline and threonine are oxidized to carbonyl derivatives [32]. One of the important sources of enzyme instability is protein oxidative modification initiating denaturation or activity loss [33].

CONCLUSION

This is the first report on the properties of a MnSOD from *S. equorum*, which was found to be stable at high temperatures and in the presence of denaturing and reducing agents, however it is relatively unstable to UVC exposure.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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REFERENCES

1. Myatt L. Review: reactive oxygen and nitrogen species and functional adaptation of the placenta. Placenta 2010;31 Suppl:S66-S9.
2. Xiang H, Zhang R, Li N, Vossbrinck CR. Searching for convergent evolution in manganese superoxidase dismutase using hydrophobic cluster analysis. Genet Mol Biol 2014;37(2):460-70.
3. Sato S, Harris JI. Superoxide dismutase from *Thermus aquaticus*. Eur J Biochem 1977;73(2):373-81.
4. Abreu IA, Cabelli DE. Superoxide dismutases-a review of the metal-associated mechanistic variations. Biochim Biophys Acta 2010;1804(2):263-74.
5. Bennion BJ, Daggett V. The molecular basis for the chemical denaturation of proteins by urea. PNAS 2003;100(9):5142-7.
6. Ken C, Lee C, Duan K, Lin C. Unusual stability of manganese superoxide dismutase from a new species, *Tatumella ptyseos* ct: its gene structure, expression, and enzyme properties. Protein Expr Purif 2005;40(1):42-50.
7. Reddy CD, Venkaiah B. Purification and characterization of Cu-Zn superoxide dismutases from mungbean (*Vigna radiata*) seedlings. J Biosci 1984;6(1):115-23.
8. Malinowski DP, Fridovich I. Subunit association and side-chain reactivities of bovine erythrocyte superoxide dismutase in denaturing solvents. Biochem 1979;18(23):5055-60.
9. Ou-yang C, Gao S, Cai F, Chung T, Wang S. Cloning, expression, and purifi cation of a Cu/Zn superoxide dismutase from *Jatropha curcas*. Z Naturforsch 2013;68(1-2):60-9.
10. Forman HJ, Fridovich I, Forman J. On the stability of bovine superoxide dismutase: the effects of metals. J Bio Chem 1973;248(8):2645-9.
11. Fukaya Y, Yamaguchi M. Regucalcin increases superoxide dismutase activity in rat liver cytosol. Biol Pharm Bull 2004;27(9):1444-6.
12. Nasibi F, M-kalantari KH. The effects of UVA, UVB and UVC on protein and ascorbate content, lipid peroxidation and biosynthesis of screening compounds in Brassica napus. Iran J Sci Technol A 2005;29(A1).
13. Yun YS, Lee YN. Purification and some properties of superoxide dismutase from *Deinococcus radiophilus*, the UV-resistant bacterium. Extremophiles 2004;83(3):237-42.
14. Liu J, Yin M, Zhu H, Lu J, Cui Z. Purification and characterization of a hyperthermostable Mn-superoxide dismutase from *Thermus thermophilus* HB27. Extremophiles 2011;15(2):221-6.
15. Pham-huy LA, He H, Pham-huy C. Free radicals, antioxidants in disease and health. J Biomed Sci 2008;4(2):89-96.
16. Gotz F, Bannerman T, Schleifer K. The genera *Staphylococcus* and *Macrococcus.* Prokaryotes; 2006. p. 5-75.
17. Irlinger F, Loux V, Bento P, Gibrat JF, Straub C, Bonnarme P, *et al*. Genome sequence of *Staphylococcus equorum subsp. equorum* Mu2, isolated from a French smear-ripened cheese. J Bacteriol 2012;194(18):5141-2.
18. Indrayati A. 16S rDNA-Based identification of novel superoxide dismutase producing bacteria isolated from Indonesia. Microbiol Indones 2011;5(2):88-93.
19. Ananthakrishnan A, Ramesh B, Hi M, Am S. Optimisation studies in the production and purification of serratiopeptidase from *Serratia marscecens* UV mutant SM3. Int J Pharm Pharm Sci 2013;5(3):2-6.
20. Kumar T, Borah D. Process optimization, partial purification and characterization of protease enzyme from *Bacillus altitudinis* (MCCB-0014). Int J Pharm Pharm Sci 2012;4(3).
21. Kumar KD, Usha KY, Satyanarayana S V, Sailaja V. Characterization of partially purified lipase from

Saccharomyces cerevisiase. Int J Pharm Pharm Sci 2014;6(8):12-5.

22. Meier B, Michel C, Saran M, Parak F, Rotilioii G, Oberschleissheim D. Kinetic and spectroscopic studies on a superoxide dismutase from *Propionibacterium shermanha* that is active with iron or manganese: pH-dependence. Biochem J 1995;310:945-50.
23. Oehler S, Amouyal M, Kolkhof P, Bergmann B, Muller-Hill B. Quality and position of the three lac operators of *E. coli* define efficiency of repression. EMBO J 1994;13(14):3348-55.
24. Areekit S, Kanjanavas P, Khawsak P, Pakpitchareon A. Cloning, expression, and characterization of thermotolerant manganese superoxide dismutase from *Bacillus* sp. MHS47. Int J Mol Sci 2011;12(1):844-56.
25. Seo SN, Lee JH, Kim YM. Molecules and characterization of an iron-and manganese-containing superoxide dismutase from *Methylobacillus* Sp. strain SK1 DSM 8269. Mol Cells 2007;23(3):370-8.
26. Zhu Y, Li H, Zhang X, Zhang C, Xiang J, Liu G. Characterization of a thermostable manganese-containing superoxide dismutase from inshore hot spring thermophile *Thermus* sp. JM1 Acta Ocean Sin 2011;30(6):95–103.
27. Obe ST, Himogaki HS, Hdera MO, Sai YA, Ba KO. Expression of Bacillus protease (Protease BYA) from Bacillus sp. Y in *Bacillus subtilis* and enhancement of Its specific activity by site-directed mutagenesis-improvement in productivity of detergent enzyme. Biol Pharm Bull 2006;29(1):26-33.
28. Hunter T, Bannister JV, Hunter GJ. Thermostability of manganese-and iron-superoxide dismutases from *Escherichia coli* is determined by the characteristic position of a glutamine residue. Eur J Biochem 2002;269(21):5137-48.
29. Lim J, Gyu Y, Choi I, Ryu J, Ahn B. Cloning and expression of superoxide dismutase from *Aquifex pyrophilus*, a hyperthermophilic bacterium. FEBS Lett 1997;406(1-2):142-6.
30. Ken CF, Hsiung TM, Huang ZX, Juang RH, Lin CT. Characterization of Fe/Mn-superoxide dismutase from diatom *Thallassiosira weissflogii*: cloning, expression, and property. J Agric Food Chem 2005;53(5):1470-4.
31. Hollosy F. Effects of ultraviolet radiation on plant cells. Micron. 2002;33(2):179-97.
32. Cabiscol E, Tamarit J, Ros J. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int Microbiol 2000;3(1):3-8.
33. Slavica A, Dib I, Nidetzky B. Single-site oxidation, cysteine 108 to cysteine sulfinic acid, in d-amino acid oxidase from *Trigonopsis variabilis* and its structural and functional consequences. Appl Environ Microbiol Appl 2005;71(12):8061-8.