MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.15, No.4, December 2021, p 128-134

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.15.4.2

REVIEW

Relationship and Structural Diversity of Bacterial Manganese Superoxide Dismutases and the Strategy for Its Application in Therapy and Cosmetics

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Manganese superoxide dismutase (MnSOD) from bacteria shares high amino acid sequence homology and nearly identical structure. Despite of that, their characteristics are diverse, which likely due to their bacterial origin and adaptation to the environment. Most importantly, their structural similarity extends to eukaryotic MnSOD, *i.e.* human. Therefore, structural study of bacterial MnSOD is relevant to its human SOD and henceforth for its use in human as a therapeutic agent or a cosmetic ingredient. Further, eukaryotic MnSOD occurs as a tetramer while almost all of the prokaryotic are dimeric. In this review, relationship between the amino acid sequences and structures of MnSOD as well as their origin and evolution is discussed. The structures of FeSOD and cambialistic SOD, which are MnSOD closest homologs, are visited as the comparison. This study provides an insight to potential safe application of bacterial MnSOD, including necessary modifications to obtain desired characteristics for applications in human.

Key words: bacterial enzyme, environmental adaptation, manganese superoxide dismutase, structure-evolution relationship

Superoksida dismutase mangan (MnSOD) bakteri memiliki homologi urutan asam amino yang tinggi dan struktur yang hampir identik. Namun demikian, karakteristiknya beragam dan kemungkinan berkaitan dengan asal dan adaptasi bakteri ke lingkungan. Terlebih lagi, kemiripan struktur MnSOD berlanjut ke tingkat eukariot, misalnya manusia. Oleh sebab itu, kajian struktur MnSOD bakteri relevan untuk digunakan dalam kajian MnSOD manusia dan selanjutnya dapat membuka peluang untuk penggunaan MnSOD bakteri di manusia sebagai bahan terapetik atau kosmetik. Lebih jauh, MnSOD eukariot berbentuk tetramer sementara hampir semua MnSOD prokariot berbentuk dimer. Pada review ini dibahas mengenai hubungan antara urutan asam amino dan struktur MnSOD termasuk asal bakteri dan evolusinya. Struktur-struktur FeSOD dan *cambialistic* SOD, yang merupakan homolog terdekat MnSOD, juga dilihat sebagai perbandingan. Kajian ini memberikan pandangan mengenai potensi pemakaian MnSOD bakteri yang aman, termasuk upaya modifikasi yang perlu dilakukan agar karakteristik enzim lebih disesuaikan untuk penggunaan pada manusia.

Kata kunci: adaptasi lingkungan, enzim bakterial, hubungan struktur-evolusi, superoksida dismutase mangan

The outbreak of the corona virus (Covid) infection has alarmed the community on the severity of infectious diseases. Such infection often occurs in silent and becomes observable only when the symptom has surfaced, which signals that the inflammation has taken place. One of the subsequent events is disruption of the nitric oxide by the inflammatory superoxide (SO) species. In nature, the presence of SO is controlled by through the action of an enzyme called superoxide dismutase (SOD). Therefore, the use of SOD or its biomimetics in therapy for Covid patients has been developed (Karlsson *et al*. 2020). However, because its activity is one of the basic of chemical reactions occurring in the body, SOD plays a far greater role to maintain the physiology of the cells and the enzyme is associated with many other diseases. Hence, SOD can be developed as a therapeutic agents for many implications. Thereby, SOD with desired properties has been sought from various sources for this purpose. This paper will discuss the structural relationship between MnSOD, one of SOD types, from various sources and the structural information that is beneficial for future development of the enzyme in therapy.

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Manganese Superoxide Dismutase. Manganese superoxide dismutase (MnSOD, SOD-2/SOD-A, E.C. 1.15.1.1) plays an important role in survival of organisms against oxidative stress (Lushchak 2011). MnSOD catalyzes disproportionation of SO to result in oxygen (O_2) and hydrogen peroxide (H_2O_2) (Abreu and Cabelli 2010), which the latter will be further transformed into O_2 and water (H_2O) by catalase (Miller 2012, Mayo, Sainz *et al.* 2018). This cooperative action maintains SO at a negligible level and regards MnSOD as the first-line of defense for detoxification. Failure to eliminate SO leads to cell mortality as shown with MnSOD gene knock-out experiment in mice or the fruit fly *Drosophila melanogaster* (Li, Huang *et al.* 1995, Duttaroy *et al.* 2003).

MnSOD can be found in prokaryote and eukaryote. The enzyme occurs mostly in mitochondria and sometimes in peroxisome (Abreu and Cabelli 2010), which is a membrane-enclosed complex in the cytoplasm that contains oxidative enzymes. Its impaired regulation has severe impact on certain organs (Shimizu *et al*. 2010). In *Escherichia coli* and *Saccharomyces cerevisiae*, MnSOD expression is stimulated by a saturated level of O_2, which harms the cells from hyperbaric oxygen condition (Holley *et al.* 2011).

In *E. coli*, MnSOD cooperates with FeSOD enabling the bacteria to survive the environmental stress (Ganini *et al.* 2015). MnSOD is expressed as a response to the environment while FeSOD expression is related to indigenous activity of the cell. Also, MnSOD is ubiquitous while FeSOD is absent in higher organism (except in chloroplast) (Miller 2012). MnSOD and FeSOD are proposed to derive from a common ancestor (Imlay 2013). However, there is a possibility that they coexist and evolve independently because MnSOD can be traced to the eukaryotic cells archaeal origin while chloroplast FeSOD to cyanobacteria and protist FeSOD to bacteria (Sheng *et al.* 2014). MnSOD and FeSOD are likely to emerge before the branching into groups based on a evolutionary study involving MnSOD and catalase in cyanobacteria (Harada *et al.* 2021). Further, MnSOD is advised to evolve from FeSOD as a consequence of diminished bioavailability of iron (Miller 2012) and SOD with either Mn or Fe in its active site (cambialistic) is found in primitive anaerobic organisms (Miller 2012) and in unicellular organism *e.g. Plasmodium* (Mayo *et al.* 2018). Mn^-, Fe^- and cambialistic SOD share medium to high level of amino acid sequence homology but their three-dimensional structures are almost identical. Therefore, they are clustered as one family in structural studies and their structures serve as a perfect example for the higher conservation of structures over amino acid sequences. Furthermore, based on their structural similarity, mechanistic reaction of human MnSOD can be extrapolated from the structures of microbial MnSOD (Azadmanesh and Borgstahl 2018); this also allows microbial MnSOD use in human as a therapeutic agent or cosmetic ingredient.

MnSOD can be found in microbes of different living milieu (Indrayati *et al.* 2011). An evolution study of oxidative enzyme suggests that MnSOD emerged during the Proterozoic period and its encoding gene was found more in terrestrial than aquatic cyanobacteria (Boden *et al.* 2021). As MnSOD is expressed and regulated as a response to the environment, the enzyme character is often associated with the source organism. For example, a thermally stable MnSOD can be isolated from *Thermus thermophilus* (Lah, Dixon *et al.* 1995) that grows in hot-spring water (Henne *et al.* 2004). Thus, discrepancy in MnSOD thermal stability is associated with thermophile or mesophile classification (Azadmanesh and Borgstahl 2018). Similarly, MnSOD from *Deinococcus radiodurans* resists inactivation by ultraviolet as the bacteria lives under extreme oxidative conditions including ultraviolet and ionizing radiation (Makarova *et al.* 2001, Slade and Radman 2011).

The Structure of MnSOD Changes with the Evolution and Adaptation to the Environment. Structural relationship of MnSOD and to its homologs is depicted in Figure 1. Microbial MnSOD is clustered in branch 1 while from higher organisms in branch 3. FeSOD is clustered in branch 2 for both prokaryote and eukaryote. Note that archaeal FeSOD is clustered with eukaryotic MnSOD although they are rather distant. There is no clear trend for cambialistic, indicating that cambialism is more of bacteria adaptation to environmental change during chronological ages.

A comparative study between the structures of *Staphylococcus aureus* SOD suggests that cambialism emerges as a consequence of evolution that led to incorporation of different metal cofactor (Barwinska-Sendra *et al.* 2020) and likely involves lateral gene transfer (Boden *et al.* 2021). This phenomenon is observed when bacteria suffered from manganese depletion in the environment (Garcia *et al.* 2017). Thus, understanding the living conditions is pivotal to

study evolutionary relationship of SOD across species. In this instance, cambialistic SOD from *S. aureus* was concluded to evolve from MnSOD (Barwinska-Sendra *et al.* 2020) and thereby the evolutionary order begins with FeSOD, MnSOD, and finally the cambialistic. The returning use of Fe by the bacteria is possible according to different metal installation in the dimeric and tetrameric FeSOD (Miller 2012). In another phylogenetic study on oxidative enzymes from cyanobacteria concluded that MnSOD had evolved from cambialistic SOD while FeSOD evolved further from MnSOD (Harada *et al.* 2021). Thus, the genealogy of MnSOD and its homologs is yet to be established.

The structure-based relationship of Mn-, Fe-, and cambialistic SOD (Fig 1) supports the earlier hypothesis as shown by the archaeal FeSOD position, which are of the least distant from the branching point to the common ancestor. Despite the dispute, the aforementioned studies exemplify the importance of maintaining SOD activity. This prevails a relationship between the MnSOD structures to bacterial adaptation to the environment and evolution. The present study exploited that tripartite relationship with benefit of the doubling number of MnSOD structures in the past decade.

Oligomeric Form of MnSOD. In nature, MnSOD occurs as either a dimer or tetramer (dimer of dimer) (Azadmanesh and Borgstahl 2018). MnSOD from archaea, actinobacteria and eukaryote are tetrameric while bacterial MnSOD is dimeric (Sheng *et al.* 2014). The only bacterial MnSOD known with tetrameric form is from *T. thermophilus* (Fig 1). On the other hands, tetrameric FeSOD belongs to archaea and amoeba (lower eukaryotic cells). This situation further supports FeSOD older position than MnSOD in the lineage.

Already realized in the 90's, MnSOD dimer has two configurations in the tetrameric form (Wagner *et al.* 1993), in which one of the dimers rotates 84° respective to its equivalent in the tetramer of the other configuration. This difference is caused by a helix-hairpin in the monomer of one dimer protrudes to the monomer from the other dimer to form a-four antiparallel-helix bundle that locks the four monomers into a tightly packed tetramer. Such structural feature is present in human MnSOD but absent in *T. thermophilus* MnSOD (Wagner *et al.* 1993). In FeSOD from *M. tuberculosis,* another tetrameric configuration occurs from interactions between the surface loop and β-strands (Cooper *et al.* 1995); this configuration does not occur in MnSOD.

MnSOD tetramer has a ball-shape structure with a central cavity (Fig 2). This structure occurs in open (tetramer-1 and tetramer-2) and close (tetramer-3) form based on the accessibility of the central cavity. The volume of the central cavity in tetramer-1, -2, and -3 are ~4500Å^3, ~5000Å^3, and ~2500Å^3, respectively. The central cavity of the close form is much smaller and hence tetramer-3 configuration is more compact.

Differences in accessibility of the central cavity may be related to the enzyme adaptation to the environment. The phylogenetic analysis (Fig 1), tetramer-3 (the central cavity is near inaccessible) (Fig 2D) occurs in FeSOD from archaeal *Pyrobaculum aerophilum* and bacterial *M. tuberculosis* and is positioned at the earliest branching point. The more accessible central cavity is in tetramer-1 (Fig 2B) and -2 (Fig 2C), which the latter is the common one in eukaryotic Mn- and FeSOD. The shift to a more open form *i.e.* tetramer-1 to -2 in MnSOD or tetramer-3 to -2 in FeSOD appears to coincide with the shift from prokaryote to eukaryote.

MnSOD Structure and Their Implications. Formation of the tetramer in MnSOD is related to alteration of the enzyme characteristic according to the necessities of the organism. A study on potential cross-reactivity involving MnSOD from human, *Aspergillus fumigatus*, *S. cerevisiae*, *Drosophila melanogaster*, and *E. coli* indicates their recognition by IgE in the individuals that are infected by *A. fumigatus* (Crameri *et al.* 1996). The surface of their monomeric structures contains many potential IgE recognition sites that become inaccessible for IgE binding upon formation of the tetramer (Fluckiger *et al.* 2002). Tetramerization may also be as the response to temperature, salinity, or other changes in the environment. Tetrameric MnSOD from *S. cerevisiae* has higher thermal and pH-stability then the dimeric cambialistic SOD from *Candida albicans* (Sheng *et al.* 2013). Next, series of mutation shows that the half-life of human MnSOD at 37 °C is much shorter when substitution of certain amino acid residues causes disintegration of tetramer into dimer (Borgstahl *et al.* 1996, Hernandez-Saavedra *et al.* 2010). Thus, tetramerization also strengthens thermal stability of MnSOD through reinforcing the interactions at the dimer interface (Sheng *et al.* 2013).

The structure of *S. equorum* MnSOD (Retnoningrum *et al.* 2018) is dimeric, in which the dimer dissociates at 52-56 °C while the monomer is denatured at 63-68 °C (Retnoningrum *et al.* 2016). *S. equorum* is a mesophilic bacterium (Indrayati *et al.*

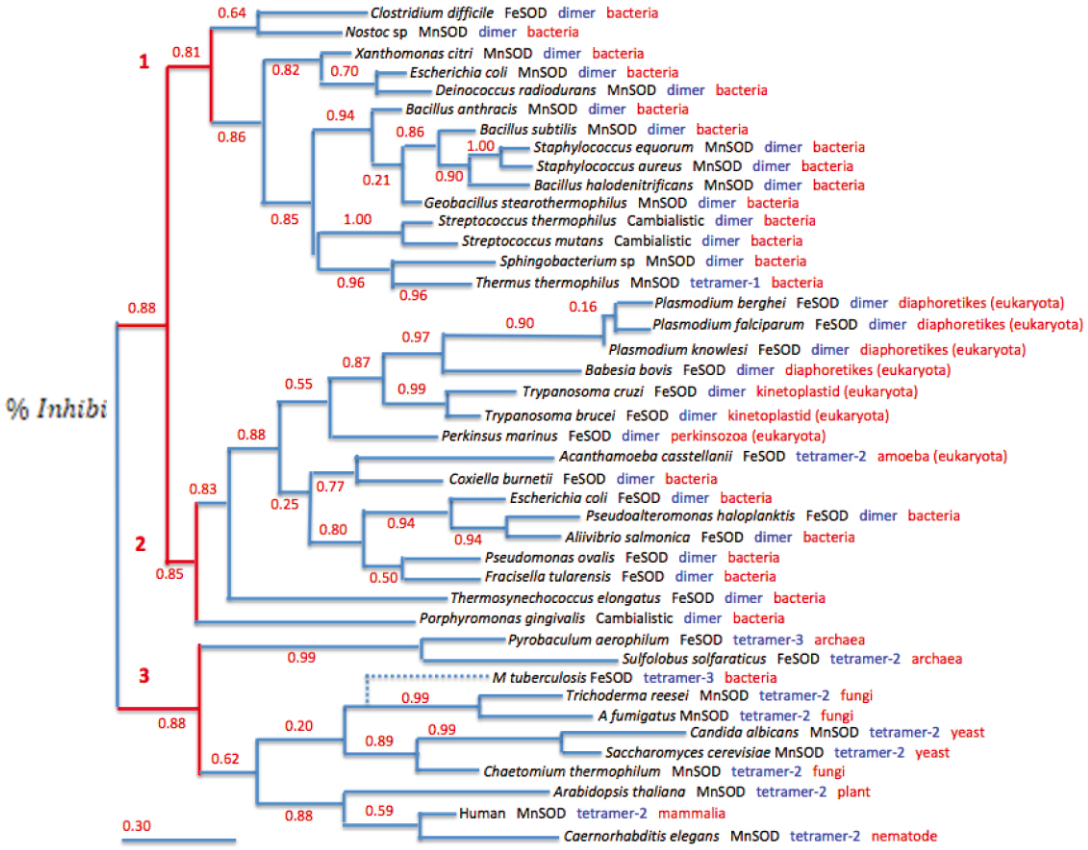

Fig 1 Phylogenetic tree of *S. equorum* MnSOD and its structural homologs based on their structures after a BLAST-P search (Altschul *et al.* 1997) using MnSOD*Seq* as the query. The output was consulted to the protein structure data bank. Relationship analysis was done with Phylogeny.fr service (Dereeper *et al.* 2008). Selected structures represent eukaryote and prokaryote. FeSOD and cambialistic SOD were also included. The number 1-3 denotes the branching points for the clustering. Tetramer-1 to tetramer-3 notation refers to the type of tetrameric form.

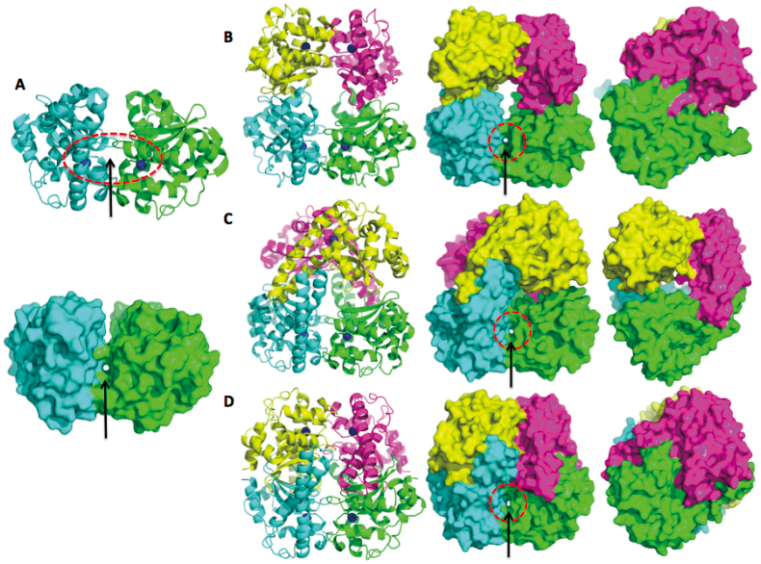

Fig 2 The structures of MnSOD dimer (A), MnSOD tetramer-1 (B), MnSOD tetramer-2 (C), and FeSOD tetramer-3 (D) are shown as ribbon and surface representation. The coloring is based on the monomer: green-A, cyan-B, purple-C (equivalent of A), and yellow-D (equivalent of B). *E. coli* MnSOD (PDB ID 1D5N, dimer), *A. castellanii* FeSOD (PDB ID 6J55, tetramer-1), *T. reesei* MnSOD (PDB ID 6DQY, tetramer-2), and *M. tuberculosis* MnSOD (PDB ID 1IDS, tetramer-3) represent different oligomers. The surface representation of the tetramers is showed at two sides of 90° rotation and oriented according to the ribbon. The dashed red circle indicates the active site in the dimer and the black arrow shows the channel for hypothetical direction of the substrate (product) to enter (leave) the active site. The pictures are prepared using PyMOL(DeLano 2008).

2011), thus the enz yme thermal stability was unique. The enzyme maintains its activity and dimeric form at either 37 °C and 52 °C, but its activity drops significantly with the loss of dimeric form, which undergoes a transition of a native-like structure at ~57 °C (Retnoningrum *et al.* 2017). Alteration of the monomer by an L169W substitution also resulted in a native-like structure that retains the enzyme activity but easily lost the dimeric form (Retnoningrum *et al.* 2018). The results indicates a "loose" dimer form that is similar to the "loose" tetrameric structure of MnSOD from *C. albicans* (Sheng *et al*. 2013). Following this revelation, a series of modification was done to strengthen the dimer, but they interfered with the enzyme activity (Retnoningrum *et al*. 2021, Retnoningrum *et al.* 2021).

SOD has been widely used as active ingredient in cosmetic and health care product, but has not received approval for therapy (Pedone *et al.* 2020). For use in human, the unwanted co-reactions *i.e.* evoking the immune system must be avoided, therefore antigenicity, immunogenicity and allergenicity must be checked to ensure safety. In this instance, antigenicity and immunogenicity of *S. equorum* MnSOD was studied *in silico* employing the program suites in the Immune Epitope Database Analysis Resource (Kim *et al.* 2012) and VaxiJen (Doytchinova and Flower 2007), using the human leukocyte antigen (HLA) group A*33:03 and B*15:02 (the highest frequency in east Asia population), also DRB1*A04:01 and DPA1*01:02/DPB1*04:02 (the most common in the world population) in the selection target (Sanchez-Mazas *et al.* 2005, Yuliwulandari *et al.* 2010). The results suggested that the fragments of the enzyme could not be recognized by the major histocompatibility complex (MHC) class II hence it would not interact with the antigen presenting cells that initiate the immune response. Thereby, *S. equorum* MnSOD was not considered antigenic or immunogenic. Next, an *in silico* testing with allergen prediction programs AllerTOP (Dimitrov *et al.* 2013), AlgPred (Saha and Raghava 2006), and AllerBase (Kadam *et al.* 2017) suggested that the enzyme was also not allergenic. However, its amino acid sequence shares over 35% homology and contain an identical fragment to the allergen in the FAO/WHO convention database (Ivanciuc *et al.* 2003, Fiers *et al.* 2004). The identical residues are part of the strictly conserved amino acid sequence 159-P-I/L-L-G-L/I-D-**V-W-E-H-A-Y-Y-L**-K/Q-Y-Q/K-175 (the allergenic potential is in underlined bold). This amino acid sequence is located in the dimer interface that facing the inner-cavity upon tetramerization. Thus, allergenicity of *S. equorum* MnSOD could be avoided when the enzyme is assembled into higher oligomeric states *i.e.* tetramer.

In conclusion, higher order structure of MnSOD from various organisms is diverse and likely arose during the evolution as a strategy in their adaptation to changes in the environment. This endogenous strategy can be adopted upon modification of the enzyme to improve its characteristics for use in therapy and cosmetics.

ACKNOWLEDGEMENTS

This work is financially funded by World Class Research scheme from Indonesian Ministry of Education, Culture, Research, and Technology.

Note: This review paper is dedicated for the late Dr. Debbie S. Retnoningrum, former Chief Editor of Microbiology Indonesia, who contributed significantly in Study of Bacterial Manganese Superoxide Dismutase in Indonesia.

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