Talaromyces marneffei Cu, Zn Superoxide Dismutase Recombinant Protein Expression in *Pichia pastoris*, Enzymatic Activity and Its Resistance to Oxidative Stress

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Abstract

Talaromyces marneffei is a dimorphic fungus that is known to cause a disease called talaromycosis, also known as penicilliosis, in immunocompromised individuals. The fungal pathogenicity and virulence factors remain unclear. Superoxide dismutase (SOD) is a neutralizing enzyme through reactive oxygen species generated by the host and has been proven to contribute to the virulence of many pathogenic bacteria and fungi. In this study, full-length *sodA* gene encoding *T. marneffei* Cu, Zn SOD was amplified, cloned into pPICzαB vector and successfully integrated into the *Pichia pastoris* yeast genome. The selected positive clone was induced for protein expression by methanol. An approximately 23 kDa molecular mass of secreted recombinant Cu, Zn SOD is enzymatically active which is like the native and standard enzyme. A rabbit polyclonal antibody raised against recombinant Cu, Zn SOD was proved to be reactive to the native enzyme by using Western blot analysis. pPICzαB/*sodA* also appeared to be more resistant than the control pPICzαB recombinant yeast in the oxidative stress conditions. This is the first study of the expression of recombinant *T. marneffei* Cu, Zn SOD protein and its enzyme activity determination. This enzyme is an important virulence factor and targeting this enzyme may be a promising strategy for developing new therapeutics.

Keywords: *Talaromyces marneffei*, Cu, Zn SOD, recombinant protein, antioxidant activity, oxidative stress

Introduction

Talaromycosis is an opportunistic mycotic disease, life-threatening especially in HIV-infected patients in areas in which the disease is endemic including northern areas of Thailand. The cause of this disease is the thermally dimorphic fungus, *Talaromyces* (*Penicillium*) *marneffei*. At room temperature or its normal environmental condition, this fungus grows into mycelial or saprophytic form. Once in the host tissue or cultured at 37°C with an appropriate medium, this fungus can transform into a yeast phase or parasitic form (Cooper & Haycocks, 2000; Kummasook et al., 2011). This transformation is thought to be an important virulence factor of the fungus, allowing it to evade the host immune system and establish a persistent infection. While much remains to be learned about the virulence factors of *T. marneffei*, the objective of ongoing research is to better understand the mechanisms by which the fungus causes disease and identify potential targets for new therapies.

Superoxide dismutase (SOD) is an important antioxidant enzyme that helps protect cells from damage caused by reactive oxygen species (ROS). It does this by catalyzing the conversion of superoxide radicals (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2), which is then further broken down to water (H_2O) by catalase

(Fridovich, 1995). This enzyme is known to be protective of many organisms from reactive oxygen intermediates (ROIs). Several different forms of SOD exist in various organisms. Prokaryotes typically have iron (Fe-SOD) and manganese (Mn-SOD) containing SOD, while eukaryotes have a manganese-containing form and a copper- and zinc-containing form (Cu, Zn SOD). SOD is one of the virulence factors in many pathogenic organisms, such as *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans* (Warrisa & Balloub, 2019; Ellett et al., 2018; Maurya & Namdeo, 2021). *sodA*-encoding Cu, Zn SOD has been characterized in *T. marneffei*, and is highly expressed in the yeast form of *T. marneffei* rather than conidia and mold form. The upregulation of *sodA* during macrophage infection assumes that this gene is important for dormant *T. marneffei* in the macrophage and spreads to other organs causing the dissemination of infection (Thirach et al., 2007).

We employed the *Pichia pastoris* expression system to produce the recombinant Cu, Zn superoxide dismutase protein from *T. marneffei*. This system offers advantages, such as fast growth and easy genetic manipulation, as well as eukaryotic features including a secretory pathway leading to correct protein processing and post-translational modifications. The recombinant protein was investigated for immunogenic properties and specific enzyme activity. Additionally, overexpressed Cu, Zn SOD yeast was tested for resistance to the various conditions of oxidative stress.

Materials and Methods

Cloning of Cu, Zn SOD gene and construction of expression vector

sodA coding sequence was amplified from cDNA clone by polymerase chain reaction (PCR) using forward primers, 5-ATCTCGAGAAAAGAGAGGCTGAAGCTGTTGTCAAGGCTGTCGCTGTCCT-3' and reverse primers, 5-CTGCGGCCGCCTAATGATGATGATGATGATGAGCAGAGATACCAATGAC-3'. The underlined sequences in forward and reverse primers encode for *Xho*I and *Not*I restriction sites, respectively. The PCR products were cut by restriction enzymes and cloned into the pPICzαB plasmid (Invitrogen, USA) to generate pPICzαB/ *sodA*. The plasmids were transformed into TOP10 *Escherichia coli* competent cells by chemical method (Thermo Scientific, USA). The positive clones were selected on LB medium containing 100 ug/ml zeocin (Invitrogen) and checked for insertion with specific primers by PCR. *Mss*I restriction enzyme was used to linearize approximately five micrograms of purified plasmid and transformed into *P. pastoris* X-33 competent yeast cells by electroporation (Bio-Rad, USA). The selected clone was isolated from yeast peptone dextrose sorbitol (YPDS) agar with 200 and 400 ug/ml of zeocin after incubated at 30°C, for 24-48 hr and checked for insertion by colony-PCR with specific primers and nucleotide sequencing.

Cu, Zn SOD expression and purification

Transformants which grew normally on the minimal dextrose with histidine (MDH) and minimal methanol with histidine (MMH) agar plates were isolated and then grown in buffered glycerol-complex medium (BMGY) for 12-15 hr until OD600 reached 2-6 to confirm the Mut^+ phenotype (Macauley-Patrick et al., 2005). The cells were harvested by centrifugation at 5,000xg for five min and then cultured in a buffered methanol-complex medium (BMMY). Methanol was added every day (day 0-3) to give 0.5% (v/v) to induce recombinant protein expression. The Cu, Zn SOD recombinant protein was secreted into a culture medium by the α-factor secretion signal of the plasmid. The culture supernatant was collected by centrifugation and filtered through a 0.22 um

membrane filter to sterilize and remove the remaining cells. The protein was concentrated using VivaSpin™ protein concentrator spin columns (GE Healthcare Life Sciences, USA). The recombinant Cu, Zn SOD protein was then purified with nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the supplier's manuals (Macherey-Nagel, USA). The eluted protein was carefully pipetted into new tubes then the eluting step was repeated twice and the proteins were then stored at -80°C. The concentrations of protein were determined by Bradford's method. The purity of the protein was checked by SDS-PAGE (15% w/v) and stained with Coomassie brilliant blue.

Protein characterization and identification

His-tagged fusion proteins were detected with Western blot by using the chromogenic Ni-NTA conjugates system (Kirkegaard and Perry Laboratories, USA). Protein identification was performed by Liquid Chromatography Mass Spectrometer (LC/MS) at the Proteomics Service Center, Mahidol University, Thailand.

Enzyme activity assay

The non-denaturing gel enzyme activity assay was performed for Cu, Zn SOD (Bertrand & Eze, 2014; Chang & So, 1999). In this assay, after electrophoresis, the gel was incubated in 1.23 mM NBT (Sigma-Aldrich, USA) in the dark for 15 min with orbital shaking at room temperature, then washed in 0.1 M KH_2PO_4, pH 7.0. The gel was incubated in 0.1 M KH_2PO_4 buffer-containing 28 μM riboflavin and 28 mM TEMED in the dark for 15 min at room temperature. The gel was exposed to a 13 W fluorescent tube for 10 min after washing in 0.1 M KH_2PO_4 and 5 mM potassium cyanide (KCN) was used for enzyme inhibition to confirm the specific activity of recombinant SOD. The positive control in this assay was bovine erythrocyte SOD (Sigma-Aldrich). The enzyme activity levels were measured with an SOD assay kit (Sigma-Aldrich).

Viability under oxidative stress conditions

The recombinant strains carrying pPICzαB/*sodA* vector and the control yeast with empty pPICzαB were cultured for protein expression for three days. The cultures were adjusted to 10^6 cells/ml and supplemented with 5 mM of paraquat, menadione, or H_2O_2 for one hr or heated at 48°C for 45 min and then serially tenfold diluted in sterile water to 10^{-1}, 10^{-2} and 10^{-3}. Samples of 20 ul of each dilution were dropped on YPD agar plates and incubated for three days at 30°C.

Western blotting

Cu, Zn SOD recombinant protein was subject to 15% gels SDS-PAGE and Western blotting as described previously (Holdom et al., 2000). The blots were developed with a rabbit anti-SOD polyclonal antiserum (1:500 and 1:1000), then incubated with peroxidase-linked goat anti-rabbit immunoglobulin G (1:1000) or peroxidase-linked goat antihuman IgG (Jackson Immunochemicals, USA) (1:250). Finally, blots were developed with 3,3,9-diaminobenzidine and 4-chloronaphthol after washing.

Results

Heterologous expression and purification of recombinant Cu, Zn SOD

The *sodA* cDNA was inserted into the yeast *P. pastoris* X-33 expression system, and the heterologous expression of Cu, Zn SOD was under transcriptional control of the *AOX1* promoter. After methanol induction, Cu, Zn SOD was successfully expressed and secreted. SDS-PAGE of the induced recombinant Cu, Zn SOD from a recombinant yeast is shown in (Fig.1). The protein band was detected after concentration using VivaSpin™

protein concentrator spin columns. The expressed protein was purified with Ni-NTA agarose. The single band of purified protein with a molecular weight of about 23 kDa was observed on SDS-PAGE and close to the fungal predicted molecular weight protein (Fig. 1A).

Properties of Cu, Zn SOD recombinant

The culture supernatant of the selected clone was collected on day three and concentrated by lyophilization. Then the single-band recombinant protein was purified by Ni-NTA agarose. The his-tag of the recombinant protein was detected by Western blot analysis, the same molecular weight of protein from the concentrate spin column and Ni-NTA agarose purification could be observed (Fig. 1B). The band of protein was cut from the gel and eluted for protein analysis by LC/MS. The results showed the matching of amino acid fragments to *T. marneffei* Cu, Zn superoxide dismutase SOD1 (ATCC18224) accession XP_002147430. The recombinant and native proteins were immunoenzyme developed with the Cu, Zn SOD polyclonal antibody showing intense reactivity. The wildtype SOD was predictably translated into 154 amino acids protein which calculable molecular weight as 16 kDa. The Western blots showed that the protein band was recognized by the rabbit polyclonal antibody raised against the recombinant protein at approximately the same relative molecular mass as the native protein (Fig. 1C). The molecular mass of the recombinant of the enzyme was detected to be 23 kDa, whereas the native form of the protein is 19 kDa.

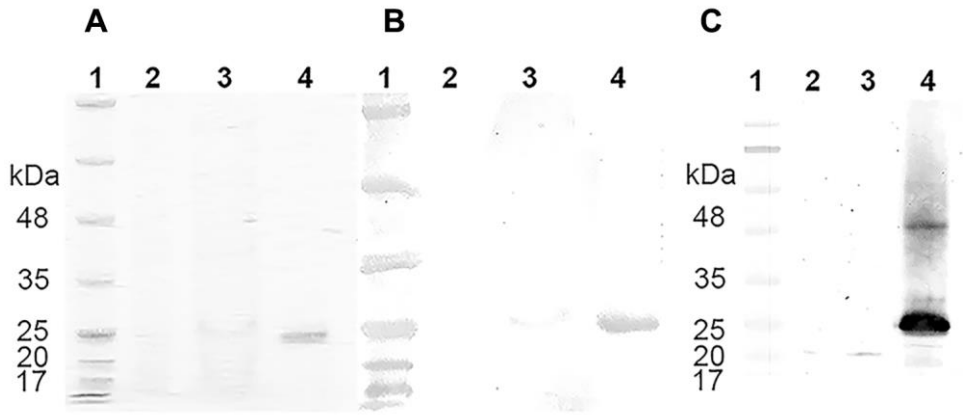

Figure 1 Analysis of recombinant *T. marneffei* Cu, Zn SOD by SDS-PAGE (A) Western blotting (B); detection of his-tagged fusion proteins with Ni-HRP and colorimetric developed with TMB. lane 1, protein marker; lane 2, original culture supernatant from positive clone; lane 3, concentrated supernatant of recombinant Cu, Zn SOD by VivaSpin™ protein concentrator spin columns; lane 4, Ni-NTA agarose purified recombinant Cu, Zn SOD. Immunoenzyme development with rabbit anti-Cu, Zn SOD polyclonal antibody (C). Lane 1, molecular size markers, lanes 2 and 3, total protein from *T. marneffei* mold and yeast; lane 4, purified recombinant Cu, Zn SOD

Enzymatic activity

The SOD recombinant protein was purified from the culture supernatant secreted by pPICzαB/*sodA* by the procedure using a Ni-NTA agarose. The initial quantity and specific activity of the recombinant Cu, Zn SOD in the culture supernatant was 60 mg and 57 U/mg. After purification, 1.83 mg of Cu, Zn SOD with a specific activity of 352 U/mg and a total activity of 644 U were obtained. Table 1 summarizes the purification protein results. The clear bands in the non-denaturing gel are the reaction of free radicals and superoxide dismutase enzyme. The results show that the SOD activity from purified recombinant protein from the culture supernatant of

the positive clone is similar to the SOD standard. The native enzyme activities were detected in total protein extracted from the mold and yeast form of *T. marneffei*. These results were correlated with the detections of the native protein with immunoenzyme (Fig. 2).

Table 1 Purification of recombinant SOD expressed by *Pichia pastoris*

Step	Volume (ml)	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)
Culture supernatant	200	60	3440	57	100
Ni-NTA agarose	4	1.83	644	352	18.7

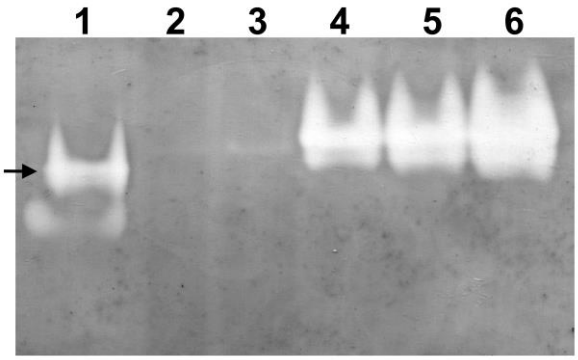

Figure 2 Cu, Zn SOD activity in native PAGE. Non-reducing proteins were run on 15% native PAGE and stained with NBT before light exposure. Lane 1, recombinant Cu, Zn SOD; Lane 2-3, 10 ug of total protein from *T. marneffei* mold and yeast; Lane 4-6, bovine erythrocyte Cu, Zn SOD standard 1, 5 and 10 U. The arrow points to the clear band of recombinant enzyme activity

Viability under oxidative stress conditions

To evaluate the protective role of the Cu, Zn SOD, the recombinant yeast contained pPICzαB/*sodA* and the control yeast contained pPICzαB were incubated with 5 mM menadione, paraquat or H_2O_2 which cause oxidative stress conditions that were then spotted on the YPD plates. After incubation, both control and recombinant yeast cells grew at comparable rates on the YPD agar plates without any stressing agents. Cu, Zn SOD recombinant yeast were more resistant to oxidative stress conditions than the control (Fig. 3).

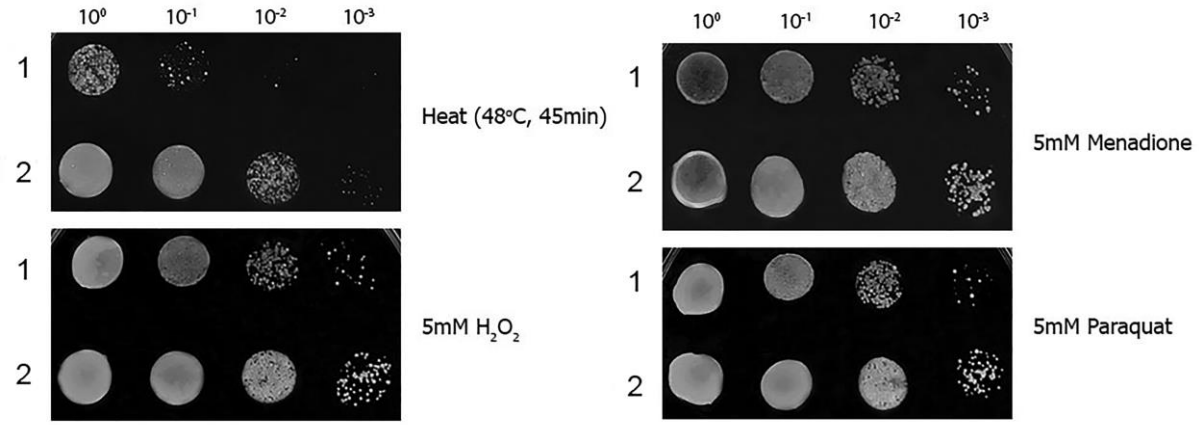

Figure 3 The oxidative stress resistance tests. The recombinant of pPICzαB control (1) and pPICzαB/*sodA* (2) were grown for expression at 30°C for 72 hours, adjusted to the same absorbance (OD600), incubated with heat at 48°C for 45 min or 5 mM menadione, paraquat, H_2O_2 for one hr, then serially ten-fold diluted in PBS and dropped on YPD plates

Discussion

The *sodA* cDNA was transformed into the *Pichia pastoris* expression system. After being induced with methanol, the Cu, Zn SOD was successfully expressed and excreted. Three days after induction, the secreted proteins were concentrated by lyophilization, and further purified with Ni-NTA agarose. Despite the low purification yield, the use of this method results in a faster and less complicated purification than other methods such as protein concentrator spin columns. Moreover, recombinant SOD in sufficient amounts of protein for subsequent characterization was obtained.

The wildtype SOD is predictably calculated to have a molecular weight of 16 kDa. Western blotting demonstrated approximately 19 kDa of the native Cu, Zn SOD that was detected by the anti-Cu, Zn SOD antibody. In addition, an approximately 23 kDa single band purified enzyme was presented on SDS-PAGE that estimated 4 kDa added up from native enzyme. The difference is because of the post-translational modification of protein such as glycosylation that was previously described for Cu, Zn SOD of *Aspergillus fumigatus* or the presence of the polyhistidine tag and signal peptides. Remarkably, the results from mass spectrophotometry and the specific activity of the enzyme that was inhibited with 5 mM KCN further confirmed that the recombinant SOD from *T. marneffei* belonged to the Cu, Zn SOD classification. The intracellular SOD (Cu, Zn SOD) are found in mammals and humans with approximately 32 kDa homodimer and 15-18 kDa monomers (Holdom et al., 2000). The standard bovine erythrocyte SOD appeared to be two subunits in non-denaturing gel enzymatic activity detection, and we could detect the activity of the native enzyme in the mold and yeast phases of *T. marneffei* corresponded to the standard. Interestingly, the activity of recombinant enzyme appears as a homodimer, separating into two distinct bands because of concentration and purification processes.

The immunoreactivity of a rabbit polyclonal antibody raised against recombinant Cu, Zn SOD to *T. marneffei* native protein from the mold and yeast form confirms the antigenic properties of this enzyme. Furthermore, the amount of Cu, Zn SOD could be detected in yeast higher than in the mold form as was expected from information in a previous study (Thirach et al., 2007). This recombinant enzyme was recognized by human immune sera to verify an antigenic property. However, we found only 20% (3 from 15 samples) of talaromycosis patients' sera reacted with recombinant Cu, Zn SOD by Western blot (data not shown). Immunocompromised patients, such as those with HIV infection, may have a weakened immune system and therefore produce a lower or undetectable level of antibodies in response to *T. marneffei* infection compared to immunocompetent individuals. In addition, the severity of immunosuppression may vary between different patients, which could lead to variations in the antibody response (Holdom et al., 1995). Another possible explanation for the differential antibody response is that this fungus may have different antigenic variants, which could lead to variations in the antibody response among different patients. Oxygen-dependent killing mechanisms are very important for removing *T. marneffei* from the infected host, including the myeloperoxidase, hydrogen peroxide, superoxide radical anion, and reactive nitrogen intermediate responses of macrophages (Kudeken et al., 1998; Kudeken et al., 1999). Therefore, the antioxidant defense systems probably are essential for organisms to resist the host immune mechanism and to exhibit full virulence itself (Zhang et al., 2011; Li & Yu, 2007). To better understand, the phagocytosis-killing assay can be used to evaluation of the protection mediating antibodies to Cu, Zn SOD during infection (Lu et al., 2013; Sapmak et al., 2016; Amsri et al., 2021).

Conclusion and Suggestions

We first constructed the recombinant yeast and produced the Cu, Zn SOD recombinant protein of pathogenic fungi, *T. marneffei*. The recombinant yeast overexpressing Cu, Zn SOD is potentially more resistant to oxidative stress than the control, which suggests that Cu, Zn SOD of *T. marneffei* play roles in protection from oxidative stresses. Further investigations to determine the fungal fate in macrophages, and its virulence in an animal infection model, are necessary to prove that Cu, Zn SOD is involved in the virulence of *T. marneffei* and to provide important information as to how the antioxidant enzymes of pathogenic fungi function in the infection process. This study provides information that elucidates the function and importance of the Cu, Zn SOD from *T. marneffei* in protecting the fungus from oxidative stress during infection. Cu, Zn SOD could also have implications for the development of new therapies for talaromycosis and other fungal infections, by identifying potential targets for drug development.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was approved by Ethical Committee of Naresuan University Institutional Review Board (NU-IRB) (the number of Ethics Committee protocol: IR.TUMS.SPH.REC.1395.25287)

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Author Contributions

Author 1 (Sophit Khanthawong): Conceptualization of the research, development of methodology, collection of data and review of the manuscript.

Author 2 (Kanruethai Wongsawan) and 3 (Ronachai Pratanaphon): Formulated the topic of the research, interpreted the analysed data, and reviewed and edited the manuscript.

Author 4 (Nongnuch Vanittanakom): Developed the methodology, reviewed and corrected the manuscript.

Conflict of Interests

The authors declare no conflicts of interest.

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