UNIVERSITI PUTRA MALAYSIA

EXPRESSION AND CHARACTERIZATION OF A RECOMBINANT SUPEROXIDE DISMUTASE FROM *LACTOCOCCUS LACTIS* M4

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EXPRESSION AND CHARACTERIZATION OF A RECOMBINANT SUPEROXIDE DISMUTASE FROM *LACTOCOCCUS LACTIS* M4

By

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EXPRESSION AND CHARACTERIZATION OF A RECOMBINANT SUPEROXIDE DISMUTASE FROM *LACTOCOCCUS LACTIS* M4

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May 2009

Chair : Raha Abdul Rahim, PhD

Faculty : Biotechnology and Biomolecular Sciences

Lactococcus lactis is widely used in the dairy industry for the production of fermented food. During industrial process, *L. lactis* is often exposed to various environmental stresses such as oxidative stress. Superoxide dismutase (SOD) plays an important role in protecting living organisms from oxidative stress by catalyzing the dismutation of superoxide radical to oxygen and hydrogen peroxide. Hence, it is essential to study the SOD from *L. lactis* in details. A full-length superoxide dismutase gene (*sod*) was amplified from a locally isolated *Lactococcus lactis* M4 strain by polymerase chain reaction (PCR). The gene was first cloned in pCR®-BluntII-TOPO® vector and then subcloned into pRSET A expresion vector. The construct was transformed into *Escherichia coli* strain BL21(DE3)pLysS for protein expression. Restriction enzyme digestion of the construct indicated the presence of

the s*od* gene. BLASTN analysis showed the DNA sequence of the query gene was 98% homologous to the published *sod*A nucleotide sequence of *L. lactis* subsp. *lactis* IL1403. This SOD gene composed of 621 nucleotides that could encode a protein of 206 amino acids. It was predicted to be a manganese-SOD (MnSOD) based on homology comparison with amino acid sequences of MnSOD from other organisms. Expression of the recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside. The recombinant superoxide dismutase was purified to homogeneity by immobilised ion affinity chromatography and gel filtration chromatography. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that the recombinant SOD had a molecular mass of approximately 27 kDa. However, the molecular mass of native enzyme was estimated to be 114.8 kDa by gel filtration chromatography, implying that the recombinant SOD is a tetramer. The purified recombinant enzyme had a pI of 4.5, exhibited maximal activity at 25°C and pH 7.2, respectively. It was also thermostable up to 45°C. SOD activity was inhibited by sodium azide, ethylene diamine tetracetic acid and sodium dodecyl sulphate. The insensitivity of this recombinant SOD to cyanide and hydrogen peroxide confirmed that it was a MnSOD. In conclusion, a gene coding for MnSOD in *L. lactis* M4 was cloned and expressed in *E. coli* as an active enzyme. The expressed recombinant MnSOD was purified to homogeneity and characterized.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk ijazah Sarjana Sains

PENZAHIRAN DAN PENCIRIAN REKOMBINAN SUPEROKSIDA DISMUTASE DARIPADA *LACTOCOCCUS LACTIS* M4

Oleh

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Lactococcus lactis digunakan secara meluas dalam industri tenusu bagi penghasilan makanan fermentasi. Semasa proses industri, *L. lactis* selalu terdedah kepada pelbagai jenis tekanan alam sekitar seperti tekanan oksidatif. Superoksida dismutase (SOD) memainkan peranan penting dalam melindungi organisma hidup daripada tekanan oksidatif dengan merangsangkan dismutasi radikal superoksida kepada oksigen dan hidrogen peroksida. Dengan demikian, kajian ke atas *L. lactis* SOD secara mendalam adalah penting. Gen SOD telah diamplifikasikan daripada strain tempatan *Lactoccocus lactis* M4 dengan menggunakan teknik Tindak balas Berantai Polimerase (PCR). Gen ini diklonkan ke dalam vector pCR®-BluntII-TOPO® terlebih dahulu dan seterusnya ke dalam vector penzahiran pRSET A. Gen ini

ditransformasikan ke dalam strain *E. coli* BL21(DES)pLysS. Pencernaan dengan enzim pembatas menunjukkan kehadiran gen *sod* dalam konstruk ini. Analisis BLASTN menunjukkan jujukan gen berkenaan adalah 98% homologi dengan jujukan nukleotida gen *sod*A daripada *L. lactis* subsp. *lactis* IL1403. Gen SOD ini terdiri daripada 621 nukleotida-nukleotida yang mengekod suatu protein yang mengandungi 206 asid amino. Ia diramal sebagai satu mangan-SOD (MnSOD) berdasarkan kepada pembandingan homologi dengan jujukan asid amino MnSOD daripada organisma-organisma lain. Penzahiran rekombinan protein ini diaruh oleh isopropil-β-D-thiogalaktopiranosida. Rekombinan SOD berjaya ditulenkan dengan menggunakan kromatografi afiniti tersekatgerak ion logam dan kromatografi penurasan gel. Analisis SDS-PAGE dan "western blot" yang dijalankan ke atas rekombinan SOD ini menganggarkan berat molekul rekombinan SOD adalah sebanyak 27 kDa. Namun demikian, kromatografi penurasan gel mendapati bahawa rekombinan SOD bersifat asli mempunyai anggaran berat molekul sebanyak 114.8 kDa dan mencadangkan bahawa rekombinan SOD ini mungkin adalah tetramer. Rekombinan enzim tulen mempunyai nilai pI 4.5, di samping menunjukkan activiti maksima pada suhu 25°C dan pH 7.2. Rekombinan enzim ini juga stabil terhadap rangsangan haba sehingga ke suhu 45°C. Aktiviti SOD disekat oleh natrium azida, asid etilena diamina tetrasetik dan natrium dodesil sulfat. Rekombinan SOD tidak sensitif terhadap rawatan sianida dan hidrogen peroksida. Ini membuktikan bahawa rekombinan SOD ini tergolong dalam keluarga MnSOD. Pada kesimpulannya, MnSOD gen daripada *L. lactis* telah diklonkan dan dizahirkan ke dalam *E. coli* sebagai satu enzim yang aktif. Penulenan and pencirian rekombinan MnSOD telah dilaksanakan selepas penzahiran.

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I certify that a Thesis Examination Committee has met on 8 May 2009 to conduct the final examination of Tan Boon Hooi on her thesis entitled "Expression and Characterization of a Recombinant Superoxide Dismutase from *Lactococcus lactis* M4" in accordance with the Universities and Universities Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Date: 29 May 2009

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledge. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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TAN BOON HOOI

Date: 13 May 2009

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LIST OF ABBREVIATIONS

bp	base pair
BLAST	Basic Local Alignment Search Tool
$CaCl_2$	calcium chloride
CM	carboxymethyl
CuZnSOD	copper-zinc-containing superoxide dismutase
D	Aspartic acid
DAB	3, 3'-diaminobenzidine
DB	dilution buffer
DBT	dilution buffer with 1% (v/v) Tween 20
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
ddH_2O	double-distilled water
dH_2O	distilled water
dNTP	deoxynucleotide triphosphate
ECSOD	extracellular superoxide dismutase
EDTA	ethylene diamine tetra acetate
EK	enterokinase
FDA	Food and Drug Administration
FeSOD	iron-containing superoxide dismutase
FPLC	Fast Protein Liquid Charomatography
GRAS	Generally Recognized as Safe
GST	glutathione-S-transferase
H_2O	water
H_2O_2	hydrogen peroxide

HCl	hydrochloric acid
His (H)	Histidine
HRP	horseradish peroxidase
IEF	Isoelectric focusing
IMAC	Immobilised metal ion affinity chromatography
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
KCN	potassium cyanide
LAB	lactic acid bacteria
LB	Luria-Bertani
L. lactis	*Lactococcus lactis*
Lys	lysine
mA	milliAmpere
MCS	multiple cloning sites
MnSOD	manganese-containing superoxide dismutase
MW	molecular weight
NaN_3	sodium azide
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
NiSOD	nickel-containing superoxide dismutase
O_2^-	superoxide radical
OD	optical density
·OH	hydroxyl radical
ori	origin
PAG	polyacrylamide gel

PAGE	polyacrylamide gel elctrophoresis
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
Pfu	*Pyrococcus furiossus*
PVDF	polyvinylidene fluoride
RE	restriction digestion
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
sod	superoxide dismutase gene
subsp.	subspecies
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	tetramethyl-ethylene diamine
T_m	melting temperature
Trx	thioredoxin
UV	ultraviolet
V	voltage

CHAPTER 1

INTRODUCTION

Most of the living organisms consume oxygen (O_2) as an important element to support their lives. However, there are several disadvantages related to the utilization of oxygen which are linked to the potential toxicities it possesses. During the partial reduction of O_2, reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are formed. These ROS impose oxidative stress which can cause oxidative damage to the cells, including deoxyribonucleic acid (DNA) strand breakage, protein inactivation and membrane lipid peroxidation (Kreig and Hoffman, 1986). Superoxide dismutase (SOD) plays a vital role in the defense mechanism against the oxidative stress by catalyzing the formation of H_2O_2 and O_2 from O_2^- (Fridovich, 1986), thus protects living organism from oxidative lethality. SOD has found applications in gene therapy, used for cardiovascular diseases as well as in the pharmaceutical and cosmetic industries. It is also of great interest to involve SOD in potential therapeutic treatments for senescence, cell impairment and carcinogenesis (Li *et al*., 2005).

SOD can be found in almost all aerobic and some anaerobic organisms. SOD can be classified into 4 groups according to their metal cofactor: manganese (MnSOD), iron (FeSOD), copper-zinc (CuZnSOD), and nickel (NiSOD). MnSOD, encoded by *sodA* (Takeda and Avila, 1986), is found in prokaryotes and in mitochondria matrix of

eukaryotes. MnSOD and FeSOD are structurally very similar whereas CuZnSOD is not related (Stallings *et al*., 1984). All previously tested streptococci (including *Lactococcus lactis* subsp. *lactis*) appear to carry a MnSOD (Zitzelsberger *et al*., 1984).

Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) by the United States Food and Drug Administration (FDA) because they contain peptides that are readily digested in the human intestines. LAB such as *Lactobacillus* sp. and *Lactococcus* sp., are widely used for the production of fermented food products. During industrial processes, LAB are often exposed to multiple environmental stresses that can cause loss or reduction of bacterial viability, reproducibility, as well as organoleptic or fermentative qualities. Oxidative stress is among the most deleterious to the cells. *L. lactis* circumvent the threats from superoxide radicals by SOD. However, most lactobaccilli lack this SOD defense system. *Lactobacillus plantarum* developed an alternative nonenzymatic defense system by accumulating high intracellular Mn^{2+} concentration which can scavenge O_2^- (Archibald and Fridovich, 1981).

The importance of LAB in human health is becoming more significant since they are considered as safe and natural. Apart from being manufactured as probiotics, LAB could also be used as vehicles for the delivery of pharmaceutical or nutraceutical agents (Kaur *et al*., 2002). LAB that express SOD can be used to prevent lipid peroxidation. The GRAS status of *L. lactis* is a distinct advantage for its use in the production and secretion of therapeutic or vaccine proteins (Wegmann, 2007; Le Loir *et al*., 2005). The lack of

endogenous SOD may account for high sensitivity of most species of *Lactobaccillus* to oxidative stress (Roy *et al*., 1993).

Impelled by the advent of recombinant DNA technology, many biology aspects, such as physiology, biochemistry and genetics of these bacteria has been greatly exploited. Several studies have been carried out to clone and express the SOD from other organisms in *Lactococcus* and *Lactobacillus* (Bruno-Bárcena *et al.*, 2004; Roy *et al*., 1993), which demonstrated the importance of SOD in protection against oxidative toxicity. Researchers have made considerable efforts during the last two decades to improve knowledge of oxidative stress in *L. lactis*. Since *L. lactis* is of great economic importance, it is essential to study the lactococcal SOD extensively in order to explore its features and properties. *L. lactis* SOD has been measured qualitatively and quantitatively under various culture conditions (Chang and So, 1999). Nucleotide sequencing of lactococal *sodA* and homology comparison of the deduced amino acids with other SODs has been done (Sanders *et al.*, 1995), but further characterization of this enzyme has yet to be performed.

In this study, a full-length SOD gene from a locally isolated *L. lactis* M4 was cloned into pRSET A expression vector that utilizes the T7 promoter system. The recombinant SOD gene was expressed in *Escherichia coli* BL21(DE3)pLysS competent cells which has a simple inducible system that can provide high-level protein expression. Purification and characterization of this SOD were performed to attain better insight of the lactoccocal SOD.

The objectives of this study were:

1. To clone and express the SOD gene from locally isolated *Lactococcus lactis* M4 in *E. coli*.
2. To purify the expressed recombinant SOD.
3. To characterize the purified recombinant SOD physico-chemically.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are phylogenetically members of the *Clostridium-Bacillus* subdivision of Gram-positive Eubacteria. They are defined as a group of microaerophilic, Gram-positive organisms that ferment hexose sugars to produce primarily lactic acid (Makarova *et al.*, 2006; Bolotin *et al*., 2001). According to Orla-Jensen (1919), the "true lactic acid bacteria" form a natural group of gram-positive, non-motile, non-sporeforming, rod- and coccus-shaped organisms that ferment carbohydrates to form mainly lactic acid and alcohol. The genera include *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Ray, 1996). Their DNA base composition is less than 50 mol % G+C. Hexose sugar such as glucose is metabolized into lactic acid via homofermentative pathway, or to lactic acid, carbon dioxide, acetic acid, and/or ethanol via heterofermentative pathway (Pot *et al*., 1994).

LAB are commonly found in foods, such as fermented meat, vegetables, fruits, beverages and dairy products. They are naturally present in the respiratory, intestinal and genital tracts of human and animals, in sewage and in plant materials (Stiles and Holzapfel, 1997; Pot *et al*., 1994). Isolates of the same species are frequently obtained from plant, dairy, and animal habitats, indicating wide

distribution and specialized adaptation to these diverse environments (Makarova *et al.*, 2006).

Due to the ability of LAB in producing large amount of lactic acid and growth inhibitory substances, they are widely used in the production of fermented food including dairy products, meat and vegetables (Miyoshi *et al*., 2003). LAB are also vital for the production of wine, coffee, silage, cocoa, sourdough, and numerous indigenous food fermentations. These bacteria are responsible for both preservation and sensory characteristics, such as colour, flavour, and texture., and thus, they have been traditionally used as the starter cultures for the fermentation of foods and beverages (De Vuyst and Vandamme, 1994). LAB also contribute to the human health such as control of tumours and promote longevity of the mankind (Adachi, 1996). The milk products fermented with LAB, such as *L. lactis* exhibited anti-tumour and anti-mutagenic activity. Some of the LAB, such as *Lactobacillus* and *Enterococcus*, are recognized as probiotic bacteria that exhibit a beneficial effect on the health of host by improving its intestinal microbial balance (Kaur *et al*., 2002). Recent researches are aimed to develop a vaccine delivery system using this non-invasive bacterium (Robinson *et al.*, 1997; Wells *et al.*, 1996; Norton *et al.*, 1995), or even as oral vaccines (Steidler *et al*., 2000).

2.2 *Lactococcus lactis*

Lactococcus is a member of the group of LAB based on the ability to produce lactic acid from hexose sugar fermentation (Pot *et al.*, 1994). They were first known as lactic streptococci. Since 1985, most of the Lancefield group N lactic streptococci

have been transferred to the genus *Lactococcus* (Stiles and Holzapfel, 1997). Currently, there are 5 species in the genus *Lactococcus*, which are *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus piscium*, *Lactococcus plantarum*, and *Lactococcus raffinolactis*. In order to distinguish the "dairy streptococci" from the streptococci that contain a number of notorious human pathogen, they were reclassified into two *L. lactis* subspecies, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris*, which were previously designated as *Streptococcus lactis* and *Streptococcus cremoris*, respectively (Schleifer *et al*., 1985).

Lactococci are Gram-positive bacteria with ovoid elongated shape at about 0.5 – 1.0µm in diameter. They are present in pairs or short chains, nonmotile, nonsporulating, facultative anaerobic to microaerophilic (Miyoshi *et al*., 2003; Stiles and Holzapfel, 1997). Their natural habitats are green vegetation, silage, dairy environment, and raw milk. In general, they grow well between 20-30^{o}C, but not at 45^{o}C. They are mesophiles but can grow at 10^{o}C. They do not grow in 6.5% NaCl or at pH 9.6. In a suitable broth, they can produce 1% L(+)-lactic acid and reduce the pH to about 4.5 (Ray, 1996).

Lactococci has been categorized as "Generally Recognized as Safe" (GRAS) by the United States Food and Drug Administration (FDA). Among the 5 species of *Lactococcus*, only *L. lactis* has been widely used in dairy fermentation, which comprise 3 subspecies: *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *hordniae*, but only the first two are involved in the making of many dairy products, for example, the production of butter, buttermilk, lactic butter, sour cream and several cheeses (De Vuyst and Vandamme, 1994). *L. lactis* subsp. *lactis* is