UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF A SULFURAMINO ACID LYASE FROM *CITROBACTER FREUNDII* (KP25)

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FSMB 2003 13

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

February 2003

DEDICATION

"Dedicated to my beloved mom, dad and bro and also to all my friends."

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: Professor Hasanah Mohd. Ghazali, Ph.D.

Faculty: Food Science and Biotechnology

L-Methionine γ-lyase (EC 4.4.1.11; LMGL) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the direct conversion of L-methionine to α-ketobutyrate, methanethiol and ammonia by an α,γ-elimination reaction. Seventy nine LMGL-producing microorganisms isolates were screened from six local sources by the 5'5-dithiobis (2-nitrobenzoic acid) (DTNB) test. The six local sources were soil samples from around the Faculty of Food Science and Biotechnology and Central Research Laboratory, Universiti Putra Malaysia and Kuantan sea coast, soil and water samples from hot springs in Ulu Legong, Baling, Kedah and Pedas, Negeri Sembilan and intestine samples from chicken. A simple and convenient colorimetric screening method, the DTNB test detects methanethiol, which reduces DTNB contained in an agar-plate medium to form yellow colour aryl mercaptan (4 thiol-2-nitro-benzoate) around the colony of a bacterium that is able to produce LMGL. LMGL was detected from 45 (57%) of the bacterial isolates by 3-methyl-2-benzothiazolone hydrazone (MBTH) assay. LMGL activity was quantitatively assayed by determining the amount of α-ketobutyrate produced spectrophotometrically at 320 nm after derivatization with MBTH. Twelve relatively high producers of LMGL were identified by Gram stain, 10

types of biochemical tests consisting of potassium hydroxide (KOH), catalase, oxidase, indole, citrate utilization, phenylalanine deaminase and urease tests and triple sugar iron agar (TSIA), nutrient agar and MacConkey agar reactions, and by using the Biolog test kits (Biolog, Inc., Hayward, Calif.). *Enterobacter nimipressuralis*, *Enterobacter intermedius*, *Pseudomonas pyrrocinia*, *Ralstonia pickettii* and *Citrobacter freundii* (*C. freundii*) were found to be new sources for LMGL while the remaining two were *Escherichia coli* and *Bacillus cereus/thuringiensis*. The methionine-utilizing enzyme was partially purified from *C. freundii* (KP25) isolated from soil samples of Kuantan sea coast, which contained the highest activity. The purification scheme, involving dialysis, removal of nucleic acid with deoxyribonuclease I (DNase I) and ammonium sulfate [$(NH_4)_2SO_4$] precipitation resulted in a purification fold of 0.6 with a recovery of 22.6% and a specific activity of 0.02 U/mg, all using methionine as the substrate. It was found that the partially purified enzyme extract from *C. freundii* (KP25) catalyzed D-amino acids better than L-amino acids and also degraded cysteine and its S-substituted derivatives such as more effectively than methionine and its S-substituted derivatives. Hence, the result on substrate specificity of the lyase present in the enzyme extract shows the probable presence of D-cysteine desulfhydrase (EC 4.4.1.15) and the absence of LMGL. Crude enzyme extract from *C. freundii* (KP25) was characterized by using D- and L-cysteine instead of DL-methionine as the substrates. The temperature and pH optimum of the crude enzyme extract were 45^{O}C and pH 9.0 in 125 mM glycine-sodium hydroxide (NaOH) buffer with each D- and L-cysteine as the substrate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN SATU LYASE ASID AMINO SULFUR DARIPADA *CITROBACTER FREUNDII* (KP25)

Oleh

LIM LENG CHOO

Februari 2003

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L-Metionin γ-lyase (EC 4.4.1.11; LMGL) adalah sejenis enzim yang bergantung kepada piridoksal 5'-fosfat (PLP) yang memangkin penukaran terus L-metionin kepada α-ketobutirat, metanaetiol dan amonia melalui tindakbalas penyingkiran α,γ. Tujuh puluh sembilan isolat mikroorganisma penghasil LMGL telah disaring dari enam sumber tempatan menggunakan ujian 5'5-ditiobis (asid 2-nitrobenzoik) (DTNB). Enam sumber tempatan tersebut adalah sampel tanah di sekeliling Fakulti Sains Makanan dan Bioteknologi dan Makmal Penyelidikan Pusat, Universiti Putra Malaysia dan pantai Kuantan, sampel tanah dan air dari telaga air panas di Ulu Legong, Baling, Kedah dan Pedas, Negeri Sembilan dan sampel usus dari ayam. Satu kaedah penyaringan yang berasaskan warna yang mudah, ujian DTNB mengesan metanaetiol yang menurunkan DTNB yang terkandung di dalam media plat agar untuk membentuk warna kuning aril mercaptan (4 tiol-2-nitro-benzoat) sekeliling koloni bakterium yang berupaya menghasilkan LMGL. LMGL dikesan dari 45 isolat bakteria dengan ujian 3-metil-2-benzotiazolon hidrazon (MBTH). Aktiviti LMGL ditentukan secara kuantitatif dengan menentukan amaun α-ketobutirat yang dihasilkan secara spekrofotometrik pada 320 nm setelah bercampur

dengan MBTH. Dua belas pengeluar LMGL yang tertinggi dikenalpasti melalui ujian Gram, 10 jenis ujian biokimia yang terdiri daripada ujian potasium hidroksida (KOH), katalase, oksidase, indol, penggunaan sitrat, diaminase fenilalanin dan urease dan tindakbalas agar 'triple sugar iron' (TSIA), nutrien dan MacConkey, dan dengan menggunakan kit ujian Biolog (Biolog, Inc., Hayward, Calif.). *Enterobacter nimipressuralis*, *Enterobacter intermedius*, *Pseudomonas pyrrocinia*, *Ralstonia pickettii* dan *Citrobacter freundii* (*C. freundii*) dikenalpasti sebagai sumber baru bagi LMGL manakala selebihnya adalah *Escherichia coli* dan *Bacillus cereus/thuringiensis*. Enzim yang berupaya menggunakan metionin ditulinkan separa dari *C. freundii* (KP25) yang dipencil dari sampel tanah pantai Kuantan kerana mengandungi aktiviti enzim yang paling tinggi. Skim penulinan yang merangkumi dialisis, penguraian asid nukleik dengan deoksiribonukleas I (DNase I) dan pemeringkatan amonium sulfat [$(NH_4)_2SO_4$] menghasilkan paras penulinan sebanyak 0.6 dengan pendapatan semula sebanyak 22.6% dan aktiviti spesifik sebanyak 0.02 U/mg, kesemuanya dengan menggunakan metionin sebagai substrat. Didapati enzim separa tulen dari *C. freundii* (KP25) berupaya memangkin D-asid amino lebih baik daripada L-asid amino dan juga menguraikan sistein dan terbitan penggantian sulfurnya lebih berkesan daripada metionin dan terbitan penggantian sulfurnya. Maka, keputusan spesifisiti substrat untuk lyase yang hadir dalam ekstrak enzim menunjukkan kemungkinan kehadiran D-sistein disulfilhidrase dan ketiadaan LMGL. Ekstrak enzim kasar *C. freundii* (KP25) kemudiannya dicirikan dengan menggunakan D- dan L-sistein dan bukan DL-metionin sebagai substrat. Suhu dan pH optimum ekstrak enzim kasar adalah 45^{O}C dan pH 9.0 dalam 125 mM penimbal glisin-sodium hidroksida (NaOH) dengan setiap D- dan L-sistein sebagai substrat.

ACKNOWLEDGEMENTS

This project is a corporation between Universiti Putra Malaysia (UPM) and Japan International Corporation Agency (JICA) under the supervision of Prof. Dr. Hasanah Mohd. Ghazali. Thus, firstly, I would like to extend my most sincere gratitude to UPM, JICA and my supervisor, Prof. Dr. Hasanah Mohd. Ghazali for the opportunity to work on this project and the assistance they provided me during the course of this study. I'm truly grateful to Prof. Dr. Hasanah Mohd. Ghazali for the opportunity to work in her well equipped Enzyme Laboratory, for her constant guidance and advice and also the confidence she has in me.

I would also like to express my special appreciation to both my co-supervisors, Assoc. Prof. Dr. Raha Abdul Rahim and Dr. Lai Oi Ming for their comments, suggestions and reviews on my project. Acknowledgement is also due to Dr. Takashi Tamura for his guidance and contributions during his three months stint in Department of Biotechnology, Faculty of Food Science and Biotechnology, UPM.

Many thanks to all my friends and colleagues for their guidance, advice, support and encouragement. I'll always appreciate and cherish the friendship we have. Finally, I thank my family, whose constant love, support and encouragement have meant so much to me in the course of my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenine triphosphate
BCNU	1,3-Bis (2-chloroethyl)-1-nitrosourea
bp	Base pair
BSA	Bovine serum albumin
CF_3SH	Trifluoromethanethiol
CSF_2	Carbonothionic difluoride
C. freundii	*Citrobacter freundii*
Da	Dalton
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleoside triphosphate
DTNB	5,5'-Dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast Protein Liquid Chromatography
g	Gram
h	Hour
H^2	Deuterium
H^3	Tritium
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HIV	Human immunodeficiency virus
kDa	KiloDalton
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
KOH	Potassium hydroxide
KP	Potassium phosphate
K_m	Michaelis constant
L	Liter
LMGL	L-Methionine γ-lyase
Lys	Lysine
M	Molar
MBTH	3-Methyl-2-benzothiazolone hydrazone
mg	Milligram
$MgSO_4$	Magnesium sulfate
min	Minute
ml	Milliliter
mM	Millimolar
MRP	Multidrug related protein
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$(NH_4)_2SO_4$	Ammonium sulfate
nm	Nanometer
No	Number
PCMB	*p*-Chloromercuribenzoic acid
PCR	Polymerase chain reaction
Pgp	P-glycoprotein

pH	Hydrogen ion concentration
PLP	Pyridoxal 5'-phosphate
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
sec	Second
TCA	Trichloroacetic acid
TMZ	Temozolomide
Tris	Tris(hydroxymethyl)-aminomethane
TSIA	Triple Sugar Iron Agar
V_{max}	Maximal velocity
µg	Microgram
µl	Microliter
µM	Micromolar
µmol	Micromol
^{O}C	Degree Celsius
%	Percentage

CHAPTER I

INTRODUCTION

Life depends on a well-orchestrated series of chemical reactions. Many of these reactions, however, proceed too slowly on their own to sustain life. Hence nature has designed catalysts, which are now referred to as enzymes, to greatly accelerate the rates of these chemical reactions. The catalytic power of enzymes facilitates life processes in essentially all life-forms from viruses to man.

Sulfur amino acid lyases such as L-methionine γ-lyase, D-cysteine desulfhydrase, L-cysteine desulfhydrase, homocysteine desulfhydrase, cystathionine γ-lyase and cystathionine β-lyase have versatile functions. The multicatalytic functions of these enzymes enable their application in various scientific fields. The application of these enzymes include synthesis of various optically active sulfur and selenium amino acids, determination of sulfur and selenium amino acids, preparation of deuterium or tritium-labeled L-amino acids, development of cheese flavour, as a novel anticancer agent and also as a drug target by prodrugs.

In this study, a LMGL producer was isolated from local sources, and the physical and chemical properties of the partially purified LMGL from it were examined. Thus, this study was divided into three chapters with the following objectives:

1. to screen, isolate and identify LMGL-producing microorganisms from local sources

2. to isolate and partially purify LMGL from the isolated and identified highest LMGL producer
3. to characterize the partially purified enzyme