UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF THERMOSTABLE ORGANIC SOLVENT TOLERANT PROTEASE FROM *BACILLUS SUBTILIS* ISOLATE RAND

RANDA ABDELKAREEM ABUSHAM

FBSB 2009 27

ISOLATION AND CHARACTERIZATION OF THERMOSTABLE ORGANIC SOLVENT TOLERANT PROTEASE FROM *BACILLUS SUBTILIS* ISOLATE RAND

By

RANDA ABDELKAREEM ABUSHAM

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

August 2009

DEDICATION

To my beloved husband Eimad Abdu and my sweetheart twins Ahmed and Shimaa.

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

ISOLATION AND CHARACTERIZATION OF THERMOSTABLE ORGANIC SOLVENT TOLERANT PROTEASE FROM *BACILLUS SUBTILIS* ISOLATE RAND

By

RANDA ABDELKAREEM ABUSHAM
August 2009

Chairman: Professor Raja Noor Zaliha Raja Abd. Rahman, PhD

Faculty : Biotechnology and Biomolecular Sciences

Currently, thermostable and organic solvent tolerant protease is not available in local and global market. In this research screening and isolation of thermostable and organic solvent tolerant protease from bacteria from spring water and contaminated soils from Selayang, Batang kali and Port Dickson, Malaysia were carried out. Nine isolates were positive on skim milk agar 10 (%). A newly isolated soil bacterium, *Bacillus subtilis* isolate Rand, which exhibited an extracellular protease activity, was identified based on 16S rRNA analysis (GenBank EU233271). Isolate Rand was isolated from contaminated soils from Port Dickson and showed the highest activity (34.9 U/ml). The crude protease activity was enhanced by *n*- hexadecane (log *P* 8.8) with 1.5 fold, *n*-tetradecane (log *P* 7.6) with 1.5 fold, *n*-dodecane (log *P* 6.0) with 1.5 fold, *n*-decane (log *P* 5.6) with 2 fold, *n*-hexane (log *P* 3.5) with 1.4 fold, *p*-xylene (log *P* 3.1) with 1.3 fold, toluen (log *P* 2.5) with 1.2 fold, benzene (log *P* 2.0) with 1.9 fold and butanol (log *P* 0.80) with 1.01 fold. Optimum activity of the crude enzyme was exhibited at 60^{o}C. The enzyme appeared to be

stable and retained its full activity after 30 minutes incubation from 4 to 55^{o}C, while 81% of the activity was still retained at 60^{o}C.

Further optimization studies were carried out to determine the best protease production condition. Maximum protease production was achieved when grown in 50 mL M2 medium (pH 7.0). Inoculum size of 5%(v/v) proved to be the best for protease production, with an optimum temperature of 37^{o}C, when grown under shaking condition of 200 rpm. All carbon sources tested decreased protease production, except lactose and melibiose whereby protease production was improved. Tryptone and ammonium heptamolybdate were found to be the best organic nitrogen and inorganic nitrogen sources, respectively. Protease production was stimulated by l-lysine and calcium. Protease from the *Bacillus subtilis* isolate Rand was purified using a combination of two purification steps, hydrophobic interaction chromatography on Octyl-Sepharose and gel filtration. Rand protease was purified by 19.3 fold purification and 60.5% recovery. Purified protease migrated as a single band with a molecular mass of ~28 kDa on SDS-PAGE.

The purified protease hydrolyzed azocasein at optimum temperature of 60ºC. However, the enzyme lost its activity with a half life of more than 20 min at 60 and 65ºC. The optimum activity of the protease was observed at pH 7.0 and it was stable in the pH range of pH 6.5 to 10. Purified Rand protease exhibited high stability in the presence of *n*-dodecane (log *P* 6.6), diethylether (log *P* 4.3), *p*-xylene (log *P* 3.1), toluene (log *P* 2.5), benzene (log *P* 2.0), acetone (log *P* 0.23), butanol (log *P* 0.8) and ethanol (log *P* 0.24). The protease activity was completely inhibited by phenylmethanesulfonyl fluoride PMSF

while 43 and 30% reduction of protease activity was observed in the presence of ethylene diamine tetraacetic acid EDTA and 1,4-Dithio-$_{DL}$-threitol DTT, respectively. Protease activity retained about 95% and 63% in the presence of aminopeptidases (Bestatin) and aspartic proteases inhibitor (pepstatin A). Among the metal ions, Zn^{2+} was found to stimulate protease activity by 175%. Protease activity was enhanced by 105%, 112% and 105%, respectively Na^{+}, K^{+} and Li^{+}. For substrate specificity, Rand protease was able to hydrolyze several native proteins such as casein, haemoglobin, albumin and azocasein.

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

PEMENCILAN DAN PENCIRIAN PROTEASE TERMOSTABIL YANG TOLERAN KEPADA PELARUT ORGANIK DARI *BACILLUS UBTILIS* ISOLAT RAND

Oleh

RANDA ABDELKAREEM ABUSHAM
Ogos 2009

Pengerusi: Profesor Raja Noor Zaliha Raja Abd Rahman, Phd

Fakulti: Bioteknologi dan Sains Biomolekul

Buat masa ini tidak terdapat protease yang termostabil dan tahan kepada pelarut organik di pasaran tempatan mahupun dunia. Dalam penyelidikan ini, penyaringan dan pemencilan bakteria yang termostabil dan toleran kepada pelarut organik telah dijalankan daripada kolam air panas dan tanah yang tercemar di Selayang, Batang kali dan Port Dickson, Malaysia. Sebanyak sembilan pencilan telah berjaya diasingkan daripada agar susu skim 10 (%). Pengasingan bakteria daripada sampel tanah dikenalpasti sebagai *Bacillus subtilis* isolate Rand, yang menunjukkan aktiviti protease di luar sel dan pengenalpastian dibuat melalui analisis 16s rRNA (GenBank EU233271). Bakteria yang dipencilkan dari sumber tanah yang tercemar dari Port Dickson ini menunjukkan aktiviti protease yang tertinggi berbanding bakteria lain iaitu (34.9 U/ml). Aktiviti protease kasar telah ditingkatkan 1.5 kali ganda oleh *n*-heksadekana (log *P* 8.8), 1.5 kali ganda oleh *n*-tetradekana (log *P* 7.6), 1.5 kali ganda oleh *n*-dodekana (log *P* 6.0), 2 kali ganda oleh *n*-dekana (log *P* 5.6), 1.4 kali ganda oleh *n*-heksana (log *P* 3.5), 1.3 kali ganda oleh *p*-xilena (log *P* 3.1), 1.2 kali ganda oleh toluena (log *P* 2.5), 1.9 kali ganda oleh benzene (log *P*

2.0) dan 1.01 kali ganda oleh butanol (log *P* 0.80). Aktiviti optimum protease kasar adalah pada suhu 60°C.

Enzim ini stabil dan mengekalkan aktiviti sepenuhnya selepas 30 minit pengeraman daripada 4 ke 55°C, sementara 81% aktiviti dikekalkan pada suhu 60°C. Seterusnya, proses untuk mengoptimumkan penghasilah protease dalam keadaan yang terbaik telah dijalankan. Penghasilan protease yang maksimum telah dicapai semasa dihidupkan di dalam media M2 berkuantiti 50 ml (pH 7.0). Penggunaan sebanyak 5% (v/v) saiz inokulasi menunjukkan penghasilan terbanyak protease dengan suhu optimum 37°C apabila ditumbuhkan dengan kelajuan goncangan 200 rpm. Penurunan penghasilan protease telah direkodkan apabila diuji dengan pelbagai sumber karbon, kecuali laktos dan melibios yang telah menunjukkan peningkatan dalam pengeluaran protease. Tripton adalah sumber nitrogen organik manakala ammonium heptamolibdat merupakan sumber nitrogen tak organik merupakan penggalak terbaik dalam penghasilan protease. Penghasilan protease telah dirangsang dengan penambahan l-lisina dan kalsium. Proses penulenan protease daripada *Bacillus subtilis* isolate Rand telah dilakukan dengan kombinasi dua langkah penulenan iaitu kromatografi tindak balas hidrofobik pada Octyl-Sepharose dan penurasan gel. Rand protease telah ditulenkan sebanyak 19.3 kali ganda dan 60.5 % pemulihan diperolehi. Jisim molekul protease yang telah ditulenkan adalah ~28 kDa dan dikesan sebagai jalur tunggal pada SDS-PAGE.

Protease tulen menghidrolisiskan azokasein pada suhu optimum 60°C. Namun begitu, enzim ini hilang aktiviti pada tempoh separuh hayat lebih dari 20 minit pada 60 dan

65°C. Aktiviti optimum telah diperhatikan pada pH 7.0 dan ia adalah stabil dalam julat pH 6.5 ke 10. Protease tulen Rand mempamerkan kestabilan tinggi dalam *n*-dodekana (log *P* 6.6), dietileter (log *P* 4.3), *p*-xilena (log *P* 3.1), toluena (log *P* 2.5), benzena (log *P* 2.0), aseton (log *P* 0.23), butanol (log *P* 0.8) dan etanol (log *P* 0.24). Aktiviti protease telah direncatkan dengan kehadiran fenilmetilsulfonil florida PMSF sementara pengurangan aktiviti diperhatikan sebanyak 43% dalam acid etilena diamina tetraacitik EDTA dan 30% dalam 1,4-Dithio-$_{DL}$-threitol DTT. Sebanyak 95% aktiviti protease kekal dalam aminopeptidases (Bestatin) dan 63% dalam perencat aspartic proteases (pepstatin A). Antara semua ion-ion logam, ion Zn^{2+} didapati telah merangsang aktiviti protease sebanyak 175%. Aktiviti protease meningkat sebanyak 105% bagi Na^{+}, 112% bagi K^{+} dan 105% bagi Li^{+}. Bagi penentuan substrat khusus, Rand protease mampu menghidrolisis beberapa protein asli iaitu casein, haemoglobin, albumin dan azokasein.

ACKNOWLEDGEMENTS

Alhamdulillah, praise to Allah on completion of my study. I would like to express profound gratitude to my advisor, Professor Dr. Raja Noor Zaliha Abd Rahman, for her invaluable support, encouragement, supervision and useful suggestions throughout the period of this research work. Her moral support and continuous guidance enabled me to complete my work successfully. I am also highly thankful to my supervisory committee members, Professor Dr. Abu Bakar Salleh and Professor Dr. Mahiran Basri for their valuable suggestions, encouragement, advice and help throughout this study.

Special thank are extended to Dr. Leow, Afshin, Sue, Baya and Salma for their help, friendship, guidance and knowledge. Special thank are also extended to all member of Enzyme and Microbial Technology Lab and chemistry group especially Fairol, Elly, Dina, Kam, Hidayah, Chee Fah, Ada, Tengku, Wani, Rofandi, Aiman, Marha, Rosa, Rauda, Sabil, Azizah, Wahidah, Elias, Shukuri, Bimo, Ina, Hana, Peiman, Ghani, Fisal, Hisham, Ampon and Zaril. Thank you for the friendship and help.

Finally, I would also like to take this opportunity to express my deep gratitude to my affectionate father Abdel Kareem Hussein, mother Fathia Ali for their fullest support, love, prayers and care, throughout the process of writing the thesis. I would like to thank my family members, brothers, sisters, aunts, uncles and my faithful friends in Malaysia and Sudan for their love and support. Last but not least, my deepest gratitude goes to my

hubby, Eimad Abdu, son, Ahmed and daughter Shimaa thanks for every thing. Without my family, I would never be here and accomplishing this work will not be possible.

I certify that an Examination Committee met on date of viva to conduct the final examination of Randa Abdelkareem Abusham on her Master thesis entitle "Isolation and Characterization of Thermostable Organic Solvent Tolerant Protease from *Bacillus subtilis* Isolate Rand" In accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Suraini Abd. Aziz, Ph.D.
Associate Professor
Department of Bioprocess Technology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Puad Abdullah, Ph.D.
Department of Cell and Molecular Biology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Dr. Sieo Chin Chin, Ph.D.
Department of Microbiology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Ainon Hamzah, Ph.D.
Associate Professor
Center of Biotechnology and Bioscience
Faculty of Science and Technology
Unniversiti Kebangsaan Malaysia
(External Examiner)

_____

BUJANG KIM HUAT, PhD.
Professor/Deputy Dean
School of Graduate Studies
University Putra Malaysia

Date: 24 November 2009

This thesis submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master. The members of the Supervisory Committee were as follows:

Raja Noor Zaliha Raja Abdul Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Abu Bakar Salleh, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mahiran Basri, PhD
Professor
Faculty of Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 December 2009

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institution.

RANDA ABDELKAREEM ABUSHAM

Date: 22 October 2009

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base pair
cm/h	Centimeter per hour
ºC	Degree centigrade
Cfu	Colony-forming unit
dH_2O	Distilled water
DNA	Deoxyribonucleic
DTT	1,4-Dithio-$_{DL}$-threitol
EDTA	Ethylene diamine tetraacetic acid
g	Gram
g/L	Gram per liter
Xg	Times gravity
h	Hour
kb	Kilo base pair
kDa	Kilo dalton
L	Liter
M	Molar
ml	Milliliter
min	Minute
nm	Nanometer
mw	Molecular weight
mM	Millimolar

M	Molar
ml/min	Milliliter per minute
mg/ml	Milligram per milliliter
mA	Milliamphere
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
rpm	Revolutions per minute
SDS	Sodium dedocyl sulfate
SMA	Skim Milk Agar
TCA	Trichloroacetic acid
TEMED	N, N, N, N' tetramethyl-ethylene diamine
%	Percentage
µg	Microgram
µl	Microliter
µm	Micrometer
V	Volt
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Enzymes have attracted the attention of the world due to their wide range of industrial applications in many fields including organic synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. Enzymes are gradually replacing the use of harsh chemicals in various industrial processes (Malathu *et al*., 2008). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. Proteases are of commercial value and find multiple applications in various industrial sectors. Proteases are widely used in detergent, food industry, leather tanning industries, pharmaceutical industry and bioremediation processes (Gupta *et al*., 2002; Rao *et al*., 1998).

The main advantage of thermostable protease is that as the temperature of the process is increased, the rate of reaction increases, which in turn decreases the amount of enzyme needed. The thermostable proteases are also able to tolerate higher temperatures, which gives a longer half-life to the enzyme. The use of higher temperatures also is inhibitory to microbial growth, decreasing the possibility of microbial contamination. The use of high temperatures in industrial enzyme processes may also be useful in mixing, causing a decrease in the viscosity of liquids and may allow for higher concentrations of low solubility materials. The mass transfer rate is also increased at higher temperatures as is the rate of many chemical reactions (Zamost *et al*., 1991).

Enzymatic reactions using protease in the presence of organic solvents have been studied extensively for the synthesis of peptides and esters. If organic solvents can be used as media for enzymatic reactions, the reaction equilibrium of hydrolytic enzymes can be shifted toward completion of the reverse reaction of hydrolysis, that is, the synthetic reaction (Ogino *et al*., 1995). The use of proteases in peptide synthesis is limited by the specificity and the instability of the enzymes in the presence of organic solvents, since reactions occurred in organic media. However, little attention has been given to the study of organic solvent-stable protease (Ghorbel *et al*., 2003).

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases can conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lyses of fibrin clots, and processing and transport of secretary proteins across the membranes. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications. Proteases represent one of the largest groups of industrial enzymes occur ubiquitously in a wide diversity of sources such as plants, animals, and microorganisms (Rao *et al*., 1998). Proteases can be classified as serine protease (EC. 3.4.21), cysteine (thiol) protease (EC 3.4.22), aspartic proteases (EC 3.4.23) and metallo-protease (EC 3.4.24) constitute one of the most important groups of industrial enzymes (Adinarayana *et al.,* 2003). Microbial proteases

play an important role in biotechnological processes accounting for approximately 59% of the total enzymes used (Shumi *et al*., 2004).

1.1 Objectives

The search for new microorganisms producing new and novel proteases for industrial purposes should be continuously pursued. The objectives of this study are:

1- To isolate and identify a bacterium producing thermostable and organic solvent tolerant protease

2- To optimize the protease production of the isolate

3- To purify and characterize the protease produced by the isolate