UNIVERSITI PUTRA MALAYSIA

CRYSTALLIZATION AND STRUCTURE ELUCIDATION OF RECOMBINANT Pseudomonas aeruginosa STRAIN K SOLVENT TOLERANT ELASTASE.

ZATTY SYAMIMI @ ADURA BT MAT SAID

FBSB 2014 37

CRYSTALLIZATION AND STRUCTURE ELUCIDATION OF RECOMBINANT *Pseudomonas aeruginosa* STRAIN K SOLVENT TOLERANT ELASTASE.

By

ZATTY SYAMIMI @ ADURA BT MAT SAID

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

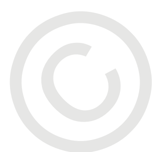
December 2014

© COPYRIGHT UPM

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs, and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright© Universiti Putra Malaysia



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

CRYSTALLIZATION AND STRUCTURE ELUCIDATION OF RECOMBINANT *Pseudomonas aeruginosa* STRAIN K SOLVENT TOLERANT ELASTASE.

By

ZATTY SYAMIMI @ ADURA BT MAT SAID

December 2014

Chairman : Mohd Shukuri Mohamad Ali, PhD
Faculty : Biotechnology and Biomolecular Sciences.

The discoveries of elastase that actively catalyse a variety of reaction in both aqueous and non-aqeous/ organic solvent are among the most important biocatalysts that constantly being sought by enzymologies. Elastase represents a class of enzyme which occupies a pivotal position with respect to their physiological role as well as commercial applications. The degradation and synthetic reaction are being more efficient with intervention of elastase. The unique properties owned by elastase become an interest to understand the three-dimensional structure of the enzyme. However, little is known on the elastase structure and function particulary tolerant in organic solvent due limited structure based information. The purpose of this study is to elucidate the three-dimensional structure of an organic solvent tolerant elastase. By investigating the structural-functions relationship of this organic solvent-tolerant enzyme using X-ray crystallography it will improve the understanding on elastase functionalities and its catalytic reactions.

This recombinant elastase strain K was successfully purified to homogeneity by combination of hydrophobic interaction chromatography and ion exchange chromatography methods. Natively folded elastase of crystallisation-scale purity, quality and quantity was demonstrated and verified by SDS-PAGE, Native PAGE and Bradford assay analysis, respectively. Elastase strain K was also confirmed to be natively homogenous in size and uniformly-charge protein by observation of a single band in native-PAGE. The final protein content obtained after final purification step was 3 mg/mL

Random crystal screening was performed using vapour diffusion methods and applied into various crystallisation formulation kits. The crystal formulation containing 1 M ammonium phosphate monobasic and 0.1 M sodium citrate tribasic dehydrate pH 5.6 shows a promising formulation producing elastase crystal. Microseeding technique has

© COPYRIGHT UPM

been chosen to improve the crystal hits. The highly purified elastase strain K with protein concentration around 3.00 mg/mL and pH 5.5 is the optimal condition for crystal growth. Besides, coupling seeding technique with capillary counter diffusion crystallization shows the improvement in size and diffraction quality of the crystals. The measurement of crystal size was 1 mm × 0.1 mm × 0.05 mm. Elastase strain K was successfully diffracted up to 1.39Å at SPring-8, Japan using synchrotron radiation. The space group has been determined to be P1211 belonged to the monoclinic space point with unit cell parameter was a = 38.99 Å, b = 90.173 Å, c = 40.60Å.

The structure of elastase strain K was refined and validated subsequently using PROCHECK and ERRAT. Crystal structure of elastase strain K showed the typical, canonical alpha-beta hydrolase fold consisting of 10-helices, 10- β-strands and other secondary structure of such as loop and coil. The elastase strain K is a zinc metalloproteinase possess His-140, His-144 and Glu-164 served as a ligand for zinc ion. The conserved catalytic triad was composed of Glu-141, Tyr-155 and His-223. Three-dimensional structure features such as calcium-binding and presence of disulphide-bridge contribute to the stabilizing the elastase strain K structure.

In conclusion, the solvent-tolerant elastase strain K has been crystallised and the three-dimensional structure of elastase strain K was successfully elucidated. Information regarding unique properties followed by the structural features of this enzyme provides a useful insight towards rational design of enzymes stable in solvents

© COPYRIGHT UPM

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

PENGHABLURAN DAN ELUSIDASI STRUKTUR ELASTASE REKOMBINAN STRAIN K RINTANG PELARUT DARI *Pseudomonas aeruginosa*

By

ZATTY SYAMIMI @ ADURA BT MAT SAID

Disember 2014

Pengerusi : Mohd Shukuri Mohamad Ali, PhD.
Fakulti : Bioteknologi dan Sains Biomolekul

Penemuan enzim elastase yang aktif memangkin pelbagai tindakbalas dalam akueus dan bukan akueus/ pelarut organik adalah antara pemangkin yang penting yang sentiasa dicari oleh pakar enzimologi. Elastase merupakan salah satu kumpulan enzim yang memainkan peranan yang penting berdasarkan peranan fisiologi dan juga kegunaannya dalam bidang komersial. Tindakbalas penguraian dan sintesis akan menjadi lebih efisen dengan menggunakan elastase. Ciri-ciri unik yang dipunyai oleh enzim elastase ini menjadi tarikan utama untuk memahami struktur tiga-dimensi enzim ini. Walaubagaimanapun, tidak banyak yang dapat diketahui mengenai struktur elastase terutamanya struktur elastase yang rintang dalam pelarut organik kerana maklumat strukturnya yang terhad. Tujuan kajian ini adalah untuk menentukn keadaan struktur tiga-dimensi elastase yang rintang terhadap pelarut organik. Dengan mengkaji hubungan di antara struktur dan fungsi enzim yang rintang pelarut organik dengan menggunakan teknik kristalografi sinar-X dapat memberi pemahaman yang jelas pada fungsi elastase dan juga tindakbalasnya.

Elastase rekombinan strain K ini telah berjaya ditulenkan menjadi homogen dengan menggabungkan kaedah kromatografi interaksi hidrofobik dan kromatografi pertukaran ion. Kadar ketulenan elastase secara kualiti dan kuantiti telah dibuktikan dan disahkan oleh SDS-PAGE, natif-PAGE dan analisis assai Bradford. Elastase strain K juga telah dibuktikan homogen dari segi saiz dan cas protein dengan memperlihatan satu jalur di natif-PAGE. Kandungan protein terakhir yang diperolehi selepas proses penulenan dan pemekatan protein adalah sebanyak 3 mg/mL.

Saringan penghabluran telah dilakukan menggunakan kaedah penyebaran wap dan diaplikasikan ke atas kit formula penghabluran. Formula penghabluran yang mengandungi 1 M ammonia fosfat monobasik dan 0.1 M natrium sitrat tribasik dihidrat pH 5.6 menunjukkan formulasi penghabluran yang berpotensi bagi penghasilan-semula penghabluran elastase. Kaedah pembenihan mikro telah dipilih untuk meningkatan

© COPYRIGHT UPM

pencapaian penghabluran elastase. Penulenan elastase strain K dengan kepekatan sekitar 3.00 mg/mL dan pelarasan pH ke pH 5.5 adalah keadaan yang optimum untuk pembentukan penghabluran elastase. Selain itu, pembentukan penghabluran elastase di dalam gel kapilari dengan mengandingkan teknik pembenihan dengan kapilari resapan berbalik menunjukkan peningkatan saiz dan kualiti pembelauan kristal. Saiz ukuran kristal elastase adalah 1 mm x 0.1 mm x 0.05mm. Elastase strain K berjaya dibelaukan sehingga 1.39 Å di SPring-8, Jepun menggunakan radiasi sinkrotron. Kumpulan ruang telah ditentukan sebagai P1211 dan kristal ini mempunyai kumpulan ruang monoklinik dengan parameter a = 38.99 Å, b = 90.173 Å, c = 40.60 Å.

Struktur penghabluran elastase strain K telah diperbaiki dan seterusnya disahkan menggunakan PROCHECK dan ERRAT. Struktur penghabluran elastase strain K menunjukkan pembentukan tipikal, konikal alfa-beta hydrolase yang terdiri daripada 10 helix, 10 - β-*pleated* dan struktur sekunder lain seperti lengkungan dan gegelung. Elastase strain K adalah metalloproteinase zink yang memiliki His-140, His-144 dan Glu-164 yang berfungsi sebagai ligand bagi ion zink. Tapak pengaktifan terdiri daripada Glu-141, Tyr-155 dan His-223. Ciri-ciri struktur tiga dimensi seperti ion kalsium dan kehadiran jambatan disulfida menyumbang kepada kestabilan struktur elastase strain K.

Kesimpulannya, elastase strain K yang rintang pelarut organik telah berjaya dihablurkan dan struktur tiga-dimensi elastase K telah berjaya ditentukan. Maklumat mengenai ciri-ciri unik diikuti oleh ciri-ciri struktur enzim ini memberikan gambaran yang berguna untuk mereka bentuk secara rasional enzim yang rintang pelarut organik yang lebih baik .

© COPYRIGHT UPM

ACKNOWLEDGEMENTS

Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this study. I would like to express my sincere appreciation to my research supervisors, Dr. Mohd Shukuri Mohamad Ali, Professor Dr. Raja Noor Zaliha Raja Abdul Rahman and Dr. Adam Leow Thean Chor for their supervision, useful comments, constructive critics, and supports throughout the course of this degree.

Special gratitude goes to our Japanese Collaborators especially Dr. Koji Inaka, for his guidance and knowledge throughout the experimental work of this research. Also, a special credits to Japanese Aerospace Exploration Agency (JAXA) and those involved in the JAXA Protein Crystal Growth space experiment for their technical support, expertises and facilities. My acknowledgement also goes to the Ministry of Science and Technology (MOSTI) Malaysia for their financial support for this project.

Sincere thanks to all my colleagues, Rudzana, Ariati, Ira Maya, Iffah, Hafizah, Azmir, Saif, Syuhada, Dina, Randa and all crystal group members; Lab 140 members, Menega, Zarir and Chee Fah for their academic discussions, kindness, moral supports and cooperations during my study. Thanks for the friendship and memories.

Last but not least, my deepest gratitude goes to my beloved parents, brother, sisters and family for their endless love, prayer and encouragement. To those who indirectly contributed in this research, your kindness means a lot to me. Thank you so much.

© COPYRIGHT UPM

I certify that a Thesis Examination Committee has met on 2 December, 2014 to conduct the final examination of Zatty Syamimi @ Adura Bt Mat Said on her thesis entitled "Crystallization and Structure Elucidation of Recombinant *Pseudomonas aeruginosa* Strain K Solvent Tolerant Elastase" in accordance with the Universities and University 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.

Members of the Thesis Examination Committee were as follows:

Mohd Arif b. Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Syahida binti Ahmad, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Muhajir b. Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Rosli Md. Illias, PhD
Professor
Faculty of Chemical and Natural Resources
Universiti Teknologi Malaysia
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School and Graduate Studies
Universiti Putra Malaysia

Date: 19 March 2015

© COPYRIGHT UPM

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

Mohd Shukuri Mohamad Ali, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha Raja Abd Rahman, D. Eng.
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Adam Leow Thean Chor, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

© COPYRIGHT UPM

Declaration by graduate student

I hereby confirm that:

- This thesis is my original work;
- Quotations, illustrations and citations have been duly referenced;
- This thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- Intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- There is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ________________________ Date: __________________

Name and Matric No.: Zatty Syamimi @ Adura Bt Mat Said, GS 141647.

© COPYRIGHT UPM

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature:	________________	Signature:	________________
Name of Chairman of Supervisory Committee:	________________	Name of Member of Supervisory Committee:	________________
Signature:	________________	Signature:	________________
Name of Member of Supervisory Committee:	________________	Name of Member of Supervisory Committee:	________________

© COPYRIGHT UPM

TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xiii
LIST OF FIGURES		xiv
LIST OF ABBREVIATIONS		Xv
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	
	2.1 Proteases	3
	2.2 Classification of proteases	4
	2.2.1 Serine proteinase	4
	2.2.2 Cysteine proteinase	5
	2.2.3 Aspartic proteinase	5
	2.2.4 Metalloproteinase	5
	2.3 Microbial protease	6
	2.4 Organic solvent tolerant protease	6
	2.5 Effect of organic solvent on enzyme	7
	2.6 Protein structure conformation in organic solvent	8
	2.7 Application of protease	10
	2.8 Pseudolysin	10
	2.9 Protein purification	12
	2.10 Protein crystallization	13
	2.11 General approach in protein crystallisation	13
	2.12 Protein crystallisation procedures	14
	2.12.1 Microbatch	15
	2.12.2 Vapour diffusion	15
	2.12.3 Seeding	15
	2.12.4 Counter diffusion	16
	2.13 Phase diagram of protein crystallization	16
	2.14 X-ray crystallography	17
3	**MATERIALS AND METHODS**	
	3.1 Material	19
	3.2 Bacterial sources	21
	3.3 Qualitative analysis of recombinant elastase	21
	3.4 Preparation of stock culture	21
	3.5 Expression of recombinant elastase strain K	21
	3.6 Purification strategy of elastase strain K	22
	3.6.1 Hydrophobic Interaction Chromatography	22
	3.6.2 Desalting and Buffer exchange	22
	3.6.3 Ion Exchange Chromatography (IEC)	22
	3.7 Protein determination	23
	3.7.1 Bovine serum albumin (BSA) standard curve	23

© COPYRIGHT UPM

3.8 Assay of elastinolytic activity 23
3.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE) analysis. 24
3.10 Native polyacrylamide gel electrophoresis (Native-PAGE) analysis 24
3.11 Protein preparation for crystallization 24
3.12 Crystallisation of elastase strain K 24
3.12.1 Sitting drop vapour diffusion techniques 25
3.12.2 Hanging drop vapour diffusion techniques 25
3.13 Optimisation of crystallisation condition 25
3.13.1 pH optimisation 25
3.13.2 Protein concentration optimisation 26
3.14 Preparation of the seed stock 26
3.15 Crystallisation of elastase strain K using seeding method 27
3.16 Crystallisation of elastase strain K using counter diffusion method 27
3.17 Harvesting protein crystal in capillary 27
3.18 Validation of protein crystal 27
3.18.1 Crystal dye 27
3.18.2 X-ray diffraction 28
3.19 X-ray crystallography 28
3.19.1 Data collection 28
3.19.2 Data processing and model building 28
3.19.3 Validation of three-dimensional crystal structure 28
3.19.4 Three-dimensional structure analysis 29

4 **RESULTS AND DISCUSSIONS**
4.1 Purification of elastase strain K 30
4.1.1 Hydrophobic Interaction Chromatography (HIC) 30
4.1.2 Ion Exchange Chromatography (IEX) 32
4.2 Purification analysis 34
4.3 Crystallisation 37
4.3.1 Crystallisation screening 37
4.4 Reproducibility of elastase crystals 43
4.5 Increasing the success rate of elastase crystal using seeding method 44
4.6 Optimisation of elastase crystallisation condition 45
4.6.1 pH optimization 45
4.6.2 Protein concentration optimization 48
4.7 Growth of elastase strain K crystal in capillary counter-diffusion 50
4.8 X-ray diffraction analysis 53
4.9 Data collection and processing of elastase strain K crystal 54
4.10 Refinement of raw data 57
4.11 Structure validation of elastase strain K 60
4.11.1 Validation using PROCHECK 60
4.11.2 Validation using ERRAT program 63
4.12 Overall structure of elastase strain K 63
4.13 Unexpected phosphate ion and glycerol in elastase strain K structure 66

© COPYRIGHT UPM

5 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH
5.1 Summary and conclusions 72
5.2 Recommendations 73

REFERENCES 74
APPENDICES 84
BIODATA OF STUDENT 100
LIST OF PUBLICATIONS 101

© COPYRIGHT UPM

LIST OF TABLES

Table		Page
1	Protein properties used during purification	13
2	Physical, chemicals and biochemical factors affecting the growth of protein crystallization experiment.	14
3	pH optimization of crystallization formulation	26
4	Protein concentration optimisation of crystallisation formulation	26
5	Purification table of elastase strain K from *E.coli* KRX/pCON2(3) by HIC and IEC	36
6	The list of formulation screening conditions and number of crystal screen (noted in bracket) that produced crystals of the elastase strain K at 20°C.	39
7	Summary of the crystallographic data collection statistics of elastase strain K	56
8	The refinement statistics for elastase strain K crystal structure.	58

© COPYRIGHT UPM

LIST OF FIGURES

Figure		Page
1	Three-dimensional structure of PST-01 protease with that thermolysin	9
2	A schematic phase diagram of protein crystallization growth	17
3	A purification profile of elastase strain K in hydrophobic interaction chromatography.	31
3	SDS-PAGE gel of the recombinanat elastase strain K fractionation of HIC purification	32
5	IEC purification profile of elastase strain K	33
6	SDS-PAGE gel of recombinant elastase strain K fractionation of IEC purification	34
7	Electrophoretic analysis of a protein purification analysed by SDS-PAGE and native-PAGE	35
8	The structure obtained during elastase strain K crystallisation screening	38
9	The crushed elastase strain K crystal in formulation 1 (11) Hampton crystal screen	42
10	X-ray diffraction pattern of elastase strain K crystal	42
11	Precipitation droplet of elastase strain K	44
12	Elastase strain K crystal	45
13	pH optimisation of crystals formulation ranging from pH 4.0 to pH 9.0	46
14	Optimisation of elastase strain K crystal at various protein concentration	50
15	Crystallisation trial of elastase strain K in agarose gel-capillary counter diffusion	51
16	Crystallisation of elastase strain K using microseeding-capillary counter diffusion.	52
17	Recombinant elastase strain K crystal grown using microseeding-capillary counter diffusion with 1 M ammonium phosphate monobasic and 0.1M sodium citrate tribasic dehydrate pH 5.6	53
18	The recombinant elastase strain K crystal mounted in a nylon loop	54
19	An X-ray diffraction pattern of elastase strain K	55
20	Snapshot of generated 2Fo-Fc electron density map of elastase strain K structure.	59
21	Ramachandran plot of elastase crystal structure	61
22	ERRAT error function against residues after minimization	62
23	Structure of elastase strain K as determined and generated using YASARA	64
24	The catalytic site of elastase strain K crystal structure which consist of Glu141, Tyr155 and His223 located between two helices.	65
25	The zinc binding site of elastase strain K	65
26	Calcium binding site of elastase strain K	66
27	The phosphate ion binding in the structure of elastase strain K	67
28	The molecule of glycerols bound in the structure of elastase strain K.	69

© COPYRIGHT UPM

LIST OF ABBREVIATIONS

Å	Angstrom
°C	Degree Celsius
DTT	Dithiothreitol
G	Gram
IPTG	Isopropyl β-D Thiogalactoside
ISS	International Space Station
kDa	Kilo Dalton
L	Liter
M	Molar
mg/mL	Milligram per millilitre
Min	Minute
mL	Mililiter
Mm	Milimeter
Nm	Nanometer
OD	Optical density
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMA	Skim milk agar
Spring-8	Super Photon Ring-8 GeV
TEMED	N, N, N, N-Tetramethyllenediamide
µl	Microliter
v/v	Volume per volume

© COPYRIGHT UPM

© COPYRIGHT UPM

CHAPTER 1

INTRODUCTION

Most of the chemical reactions in living organisms are catalysed by enzymes. Without enzymes, all of the chemical reactions take place in a slow rate or there will be no reactions occur at all. The presence of enzymes can speed up the biochemical reactions in living organism. This biological catalyst often increases the rate of a chemical reaction millions of time compared to reaction uncatalysed. Enzymes are very specific as they are able to promote only one type of chemical reaction for each of the enzymes. Nowadays, enzymes have been studied extensively due to its commercial ability and abundant sources available.

Proteases are one of the groups of enzyme that has been widely studied. Since they are physiologically necessary for living organism, proteases can be found in diverse sources such as plants, animals and microorganisms. However, proteases from microbes are an attractive source due to the limited space used for their cultivation and their ready susceptibility to genetic manipulation. *Pseudomonas aeruginosa* is the most widely studied group of protease-producing microorganism (Engel *et al*., 1998). *Pseudomonas* sp. have their ability to produce proteolytic enzyme: elastase and alkaline protease (Ryan, 1984; Iglewski, 1988). Alkaline protease has broad cleavage specificity but it is not as potent as elastase and not hydrolyse elastin. Elastase, on the other hand, able to degrade proteins at multiple site and host protein in addition to elastin. Since, it is active on elastin, this type of protease is called 'elastase'. The elastase is belongs to the natural metalloprotease (Morihara *et.al*, 1965) which require Zn^{2+} for its activity. Elastase was said behave in a same manner as thermolysin and *Bacillus subtilis* neutral proteinase due to the high degree of sequence similarity and functional homology (Morihara *et.al*. 1965).

Elastase is another important member of protease family as it contributed equally impressive in industries. As elastase can degrade elastin (Morihara, 1967) which other protease cannot, it has broad applications in medical therapy and food processing as well as daily use chemical industries. This enzyme also was adapted for proteomic applications for digestion of proteolytically resistant and inaccessible site (Saveliev *et al*., 2012). Besides, elastase also executes a variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as homeostasizs and inflammation.

The discovery of elastase that is active in organic solvent media has great potential in the synthesis of useful products. As elastase is a nonspecific protease with a preference for digestion hydrophobic residue, its can perform digestion of hydrophobic substrate. This kind of enzymes gives a major contribution in bioremediation industries because most of the environment pollutants can be classified as hydrophobic solvents and degradation of these solvents can be done by this microbial enzyme (Cruden *et al.,* 1992). Apart from that, the capability to catalyze a variety of reactions in organic solvent media expended a great potential used in solvent-used industrial processes as

© COPYRIGHT UPM

well as able to shift the reaction equilibrium toward protein synthetic directions. Elastases are alternatively used in molecular biology aspect whenever other proteases applied are not informative in the process of protein synthesis. Elastase was chosen as it capable to catalyse multiple sites of protein rather than other proteases. Thus, studies details on elastase that stable in the presence of organic solvents become a commercially important for industrial and biotechnological applications.

Since elastases are potential enzyme in biochemical and biotechnological aspects, there are a number of elastase from animal (porcine and pancerase) and microbial (*Pseudomonas sp*.) have been identified, purified and characterized. The interest of reseracher s develops from discovering the characterstics of elastase to understand these diverse characteristics by investigating the structure of elastase up to atomic level. Thus, the employment of X-ray crystallography is definitely the perfect method to describe the behaviour of the enzyme. Having a three-dimensional structure of a protein at atomic level is important for several reasons such as enable to gather information about how protein works in biology; the function and the structure of a protein dictate what that protein is capable of doing.

The recombinant elastase strain K has demonstrated a stability and enhancement of the activity in hydrophilic organics solvents such as DMSO, methanol, ethanol and 1-propanol (Wong *et al*., 2010). In this study, elastase strain K was subjected to elucidate its native three dimensional structures. Analysis of the three-dimensional structure of elastase strain K was performed in order to understand the properties of elastase strain K as one of the organic solvent tolerant elastase. Hence, this research was undertaken with the following sub-objectives:

- To purify the organic solvent tolerant elastase strain K
- To optimise the crystallisation conditions of the purified elastase strain K
- To elucidate the three-dimensional structure of the crystallised protein.

© COPYRIGHT UPM

REFERENCES

Abad-Zapatero, C., Goldman, R., Muchmore, S.W., Hutchins, C., Stewart, K., Navaza, J., Payne, C.D. and Thomas, L.R. 1996. Structure of a secreted aspartic protease from C. albicans complexed with a potent inhibitor: Implications for the design of antifugal agents. *Proteins sciences*. 5(4): 640- 652.

Ahmad, S., Gromiha, M., Faraweh, H. and Sarai, A. 2004. ASAView: Database and tool for solvent accessibility representations in proteins. *BMC Bioinformatics*. 5:51.

Anthonsen, T. and Sjursnes, B.J. 2000. Importance of water activity for enzyme catalysis in non-aqueous organic systems. In: *Methods in non-aqueous enzymology*. (ed) Gupta, M.N., Birkhauser Verlag, Basel. pp: 14-35

Anwar, A. and Saleemuddin, M. 1998. Alkaline protease. A Review. Bioresource Technology, 6: 175-183.

Asherie, N. 2004. Protein crystallization and phase diagram. *Methods*. 34: 266-272.

Ataka, M. 1993. Protein crystal growth: an approach based on phase diagram determination. *Phase Transition*. 45: 205-219.

Barret, A.J., 1986. Introduction to the proteinases. In: Protease Inhibitors, (eds.) Barret, A. J., and Salvesen, G., Elsevier, Amsterdam. pp: 3-22.

Barret, A.J and McDonald, J.K. 1985. Nomenclature: protease, proteinase and peptidase. *Biochemical Journal*. 1986. 237(3): 935.

Beaufort, N., Corvazier, E., Mlanaoindrou, S., de Bentzmann, S. and Pidard, D. 2013. Distruption of the endothelial barrier by proteases from the bacterial pathogen *Pseudomonas aeruginosa:*Implication of matrilysis and receptor cleavage. *PLoS ONE*. 8:9

Bergfors, T. 2003. Seeds to crystals. *Journal of structural Biology*. 142: 66-76.

Bergfors, T. 1999. Protein crystallization: Techniques, strategies, and tips. (International University Line, La Jolla, California.. ISBN 0963681753.

Bernal, J.D., and Crowfoot, D. 1934. X-ray photographs of crystalline pepsin. *Nature*. 133: 794- 795.

Bever, R.A. and Iglewski, B.H., 1988. Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. *Journal of Bacteriology*. 170(9): 4309-4314.

Biertumpfel, C., Basquia, J., Suck, D. and Sauter, C. 2002. Crystallization of biological macromolecules using agarose gel. *Acta Crystallographica Section* D. D58: 1657-1659.

Braun, P., Bitter, W., and Thommasen, J., 2000. Activation of *Pseudomonas eruginosa* elastase in *Pseudomonas putida* by triggering dissociation of the propeptide-enzyme complex. *Microbiology*, 146: 2565-2572.

Cappucino, J.G., and Sherman, N., 1992. Microbiology. A laboratory manual, Benjamin/Cummings Publishing Co, Redwood City, California. 125-128.

Careri, G., 1998. Cooperative chatge fluctuations by migrating protons in globular proteins. *Progress in Biophysics and Molecular Biology*, 70: 223-249.

Chayen, N.E. 1996. A novel technique for containerless protein crystallization. *Protein Engineering*. 9: 927-929.

Chayen, N.E. 1998. Comparative studies of protein crystallisation by vapour-diffusion and microbatch techniques. *Acta Crystallographica* D. 54: 8- 15.

© COPYRIGHT UPM

Chayen, N.E. 1999. Recent advances in the methodology for the crystallization of biological macromolecules. *Journal of Crystal Growth*. 199: 649-655.

Chayen, N.E. 2005. Methods for separating nucleation and growth in protein crystallization. *Progress in Biophysics and Molecular Biology.* 88: 329-337.

Chayen, N.E., and Saridakis, E. 2008. Protein crystallization: from purified protein to diffraction-quality crystal. *Nature Methods*. 5: 147-153.

Chiplonkar, J.M., Gangodkar, S.V., Wagh, U.V., Ghadge, G.D., Rele, M.V. and Srinivasan, M.C., 1985. Application of alkaline protease *Conidiobolus* in animal cell culture. *Biotechnology letters*, 7: 665-668.

Cho, Y., Gorina, S., Jeffrey, P.D. and Pavletich, N.P. 1994. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science.* 265: 346-355.

Colman, P.M., Jonsonius, J.N. and Matthews, B.W. 1972. The structure of thermolysin: an electron density map at 2.3 Å resolution. *Journal of Molecular Biology*, 70: 701-724.

Colovos, C. and Yeates, T.O. 1993. Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science*. 2(9): 1511-1519.

Cowtan, K. 1994. Joint CCP4 and ESF-EACBM. Newsletter on protein crystallography. 31: 34-38.

Cruden, D.L., Wolfram, J.H., Rogers, R.D., Gibson, D.T. 1992. Physiological properties of a Pseudomonas strain which grows with p-xylene in a two-phasw (organic-aqueous) medium. Applied and Environmental Microbiology. 58: 2723-2729.

Doukyu, N. and Ogino, H. 2010. Organic solvent-tolerant enzymes. *Biochemical Engineering Journal*, 48: 270-282.

Ducruix, A., and Geige, R. 1992. Crystallization of nucleic acid and proteins: a practical approach. Oxford Press. 86-360.

Ducruix, A. and Giege´, R. 1999. Crystallization of Nucleic Acids and Proteins. A Practical Approach, 2nd edition. Oxford University Press.

Durbin, S.D. and Feher, G. 1996. Protein crystallization. *Annual Review of Physical Chemistry*. 47: 171-204.

Emsley, P. and Cowtan, K. 2004. Coot: mode-building tools for molecular graphics. *Acta Crystallogaphica D*. 12(1): 2126-2132.

Engel, L.S., Hill, J.M., Caballero, A.R., Green, L.C. and O'Callaghan, R.J. 1998. Protease IV, a unique extracellular protease and virulence factor from Pseudomonas aeruginosa. *The Journal of Biological Chemistry*. 273(27): 16792-16796

Fu, D., Ballesteros, J.A., Weinstein, H., Chen, J. and Javitch, J.A. 1996. Residues in the seventh membrane-spanning segment of the dopamine D2 receptor accessible in the binding-site crevice. *Biochemistry*. 35: 11278-11285.

Fukushima, J., Yamamoto, S., Morihara, K., Atsumi, Y., Takeuchi, H., Kawamoto, S. and Okuda, K., 1898. Structural gene and complete amino acid sequence of *Pseudomonas aeruginosa* IFO 3455 elastase. *Journal of Bacteriology*. 171(3): 1698-1704.

Galloway, D.R. 1991. *Pseudomonas aeruginosa* elastase and elastinolysis revisited: Recent development. *Molecular Microbiology*, 5: 2315- 2321.

Garcia-Carreno, F.L. and Toro, M.A.N.D. 1997.Classification of proteases without tears. *Biochemical Education.* 25: 161-167.

Garcia-Ruiz, J.M. 2003. Counter-diffusion methods for macromolecular crystallization. *Methods in Enzymology.* 36: 130-154.

© COPYRIGHT UPM

Garman, E.F. 2010. Radiation damage in macromolecular crystallography what is it and why should we care?. *Acta Crystallographica D*. 64: 339-351.
Garman, E.F. and Owen, R.L. 2005. Cryocooling and radiation damage in macromolecular crystallography. *Acta crystallographica Section D*. 62: 32-47.
Garman, E. F., and Schneider, T. R. 1997. Macromolecular cryocrystallography. *Journal of Applied Crystallography*. 30: 211-237.
Gavira, J.A., Hernandez-Hernandez, M.A., Gonzalez-Ramirez, L.A., Briggs, R.A., Kolek, S.A. and Stewart, P.D.S. 2011. Combining counter-diffusion and microseeding to increase the success rate in protein crystallization. *Crystal Growth and Design*. 11(6): 2122-2126.
Geok, L.P., Razak, C.N.A., Rahman, R.N.Z.R.A., Basri,M. and Salleh, A.B. 2003. Isolation and screening of an extracellular organic solvent-tolerant protease producer. *Biochemical Engineering Journal*. 13: 73-77.
Ghorbel, B., Kamoun, A.S. and Nasri, M. 2003. Stability studies of protease from *Bacillus cereus* BGI. *Enzyme and Microbial Technology*. 32: 531.
Gilliland, G.L., Tung, M. and ladner, J. 1996. The biological macromolecule crystallization database and NASA protein crystal growth archeive. *Journal of Research of the National Institute of Stewards and Technology*. 101(3).
Godfrey, T. and West, S., 1996. Introduction to industrial enzymology. Industrial enzymology, 2nd edition. London: Macmillan Press. 1- 8.
Gupta, A., and Khare, S.K. 2009. Enzymes from solvent tolerant microbes useful biocatalysts for non-aqueous enzymology. *Critical Reviews in Biotechnology*. 29: 44- 54.
Gupta, A., Khare, S.K.. and Gupta, M.N. 2005. Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa PseA*. *Journal of Chromatography*. 1069: 155-1561.
Gupta, R., Beg, Q.K. and Lorenz, P. 2002. Bacterial alkaline protease: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, 59:15-32
Hase, C. and Finkelstein, R.A. 1993. Bacterial extracellular zinc-containing metalloprotease. *Microbiological Reviews*. 57: 823-837.
Hedstrom, L., 2002. Serine protease mechanism and specificity. *Chemical Reviews*. 102(12): 4501-4524.
Helliwell, J.R. and Chayen, N.E. 2007. Crytallography: a down to earth approach. *Nature*. 488: 658-659.
Holtje, H.D. and Folkers, G. 2008. Introduction to proten modelling. *In Molecular Modelling: Basic Principles and Application.* Wiley-VCH Verlag GmbH, Weinheim, Germany.
Ibrahim-Granet, O. and Bertrand, O. 1996. Seperation of proteases: old and new approaches. *Journal of Chromatography B*. 684: 239-263.
Iglewski, B.H., 1988. *Pseudomonas* toxins. In: Handbook of Natural Toxins. (eds.) Hardegree, M.C., and Tu, A.T., Marcel Dekker, New York, pp.249-265
Janson, J.C. and Ryden, L. 1998. Protein purification: principles, high-resolution methods and applications. 2nd Edition, New York: John Wiley and Sons. 645-696
Jones, T.A. 1978. A graphics model building and refinement system for macromolecules. *Journal of Applied Crystallography*. 11: 268-272.
Jones,T.A., Zou, J.Y., Cowan, S.W., and Kjeldgaard, M. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallography*. A47: 110-119.

© COPYRIGHT UPM

Karadzic, I., Masui, A. and Fujiwara, N., 2004. Purification and characterization of a protease from *Pseudomonas aeruginosa* grown in cutting oil. *Journal of Bioscience and Bioengineering*, 98: 145-152.

Kashchiev, D. 2000. Nucleation: Basic theory with applications. Oxford edition. Butterworth-Heinemann/ Oxford Press, New York.

Kawamoto, S., Shibano, Y., Fukushima, J., Ishii, N., Morihara, K. and Okuda, K. 1993. Site-directed mutagenesis of Glu-141 and His-223 in *Pseudomonas aeruginosa* elastase: catalytic activity, processing and protective activity of the elastase against Pseudomonas infection. *Infection and Immunity*. 61(4): 1400-1405.

Kessler, E., Kennah, H.E. and Brown, S.I., 1977. *Pseudomonas* protease. Purification, partial characterization and its effect on collagen, proteoglycan and rabbit cornea. *Investigative Opthalmology and Visual Science*. 16: 488-497.

Kessler, E., Safrin, M. and Burstein, Y. 1992. Identification of cleavage sites involved in proteolytic processing of pseudomonas aeruginosa preproelastase. *Federation of European Biochemical Societies.* 299: 291-293.

Kessler, E., Safrin, M., Gustin, M. and Ohman, D.E., 1998. Elastase and LasA protease of *Pseudomonas aeruginosa* are secreted with their propeptides. *Journal of Biological Chemistry*, 273: 30225-30231.

Kirk, O., Borchert, T.V. and Fuglsang, C.C. 2002. Industrial enzume applications. *Current Opinion in Biotechnology*, 13: 345-351.

Klibanov, A.M., 1997 Why are enzymes less active in organic solvents than in water?. *Trends in Biotechnology*, 15: 97-101.

Kumar, D., and Bhalla, T.C., 2005. Microbial protease in peptide synthesis: approaches and applications. *Applied Microbiology and Biotechnology*, 68: 726-736.

Laemmli, U. K., 1970. Cleavage of structure protein during assembly of the head of bacteriophage T_4. *Nature*, 277: 680-685.

Larson, S., Day, J., Greenwood, A., Oliver, J., Rubingh, D. and McPherson, A. 1991. Preliminary investigation of crystals of the neutral lipase from *Pseudomonas fluorescens. Journal of Molecular Biology.* 222: 21-22.

Laskowski, R.A., MasArthur, M.W., Moss, D.S. and Thornton, J.M., 1993. PROCHECK: a program to check the streochemical quality of protein structures. *Journal of Applied Crystallography*. 26: 283-291.

Laskowski, R.A., Watson, J.D., and Thornton, J.M. 2005. ProFunc: a server for predicting protein function from 3D structure. *Nucleic Acids Research*. 33: 89-93.

Leow, T.C., Rahman, R.N.Z.R.A., Basri, M. and Salleh, A.B. 2007. A thermoalkaliphilic lipase of *Geobacillus sp.* T1. *Extremophiles*. 11: 527-535.

Luft, J.R. and DeTitta, G.T. 1999. A method to produce microseed stock for use in the crystallization of biological macromolecules. *Acta Crystallographica D*. D55: 988-9993.

Luthy, R., Bowie, J.U. and Eisenberg, D. 1992. Assessment of protein models with three-dimensional profiles. *Nature*. 356: 83-85.

Maes, D., Gonzalez-Ramirez, L.A., Lopez-Jaramillo, J., Yu, B., DeBondt, H., Zegers, I., Afonina, E., Garcia-Ruiz, J.M., and Gulnik, S. 2004. Structural study of the type II 3-dehydroquinate dehydratase from Actinobacillus pleuropneumoniae. *Acta Crystallographica Section D*. 60(3): 463-471.

Matthews, B.W. 1968. Solvent content of protein crystals. *Journal of Molecular Biology*. 33: 491- 497.

Matthews, B.W. 1998. Structural basis of the action of Thermolysin and related zinc peptidase. Account of Chemical Research. 21(9): 333-340.

McGarth, M.E., 1999. The lysosomal cysteine protease. *Annual Reviews*. 28: 181-204.

McIver, K., Kessler, E. and Ohman, D.E. 1991. Substitution of active-site His-223 in *Pseudomonas aeruginosa* elastase and expression of the mutated lasB alleles in *Escherica coli* show evidence for autoproteolytic processing of proelastase. *Journal of Bacteriology.* 173: 4008-4015.

McPherson, A. 1995. Increasing the size of microcrystals by fine sampling of pH limits. *Journal of Biological Chemistry.* 251: 6300-6303.

McPherson, A. 1999. Crystallization of biological macromolecules (edn 1). Cold Spring Harbor Laboratory Press, Woodbury, New York.

McPherson, A. 2004. Introduction to protein crystallization. *Methods*. 34: 254-265.

McPherson, A. and Rich, A. 1973. X-ray crystallographic study of the quaternary structure of canavalin. *Journal of Biochemistry*. 74(1): 155-160.

Moreno, A., Saridakis, E. and Chayen, N.E. 2002. Combination of oils and gels for enhancing the growth of protein crystals. *Journal of Applied Crystallography*. 35: 140-142.

Moreno, A., The'obald-Dietrich, A., Lorber, B., Sauter, C. and Giege´, R. 2005. Effect of macromolecular impurities and of crystallization method on the quality of eubacterial aspartyl-tRNA synthetase crystals. *Acta Crystallography* Section *D*. D61: 789–792

Morihara, K., 1995. Pseudolysin and other pathogen endopeptidases of thermolysin family. *Method of Enzymology*. 248: 242-253.

Morihara, K., Tsuzuki, H., Oka, T., Inoue, H. and Ebata, M., 1965. *Pseudomonas aeruginosa* elastase: Isolation, crystallization and preliminary characterization. *The Journal of Biological Chemistry.* 240: 3295-3304.

Murshudov, G.N., Skubak, P., Lebedev, A.A., Pannu, N.S., Steiner, R.A., Nicholls, R.A., Winn, M.D., Long, F. and Vagin, A.A. 2011. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallographica Section D*. 67: 355-367.

Murshudov, G.N., Vagin, A.A., and Dodson, E.J. 1997. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallographica D*. D53: 240-255.

Ng, J.D., Gavira, J.A., and Garcia-Ruiz, J.M. 2003. Protein crystallization by capillary counterdiffusion for applied crystallographic structure determination. *Journal of Structural Biology*. 142(1): 218-231.

Ogino, H., Gemba, Y., Yamada, M., Shizuka, M., Yasuda, M. and Ishikawa, H. 2000a. The synthetic rate of dipeptide catalyzed by organic solvent-stable protease from *Pseudomonas aeruginosa* PST-01 in the presence of water-soluble organic solvents. *Biochemical Engineering Journal,* 5:519-223.

Ogino, H., Uchiho, T., Doukyu, N., Yasuda, M., Ishimi, K.. and Ishikawa, H. 2007b. Effect of exchange of amino acid residues of the surface region of the PST-01 protease on its organic solvent-stability. *Biochemical and Biophysical Research*, 358: 1028-1033.

Ogino, H., Uchiho, T., Yokoo, J., Kobayashi, R., Ichise, R. and Ishikawa, H., 2001. Role of intermolecular disulfide bonds of the organic solvent-stable PST-01 protease in its organic solvent stability. *Applied and Environmental Microbiology*, 67: 942-947.

Ogino, H., Yasui, K., Shiotani, T., Ishihara, T., and Ishikawa, H., 1995. Organic solvent stable-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme. *Applied and Environmental Microbiology*, 61(12): 4258-4262.

© COPYRIGHT UPM

Ohman, D.E., Cryz, S.J. and Iglewski, B.H. 1890. Isolation and characterization of a *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. *Journal of Bacteriology,* 142: 836-842.

Ogino, H., Yamada, M., Watanabe, F., Ichinose, H., Yasuda, M. and Ishikawa, H. 1999b. Peptide synthesis catalysed by organic solvent-stable protease from *Pseudomonas aeruginosa* PST-01 in monophasic aqueous-organic solvent syatems. *Journal of Bioscience and Bioengineering.* 88: 513-518.

Ogino, H., Gemba, Y. Yamada, M., Shizuka, M., Yasuda, M. and Ishikawa, H. 2000a. The synthetic rate of dipeptide catalysed by organic solvent-stable protease from Pseudomonas aeruginosa PST-01 in the presence of water-soluble organic solvents. *Journal of Biochemical Engineering*. 5:219-223.

Ooshima, H., Mori, H. and Harano, Y. 1985. Synthesis of aspartame precursor by solid thermolysin in oganic solvent. *Journal of Biotechnology Letters*. 7: 789- 792.

Otalora, F., Gavira, J.A., Ng, J.D., and Garcia-Ruiz, J.M. 2009. Counterdiffusion methods applied to protein crystallization. *Progress in Biophysics and Molecular Biology*. 101: 26-37.

Otto, H.H. and Schirrmeister, T. 1997. Cysteine proteases and their inhibitors. *Chemical Reviews*. 97: 133-171.

Otwinowski, Z. and Minor, W. 1997. Processing of X-ray diffraction data collected in oscillation mode. In *Methods in Enzymology*. 276: 307-326.

Page, M.J., and DiCera, E. 2008. Evolution of peptidase diversity. *Journal of Biological Chemistry*. 283: 30010-30014.

Parker, M.W. 2003. Protein structure from X-ray diffraction. *Journal of Biological Physics*. 29: 341- 362.

Perutz, M.F. 1964. Preparation of haemoglobin crystals. *Journal of Crystal Growth*. 2(1): 54-56.

Polgar, L. and Halasz, P., 1982. Current problems in mechanistic studies of serine and cycteine proteinase. *Biochemical Journal*. 207(1): 1-10.

Rahman, R.N.Z.R.A., Geok, L.P., Basri, M. and Salleh, A.B. 2006. An organics solvent-stable alkaline proteases from *Pseudomonas aeruginosa* strain K: Enzyme purification and characterization. *Enzyme and Microbial Technology.* 39: 1984- 1991.

Rahman, R.N.Z.R.A., Mahamad, S., Salleh, A.B. and Basri, M., 2007. A new organic solvent tolerant protease from *Bacillus pumilus* 115b. *Journal of Industrial Microbiology and Biotechnology*, 34: 509-517.

Rahman, R.N.Z.R.A., Salleh, A.B., Basri,M. and Wong, C.F. 2011. Role of α- Helical structure in Organic solvent-activated homodimer of elastase strain K. *International Journal of Molecular Sciences*. 12(9): 5793- 5814.

Rahman, R.N.Z.R.A., Geok, L.P., Basri, M. and Salleh, A.B. 2005. Physical factors affecting the production of organic solvent-tolerant protease by Pseudomonas aeruginosa strain K. *Bioresource and technology*. 96: 429–436.

Rahman, R.N.Z.R.A.; Geok, L.P.; Basri, M.; Salleh, A.B. An organic solvent-tolerant protease from Pseudomonas aeruginosa strain K: nutritional factors affecting protease production. *Enzyme and Microbial technology*. 36: 749–757

Rao, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V V. 1998. Molecular and Biotechnology aspects of microbial proteases. *Microbiology and Molecular Biology Reviews,* 62: 597-635.

Rawlings, N.D. and Barrett, A.J. 1999. MEROPS: the peptidase database. *Nucleic Acids Reviews*. 27: 325- 331.

Rawlings, N.D., Morton, F.R., Kok, C.Y.K. and Barrett, A.J. 2008. MEROPS: the peptidase database. *Nucleic Acids Research*. 36: D320- D325.

© COPYRIGHT UPM

Rawlings, N.D., Tolle, D.P. and Barrett, A.J. 2004. MEROPS: the peptidase database. *Nucleic Acids Research.* 32*:* 160-164.
Rhodes, G. 1993. Crystallography made crystal clear. San Deigo: Academic Press. 8-38.
Rodgers, D.W. 2001. Cryocrystallography techniques and devices. *International Tables for Crystallography Volume F*. 202-208.
Rost, B. 1999. Twilight zone of protein sequence alignments. *Protein Engineering*. 12: 85-94.
Ru, M.T., Dordick, J.S., Reimer, J.A., and Clark, D.S. 1999. Optimizing the salt-induced activation of enzymes in organic solvents: Effect of lyophilization time and water content. *Biotechnology and Bioengineering*, 63: 233-241.
Rupp, B. 2010. Biomolecular crystallography: Principles, practice, and application to structural biology. New York: Garland Science, Taylor and Francis Grouo. 808.
Rust, L., Messing, C.R. and Iglewski, B.H., 1994. Elastase assays. *Methods in Enzymology*, 235: 554-562.
Ryan, K., 1984. *Pseudomonas* and other opportunistic gram-negative bacilli. *In:Medical Microbiology* (ed.) Sherris, J.C., Elsevier, New York, pp. 264-270
Salemme, F.R. 1972. A free interface diffusion technique for the crystallization of proteins for X-ray crystallography. *Archives of Biochemistry and Biophysics*. 151(2): 533-539.
Salleh, A.B., Razak, C.N.A., Rahman, R.N.Z.R.A., and Basri. M. 2006. Protease: Introduction. In: New lipases and proteases. (eds.) Salleh, A. B., Rahman, R. N. Z. R. A., and Basri, M., Nova Science Publishers, New York. pp: 1-7.
Sauter, C., Lorber, B. and Geige, R. 2002. Towards atomic resolution with crystals grown in gel: The case of thaumatin seen at room temperature. *Protein. Structure, Function, and Genetics.* 48: 146-150.
Saveliev, S., Engel, L., Strauss, E., Jones., and Rosenblatt, M. The advantages to using Arg-C, elastase, thermolysin and pepsin for protein analysis. Promega corporation web site.2012. http:// worldwide.romega.com/resouces/pubhub/. Accessed January 23, 2015.
Schwarzenbacher, R., Godzik, A., Grzechnik, S.K. and Jaroszewski, L. 2004. The important of alignment accuracy for molecular replacement. *Acta Crystallographica D*. 60: 1229-1236.A
Serdakowski, A.L., and Dordick, J.S., 2008. Enzyme activation in organic solvents made easy. *Trends in Biotechnology*, 26: 48-54.
Shen, H.B., and Chou, K.C., 2009. Identification of proteases and their types. *Analytical Biochemistry*. 385(1): 153-160.
Shurki, A., Strajbl, M., Villa, J. and Warshel, A., 2002. How much do enzymes really gain by restraining their reacting fragments. *Journal of the American Chemical Society*, 124: 4097-4107.
Stewart, P.D.S., Kolek, S.A., Briggs, R.A., Chayen, N.E. and Baldock, P.F.M. 2011. Random microseeding: a theoretical and practical exploration of seed stability and seeding techniques for successful protein crystallization. *Crystal Growth and Design*. 11(8): 3432-3441.
Storer, A.C. and Menard, R. 1994. Catalytic mechanism in papain family of cysteine prptidases. *Methods in Enzymology*. 244: 486-500.
Stura, E., Wilson, I. 1991. Application of the streak seeding technique in protein crystallization. *Journal of Crystal Growth.* 110: 270-282.
Suhartono, M.T., Satiawihardja, B., Widjaja, I.P.N., and Handoko, T.W.T., 1997. Characterization of protease enzyme produced produced from *Bacillus*

© COPYRIGHT UPM

pumilus Y1 isolated from liquid tofu waste. *Journal of Molecular Biology Biotechnology*, 5(2): 72-80.

Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y., Forrester, C. and Shi, Y. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proceeding of the National Academy of Sciences.* 99(8): 5515- 5520.

Suter,S. 1994. The role of bacterial proteases in the pathogenesis of cystic fibrosis. American Journal of Respiratory and Critical Care Medicine. 150:118-122.

Talreja, S., Kim, D.Y., Mirarefi, A.Y., Zukoski, C.F., and Kenis, P.J.A. 2005. Screening and optimization of protein crystallization conditions through gradual evaporation using a novel crystallization platform. *Journal of Applied Crystallography*. 38: 988- 995.

Tanaka, M., Chien, P., Naber, N., Cooke, R. and Weissman, J. S. 2004. Conformational variations in an infectious protein determine prion strain differences. *Nature*. 428: 323-328.

Tang, X.Y., Pan, Y., Li, S. and He, B.F., 2008. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. *Bioresource Technology*. 99(15): 7388- 7392.

Thayer, M.M., Flaherty, K.M., and McKay, D.B., 1991. Three-dimensional structure of elastase of *Pseudomonas aeruginosa* at 1.5 Å resolution. *The Journal of Biological Chemistry*, 5: 2864-2871.

Vagin, A. and Teplyakov, A. 1997. “MOLREP”: An automated program for molecular replacement. *Journal of Applied Crystallography*. 30:1022-1025.

Varela, H., Ferrari, M. D., Belobrajdic, L., Vazquez, A. and Loperena, M.L., 1997. Skin unhairing proteases of *Bacillus subtilis:* Production and partial characterization. *Biotechnology Letter*. 19:755-758.

Weber, B.H. and Goodkin, P.E. 1970. A modified microdiffusion procedure for the growth of single protein. *Archives of Biochemistry and Biophysics*. 141(2): 489-498.

Wong, C.F., Salleh, A.B., Basri, M. and Rahman, R.N.Z.R.A., 2010. Organic-solvent stability of elastase strain K overexpressed in an *Eschericia-Pseudomonas* expression system. *Biotechnology and Applied Biochemistry*. 57: 1-7.

Xindu, G. and Lili, W. 2008. Liquid chromatography of recombinant proteins and protein drugs. *Journal of Chromatography B*. 866: 133-153.

Xu, D., Alipio, Z., Fink, L.M., Adcock, D.M., Yang, J., Ward, D.C., and Ma, Y., 2009. Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. *Proceeding of the National Academy of Sciences.* 106: 808-813.

Yang, J., Teplyakov, A., and Quail, J.W. 1997. Crystal structure of the aspartic proteinase from Rhizomucor meihei at 2.15 Å resolution. *Journal of Molecular Biology*. 268: 449-459

© COPYRIGHT UPM