STRUCTURAL ELUCIDATION OF GDSL ESTERASE OF Photobacterium sp. J15

SHARIFAH NUR HIDAYAH BINTI SYED MAZLAN

FBSB 2017 44

STRUCTURAL ELUCIDATION OF GDSL ESTERASE OF *Photobacterium* sp. J15

By

SHARIFAH NUR HIDAYAH BINTI SYED MAZLAN

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

September 2017

CORYRIGHT

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

STRUCTURAL ELUCIDATION OF GDSL ESTERASE OF *Photobacterium* sp. J15

By

SHARIFAH NUR HIDAYAH BINTI SYED MAZLAN

September 2017

Chairman : Adam Leow Thean Chor, PhD
Faculty : Biotechnology and Biomolecular Sciences

GDSL esterase is a member of Family II of lipolytic enzyme that catalyses the synthesis and hydrolysis of ester. The enzyme was further classified in subgroup SGNH hydrolases due to the presence of highly conserved motif, Ser-Gly-Asn-His in four conserved blocks I, II, III, and V, respectively. The broad substrate specificity and regiospecificy make these enzymes as attractive candidates for various applications. To date, the number of structure of GDSL motif enzymes deposited in the Protein Data Bank (PDB) is less than 50 and none of them came from *Photobacterium*. Besides, the low amino acid sequence identity of only 23% with a passenger domain of autotransporter EstA of *Pseudomonas aeruginosa* makes this enzyme to be more interesting to be studied structurally. Hence, in order to solve the structure of GDSL esterase (EstJ15), X-ray crystallography was conducted. The recombinant *E.* coli Rosetta-gami (DE3) pLysS (pET::*EstJ15*) was overexpressed at 20°C using 0.1 mM IPTG. The crude protein was then undergo two-step chromatography which were affinity chromatography and ion exchange chromatography to produce a highly purified EstJ15 with the tags were cleaved using thrombin. Crystallisation of the purified EstJ15 was carried out via sitting drop vapor diffusion method using 5 mg/ml protein incubated at 15°C. Crystal optimisation of EsJ15 was conducted in order to improve the crystal quality by using different concentrations of salt (ammonium sulphate) and precipitant (PEG 8000). Crystal of EstJ15 was obtained from optimised formulation containing 0.10 M ammonium sulphate, 0.15 M sodium cacodylate trihydrate pH 6.5, and 20% PEG 8000 with crystal dimension of 0.6 mm x 0.2 mm x 0.3 mm. The phase problem was initially solved by using single-wavelength anomalous dispersion (SAD) method using iodide ions as the anomalous scatterer. The crystal was diffracted at 1.78 Å using an in-house X-ray beam and the crystal belongs to primitive

orthorhombic space group $P2_12_12_1$, with unit cell parameter of a = 49.43, b = 66.28, c = 105.46 Å, $\alpha = \beta = \gamma = 90°$. There is one molecule per asymmetric unit with solvent content of 47.33%. Four iodide ions were located in the structure. The model was then used to solve the native EstJ15 data with higher resolution of 1.38 Å via molecular replacement method. The crystal also belong to primitive orthrombic space group $P2_12_12_1$, with unit cell parameter of a = 49.18, b = 66.46, c = 105.47 Å, $\alpha = \beta = \gamma = 90°$. The solvent content is 47.23% with one molecule per asymmetric unit. The crystal structure of Est15 was refined and the final model was evaluated using Ramachandran plot, Verify3D and Errat. The Ramachandran plot evaluation showed that 89.9% of the residues in the crystal structure of EstJ15 are in the most favoured region. Evaluations of the crystal structures by using Errat and Verify3D also showed that the EstJ15 model gave values that were in acceptable range of good structures. The final R_{factor} obtained was 0.1580 with R_{free} value of 0.1810. The 3D structure of EstJ15 revealed topological organisation of α/β-hydrolase fold of 38.7% helix, 21.3% sheet, 11.0% turn and 29.0% coil. There is one phosphate ion and one chloride ion in the structure. Based on the structural alignment of EstJ15 with other structurally known GDSL motif enzymes, Ser12, Asp302 and His305 were assigned as the catalytic triad with the nucleophile, Ser12 is located near to the N-terminus. Besides, EstJ15 is also grouped under the subgroup of SGNH family with the presence of four conserved blocks I, II, III, and V composed of Ser12, Gly105, Asn161 and His305, respectively. The active site of EstJ15 is located at the centre of the structure, formed a narrow tunnel, channelling to the core. The active site residues including the catalytic triad have low B-factors of less than 10 $Å^2$ contributed to the high stability of the enzyme that can withstand harsh conditions better. This explain the high salt tolerance of EstJ15 at which the enzyme remained stable and functional in the presence of high salt concentration besides remain stable in various buffers. Besides, the high specificity of EstJ15 toward short chain length substrates (up to C4) was successfully unveiled via protein-ligand docking analysis. The structure elucidation of EstJ15 also provide insights on its catalytic mechanism. In conclusion, the solved structure of EstJ15 revealed exclusive features as compared to other available structure of GDSL motif enzymes. The structure was the first GDSL motif enzyme deposited from *Photobacterium* and highly valuable due to its tolerance to harsh conditions. Besides, the structure may contribute hugely in the field of structural biology where it may serve as a template in solving the phase problem of proteins with decent amino acid sequence identity to it via molecular replacement, where they are remain unsolvable due to low identity to the current available protein structures.

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

PENERANGAN STRUKTUR GDSL ESTERASE DARI *Photobacterium* sp. J15

Oleh

SHARIFAH NUR HIDAYAH BINTI SYED MAZLAN

September 2017

Pengerusi : Adam Leow Thean Chor, PhD
Faculti : Bioteknologi dan Sains Biomolekul

GDSL esterase adalah ahli Keluarga II enzim lipolitik yang memangkin proses sintesis dan hidrolisis ester. Enzim berkenaan kemudiannya dikategorikan ke dalam subkumpulan SGNH hidrolis dengan adanya motif yang sangat terpelihara, Ser-Gly-Asn-His dalam empat blok I, II, III, dan V. Kekhususan substrat dan kekhususan rantau yang luas membuatkan enzim-enzim ini sesuai dalam pelbagai aplikasi. Sehingga kini, kurang dari 50 struktur enzim motif GDSL telah didepositkan dalam Protein Data Bank (PDB) yang mana tiada satu pun berasal dari *Photobacterium*. Selain itu, identiti rangkaian asid amino yang rendah, 23% dengan domain penumpang pengangkut auto EstA dari *Pseudomonas aeruginosa* menjadikan struktur enzim ini lebih menarik untuk dikaji. Oleh itu, untuk menyelesaikan struktur GDSL esterase (EstJ15), kristalografi sinar-X dijalankan. Penghasilan protein EstJ15 dalam rekombinan *E.* coli Rosetta-gami (DE3) pLysS (pET::*EstJ15*) telah dijalankan pada suhu 20°C menggunakan 0.1 mM IPTG. Dua langkah kromatografi iaitu kromatografi afiniti dan kromatografi pertukaran ion telah dijalankan untuk menghasilkan EstJ15 yang tulen dan tag-tag dibuang menggunakan thrombin. Penghabluran EstJ15 yang sangat tulen telah dijalankan menggunakan kaedah resapan wap titisan menggunakan 5 mg/ml protein dan diinkubasi pada 15°C. Pengoptimuman hablur EstJ15 telah dilakukan dalam usaha untuk meningkatkan kualiti kristal dengan menggunakan kepekatan garam (ammonium sulfat) dan pemendak (PEG 8000) yang berbeza. Kristal EstJ15 berkualiti sinar-X telah diperolehi daripada formulasi yang mengandungi 0,10 M ammonium sulfat, 0.15 M natrium kakodilat trihidrat pH 6,5, dan 20% PEG 8000 dengan dimensi kristal 0.6 mm x 0.2 mm x 0.3 mm. Masalah fasa pada mulanya diselesaikan dengan menggunakan penyebaran ganjil gelombang tunggal (SAD) menggunakan ion iodida sebagai penyebar ganjil. Kristal itu dibelaukan pada

resolusi 1.78 Å menggunakan sumber sinar-X dalaman dan kristal tergolong dalam kumpulan primitif otorombik $P2_12_12_1$, dengan parameter unit sel *a* = 49.43, *b* = 66.28, *c* = 105.46 Å, $\alpha = \beta = \gamma = 90$ °. Terdapat satu molekul per unit simetri dengan kandungan pelarut sebanyak 47.33%. Terdapat empat ion iodida di dalam struktur. Model itu kemudian digunakan untuk menyelesaikan data EstJ15 asli dengan resolusi yang lebih tinggi iaitu 1.38 Å melalui kaedah penggantian molekul. Kristal ini juga tergolong dalam kumpulan primitif otorombik $P2_12_12_1$, dengan parameter unit sel *a* = 49.18, *b* = 66.46, *c* = 105.47 Å, $\alpha = \beta = \gamma = 90$ °. Kandungan pelarut adalah 47.23% dengan satu molekul per unit simetri. Struktur kristal Est15 telah ditapis dan model akhir telah dinilai menggunakan plot Ramachandran, Verify3D dan Errat. Penilaian melalui plot Ramachandran menunjukkan 89.9% daripada residu di dalam struktur kristal EstJ15 terletak di kawasan yang paling disukai. Penilaian struktur kristal dengan menggunakan Errat dan Verify3D juga menunjukkan bahawa model EstJ15 memberikan nilai-nilai yang tergolong dalam lingkungan struktur protein yang boleh diterima. Nilai R_{factor} yang diperoleh 0.1580 manakala nilai R_{free} yang diperoleh adalah 0.1810. Struktur 3D EstJ15 mendedahkan organisasi topologi lipatan α/β-hidrolis dengan 38.7% helik, 21.3% lembaran, 11.0% pusingan dan 29.0% gegelung. Terdapat satu ion fosfat dan satu ion klorida di dalam struktur. Dari penjajaran struktur dengan lain-lain enzim motif GDSL, Ser12, Asp302 dan His305 telah dikenalpasti sebagai triad katalitik dengan nukleofil Ser12 berada berdekatan dengan terminal-N. EstJ15 juga dikelaskan dibawah subkumpulan keluarga SGNH dengan kehadiran empat blok terpelihara I, II, III dan V yang terdiri daripada Ser12, Gly105, Asn161 dan His305. Rongga aktif EstJ15 terletak di tengah struktur, menghasilkan terowong sempit menyalur ke teras. Residu-residu rongga aktif termasuk triad katalitik mempunyai nilai B-faktor yang rendah daripada 10 $Å^2$ yang menyumbang kepada tahap stabliti yang tinggi yang membolehkan enzim ini bertahan dalam keadaan persekitaran yang terlampau. Hal ini menerangkan tahap toleransi EstJ15 terhadap garam yang tinggi yang mana enzim ini tetap stabil dan berfungsi dalam tahap kepekatan garam yang tinggi selain tetap stabil dalam berbagai penampan. Selain itu, kekhususan substrat yang tinggi terhadap substrat yang pendek berjaya dirungkaikan melalui analisa dok protein-ligan. Penjelasan struktur EstJ15 ini juga dapat menberikan pemahaman tentang mekanisma katalitik enzim ini. Kesimpulannya, penyelesaian struktur EstJ15. Struktur ini adalah yang pertama dideposit dari *Photobacterium* dan sangat bernilai kerana ia mampu bertahan dalam persekitaran yang terlampau. Kesimpulannya, struktur EstJ15 mendedahkan ciri eksklusif berbanding dengan struktur-struktur enzim motif GDSL yang sedia ada. Struktur ini boleh menberi sumbangan besar dalam bidang biologi struktur yang mana ia boleh dijadikan sebagai templat dalam penyelesaian masalah fasa bagi protein-protein yang mempunyai identiti rangkaian asid amino yang sesuai melalui kaedah penggantian molekul, yang mana protein-protein ini masih tidak berjaya diselesaikan berikutan identiti yang rendah berbanding dengan struktur-struktur protein yang sedia ada.

ACKNOWLEDGEMENTS

All praises to Allah for His blessings, giving me a beautiful life and good health throughout the years of finishing my study. Special appreciation to my supervisor, Associate Professor Dr. Adam Leow Thean Chor for giving me the opportunity to work on this project, for the invaluable guidance, encouragement and constant support while completing this study.

I also would like to thank my supervisory committee, Professor Dr. Raja Noor Zaliha Raja Abd. Rahman and Associate Professor Dr. Mohd Shukuri Mohamad Ali and the other principal investigators in EMTech research group for their encouragement, insightful comments and helpful discussions. My sincere thanks also dedicated to Dr. Mohd Anuar Jonet from Malaysia Genome Institute (MGI) for his help, suggestion and cooperation in data collection. Their suggestions and encouragement helped greatly in accomplishing my work.

Special thanks to all the members of EMTech research group especially my closest collegues, Sayangku Nor Ariati, Noordiyanah Nadhirah, Ang Thiau Fu, Jonathan Miangwa and Ashwaani for their supports, knowledge and friendship. Special thanks also extended to the staff of Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia for their help and cooperation.

Last but not least, I am eternally grateful to my beloved parents, Syed Mazlan Syed Osman and Normah Zainan, and my siblings, Kakak and Abang for their prayers, encouragement and utmost support. Also not to forget, Sadiq Ilman for always be there for me as my biggest supporter throughout the bumpy road. To those who indirectly contributed in this research, your kindness means a lot to me. Thank you.

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

Adam Leow Thean Chor, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

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

Mohd Shukuri Mohamad Ali, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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.: Sharifah Nur Hidayah Binti Syed Mazlan, GS41087

Declaration by the Members of Supervisory Committee

This is to certify that:

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

Signature: _____
Name of Chairman of Supervisory Committee: Associate Professor Dr. Adam Leow Thean Chor

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Raja Noor Zaliha Raja Abd. Rahman

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Mohd Shukuri Mohamad Ali

TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLERATION		viii
LIST OF TABLES		xiii
LIST OF FIGURES		xiv
LIST OF ABBREVIATIONS		xvi
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	3
	2.1 Lipolytic enzymes	3
	2.2 Esterases and their applications	4
	2.3 GDSL motif enzymes	5
	2.4 Sources and functions of GDSL motif enzymes	6
	2.5 GDSL esterase from Photobacterium	8
	2.6 Protein purification strategies	9
	2.6.1 Affinity chromatography	9
	2.6.2 Affinity tag and its removal	10
	2.6.3 Ion exchange chromatography	12
	2.7 Protein crystallisation	13
	2.7.1 Properties of protein crystals	13
	2.7.2 The thermodynamic of crystal formation	14
	2.7.3 Parameters affecting protein crystal growth	15
	2.7.4 Crystallisation techniques	16
	2.8 Structural elucidation	17
	2.9 X-ray crystallography	17
	2.10 Phase determination	18
	2.10.1 Molecular replacement (MR)	19
	2.10.2 Single-wavelength anomalous dispersion (SAD)	19
	2.11 Refinement and validation	20
	2.12 Protein structure and function	21
3	**MATERIALS AND METHODS**	22
	3.1 Bacterial source	22
	3.2 Protein concentration determination	22
	3.3 Molecular weight determination by SDS-PAGE	22
	3.4 Quantitative determination of esterase activity	23
	3.4.1 Preparation of ρ-nitrophenol standard curve	23
	3.4.2 Esterase assay	23

3.5 Overexpression of recombinant EstJ15 23
3.6 Purification of recombinant EstJ15 24
3.6.1 Purification of EstJ15 by affinity chromatography 24
3.6.2 Thrombin cleavage 24
3.6.3 Purification of EstJ15 by anion exchange chromatography 24
3.7 Crystal screening 25
3.8 Optimisation of protein crystal growth 25
3.8.1 Seeding 25
3.8.2 Precipitant concentration 25
3.8.3 Salt concentration 26
3.8.4 Hanging drop vapor diffusion method 26
3.9 X-ray diffraction and structure elucidation 27
3.9.1 Cryosoaking procedure for derivatization with halide ion 27
3.9.2 X-ray diffraction data collection 27
3.9.3 Phase determination 27
3.9.3.1 Single-wavelength anomalous dispersion (SAD) 27
3.9.3.2 Molecular replacement (MR) 28
3.9.4 Model building and refinement 28
3.9.5 Structure validation 28
3.9.6 Structural analysis 29
3.9.6.1 Primary structure analysis 29
3.9.6.2 Secondary structure analysis 29
3.9.6.3 Overall structure analysis 29
3.9.7 Postulation of catalytic mechanism of EstJ15 30
3.9.8 Structural comparison of EstJ15 with its close homologs 30

4 RESULTS AND DISCUSSION 31
4.1 Purification of the recombinant EstJ15 31
4.1.1 Purification of EstJ15 using affinity chromatography 31
4.1.2 Purification of EstJ15 with anion exchange chromatography 34
4.2 Protein crystal screening 36
4.3 Optimisation of protein crystallisation 39
4.3.1 Microseeding 39
4.3.2 Optimisation of precipitant concentration 40
4.3.3 Optimisation of salt concentration 43
4.3.4 Hanging drop vapor diffusion method 45
4.4 X-ray diffraction analysis 46
4.4.1 Data collection of native EstJ15 47
4.4.2 Data collection of iodide-soaked EstJ15 crystal 49
4.5 Phase determination 51
4.5.1 Single-wavelength anomalous dispersion (SAD) 52
4.5.2 Molecular replacement 54

4.6 Evaluation of EstJ15 crystal structure 56
4.7 Structural analysis 62
4.7.1 Primary structure 62
4.7.2 Secondary structure 65
4.7.3 Overall structure analysis 67
4.7.3.1 B-factor 68
4.7.3.2 Chemical interactions 72
4.7.3.3 Docking analysis 74
4.8 Postulation of EstJ15 catalytic mechanism 78
4.9 Structural comparison of EstJ15 with its closest homologs 80

5 CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 84
5.1 Conclusion 84
5.2 Recommendation for future research 85

REFERENCES 86
APPENDICES 98
BIODATA OF STUDENT 111
PUBLICATION 112

LIST OF TABLES

Table		Page
1	GDSL/SGNH hydrolases family members and their functions	7
2	Purification techniques based on protein properties	9
3	Sequence and size of affinity tag	11
4	Enzymatic methods of tag removal	12
5	Crystallisation parameters	15
6	Optimisation of precipitant concentration (PEG 8000)	26
7	Optimisation of salt concentration (ammonium sulphate)	26
8	Hanging drop vapor diffusion method	27
9	Summary of EstJ15 purification using two steps chromatography	36
10	Protein crystal hits using various crystal screening kits	37
11	Statistics of data collection for native EstJ15 and iodide-soaked EstJ15 crystals	49
12	Refinement statistics of EstJ15 crystal structures.	55
13	Amino acid composition of EstJ15	63
14	Intramolecular and intermolecular interaction of EstJ15	72
15	Ion pair interactions	74
16	Enzymes identified as structurally homologous to EstJ15 through DALI	81

LIST OF FIGURES

Figure		Page
1	Secondary structure diagram of α/β hydrolase folds	4
2	Phase diagram of crystal growth	14
3	Experimental setup for vapour diffusion crystallisation	17
4	Purification profile of recombinant EstJ15 via affinity chromatography using Ni^{2+}-Sepharose	32
5	SDS-PAGE analysis of partially purified EstJ15 via affinity chromatography using Ni^{2+}-Sepharose	33
6	SDS-PAGE analysis of comparison between tagged-EstJ15 and tag-cleaved EstJ15	34
7	SDS-PAGE analysis of purified EstJ15 via ion exchange chromatography using Q-Sepharose	35
8	Purification profile of recombinant EstJ15 by ion exchange chromatography using Q-Sepharose	35
9	Protein crystal verification	39
10	EstJ15 crystals grown using concentrated microseeds	40
11	Optimisation of precipitant concentration (PEG 8000) in the range of 15% to 35%	42
12	Optimisation of salt concentration (ammonium sulphate) in the ranges of 0.05 M to 0.30 M	44
13	Hanging drop vapor diffusion method of different drop volume	45
14	X-ray data collection of EstJ15 crystal that failed to process due to heavily twinned diffraction spots	47
15	X-ray data collection of EstJ15 crystal at the resolution of 1.38 Å	48
16	X-ray data collection of iodide-soaked EstJ15 crystal at the resolution of 1.78 Å	50

17	Molecular replacement using crystal structure of the full-length autotransporter EstA from *Pseudomonas aeruginosa* as search template	52
18	Four iodide ions sites in the EstJ15 structure	53
19	EstJ15 structure at 1.78 Å generated from SAD phase data	54
20	Alternate conformations of five amino acid residue in EstJ15 crystal structure	55
21	Ramachandran plot of EstJ15 crystal structure	57
22	Verify 3D plot	59
23	ERRAT plot	61
24	Amino acid sequence of EstJ15	62
25	Evolutionary tree of EstJ15	64
26	Secondary structure of EstJ15	66
27	Crystal structure of EstJ15 solved at a resolution of 1.38 Å	68
28	Low B-factor regions in EstJ15 crystal structure.	69
29	Plot of the average B-factor of EstJ15 structure	71
30	Docking analysis of EstJ15 with ρ-nitrophenyl substrates	76
31	The oxyanion hole of EstJ15	78
32	Proposed mechanism of action of EstJ15	79
33	Structural alignment of EstJ15 and the passanger domain of autotransporter EstA of *Pseudomonas aeruginosa*	82
34	Structural sequence alignment between EstJ15 and core domain of EstA from *Pseudomonas aeruginosa*	83

LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
β	Beta
°C	Degree Celsius
%	Percentage
A_{600nm}	Absorbance at wavelength 600 nanometer
µL	Microliter
µm	Micrometer
APS	Ammonium persulfate
bp	Base pair
$CaCl_2$	Calcium chloride
E. coli	*Escherichia coli*
EC	Enzyme Commission
g	Gram
h	Hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDA	kilodaltons
L	Litre
LB	Luria-Bertani
M	Molar
mA	MilliAmps
mg/ml	Miligram per mililiter
min	Minute
ml	Mililiter
mm	Milimeter
nm	Nanometer
OD	Optical density
SDS	Sodium dedocyl sulphate

SDS-PAGE	Sodium dedocyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N- Tetramethyllenediamide
U	Unit
U/ml	Unit per mililiter
U/mg	Unit per milligram
µl	Microliter
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Esterases and lipases are hydrolytic enzymes that catalyse the cleavage and formation of ester bonds. These enzymes can be isolated from various sources including animals, plants and microorganisms. These enzymes are highly attractive in various applications, mainly in food and beverage, fine chemicals and pharmaceuticals due to their broad substrate specificity and regiospecificity (Pandey et al., 1999; Jaeger and Reetz, 1998).

GDSL hydrolases are grouped in Family II of lipolytic family. These enzymes exhibit α/β hydrolase folds and share the same catalytic process via Ser-Asp/Glu-His triad. Unlike other hydrolases, the members of Family II have the nucleophile, Ser located at Gly-Asp-Ser-Leu (GDSL) motif closer to the N-terminus, different from other ordinary esterases and lipases (Akoh *et al.*, 2004).

GDSL motif enzymes can be found in various sources including microbes and plants with well diversed functions due to their broad substrate specificities and diverse catalytic activities. Although these enzymes readily available to be explored, the study on these enzymes is still limited. To date, less than 50 structures of GDSL motif enzymes have been deposited in the Protein Data Bank (PDB). In fact, only four hydrolase structures originated from *Photobacterium* sp. are available at which none is GDSL motif enzyme (Rose *et al.*, 2017).

GDSL esterase J15 (EstJ15) is an enzyme originated from *Photobacterium* sp. J15, a bacterium isolated from seawater of Tanjung Pelepas, Johor, Malaysia. The open reading frame of *EstJ15* gene is 1,044 bp in length which encodes for 347 amino acids. The molecular mass and pI were predicted to be 35.6 kDa and 6.3, respectively. EstJ15 shows high activity towards short carbon chain length substrates with the highest activity on ρNP-butyrate (C4) but no activity was observed for substrate longer than C4 (Shakiba *et al*., 2016).

EstJ15 shares the highest amino acid sequence identity of 23% with the passenger domain of an autotransporter EstA (Chain A) from *Pseudomonas aeruginosa* (PDB ID: 3kvn). The full-length of autotransporter EstA comprises of an autotransporter and a passenger domain that was solved at a resolution of 2.5 Å. The autotransporter domain comprises of a relatively narrow, 12-stranded β-barrel that is covalently attached to the passenger

domain via a long, curved helix that occupies the internal space of the β-barrel (van den Berg, 2010).

Due to the low amino acid sequence identity of less than 30%, these two enzymes cannot be grouped into the same family. Hence, the study of the EstJ15 structure is important to know the structural differences of these two GDSL motif enzymes besides to investigate the catalytic mechanism of EstJ15 and more importantly is to understand why it selectively catalyses only short chain length substrates.

X-ray crystallography is one of the means in determining the three dimensional structure of biological macromolecules. In order to understand the fact behind the selectivity of EstJ15 towards short carbon chain length substrates, X-ray crystallography was conducted to study the 3D structure of EstJ15 and enable the establishment of the structure-function relationships and contribute for a better understanding of the kinetic mechanism of the enzymes.

Unlocking the structure of EstJ15 could help in the understanding of the selectivity of EstJ15 on short carbon chain length substrates and postulating its catalytic mechanism. Therefore, this study was undertaken with the following objectives:

i. To express and purify GDSL esterase enzyme
ii. To crystallise GDSL esterase enzyme
iii. To solve the 3-dimensional structure of GDSL esterase via X-ray crystallography
iv. To analyse the GDSL esterase structure.

REFERENCES

Abdelkafi, S., Ogata, H., Barouh, N., Fouquet, B., Lebrun, R., Pina, M., Scheirlinckx, F., Villeneuve, P., and Carrière, F. (2009). Identification and biochemical characterization of a GDSL-motif carboxylester hydrolase from *Carica papaya* latex. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*. *1791*(11), 1048-1056.

Abendroth, J., Gardberg, A. S., Robinson, J. I., Christensen, J. S., Staker, B. L., Myler, P. J. and Edwards, T. E. (2011). SAD phasing using iodide ions in a high-throughput structural genomics environment. *Journal of structural and functional genomics*. *12*(2), 83-95.

Afonine, P. V., Grosse-Kunstleve, R. W., Adams, P. D., and Urzhumtsev, A. (2013). Bulk-solvent and overall scaling revisited: faster calculations, improved results. *Acta Crystallographica Section D: Biological Crystallography*. *69*(4), 625-634.

Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., and Shaw, J. F. (2004). GDSL family of serine esterases/lipases. *Prog Lipid Res*. *43*(6):534–552.

Akutsu, Y., Nakajima-Kambe, T., Nomura, N., and Nakahara, T. (1998). Purification and properties of a polyester polyurethane-degrading enzyme from *Comamonas acidovorans* TB-35. *Applied and environmental microbiology*. *64*(1), 62-67.

Andualema, B. and Gessesse, A. (2012). Microbial lipases and their industrial applications: Review. *Biotechnology*. 11:100-118.

Arnau, J., Lauritzen, C., Petersen, G. E., and Pedersen, J. (2006). Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein expression and purification. 48*(1), 1-13.

Arpigny, J. and Jaeger, K. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochem. J. 343*, 177-183.

Atha, D. H., and Ingham, K. C. (1981). Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. *Journal of Biological Chemistry*. *256*(23), 12108-12117.

Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002). Secondary structure: Polypeptide chains can fold into regular structures such as the alpha helix, the beta sheet, and turns and loops. *Biochemistry* (5th ed., section 3.3). New York, NY: W. H. Freeman.

Bergfors, T. (2003). Seeds to crystals. *Journal of structural biology*. *142*(1), 66-76.

Bernal, J. D. and Crowfoot, D. (1934). *Nature*. 133:794.

Blow, D. M., Birktoft, J. and Hartley, B. (1969). *Nature*. *221*, 337-339.

Bollag, D. M. (1994). Ion-exchange chromatography. *Methods Mol Biol*. *36*, 11–22.

Bornscheuer, U. T. (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol Rev*. *26*, 73-81.

Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., and Dodson, G. (1990). A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature*. *343*(6260), 767.

Brown, T. L., LeMay, H. E., Bursten, B. E., and Brunauer, L. S. (1994). *Chemistry: the central science* (Vol. 8, pp. 3-4). Englewood Cliffs, NJ: Prentice Hall.

Bugg, C. E. (1986). The future of protein crystal growth. *Journal of Crystal Growth*. *76*(3), 535-544.

Chapman, M. S. and Blanc, E. (1997). Potential use of real-space refinement in protein structure determination. *Acta Crystallographica Section D: Biological Crystallography*. *53*(2), 203-206.

Choi, Y. J. and Lee, B. H. (2001) Culture conditions for the production of esterase from *Lactobacillus casei* CL 96. *Bioprocess Biosyst Eng*. *24*, 59–63.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst*. *D50*, 760-763.

Colovos, C., and Yeates, T. O. (1993). ERRAT: an empirical atom-based method for validating protein structures. *Protein Sci*, *2*, 1511-1519.

Cousin, X., Hotelier, T., Giles, K., Pierre Toutant, J., and Chatonnet, A. (1998). aCHEdb: the database system for ESTHER, the α/β fold family of proteins and the Cholinesterase gene server. *Nucleic acids research*, *26*(1), 226-228.

Cowtan, K. (1994). DM: an automated procedure for phase improvement by density modification. *Joint CCP4 and ESF-EACBM newsletter on protein crystallography*, *31*, 34-38.

Cowtan, K. (2003). Phase Problem in X-ray Crystallography, and Its Solution. *eLS*.

Crick, F. H. C. (1957). X-ray diffraction of protein crystals. In Colowick, S. P., and Kaplan, N. O. (1957). *Special techniques for the enzymologist*. (pp. 127-144). Netherlands: Elsevier.

Cromer, D.T. (1983). Calculation of anomalous scattering factors at arbitrary wavelengths. *J. Appl. Crystallogr*. *16*, 437–438.

Dauter, Z., and Dauter, M. (1999). Anomalous signal of solvent bromides used for phasing of lysozyme. *Journal of molecular biology*. *289*(1), 93-101.

Dauter, Z., Dauter, M., and Rajashankar, K. R. (2000). Novel approach to phasing proteins: derivatization by short cryo-soaking with halides. *Acta Crystallographica Section D: Biological Crystallography*. *56*(2), 232-237.

Dauter, Z., and Dauter, M. (2001). Entering a new phase: using solvent halide ions in protein structure determination. *Structure*. *9*(2), R21-R26.

DeLano, W. L. (2002). PyMOL.

Dessau, M. A., and Modis, Y. (2011). Protein crystallisation for X-ray crystallography. *JoVE (Journal of Visualized Experiments).* (47), e2285-e2285.

Dewan, S. S., and Setia, K. A. (2014). Global markets for enzymes in industrial applications. *Wellesley, MA: BCC Reseaerch*.

Drenth, J. (2007). *Principles of protein X-ray crystallography*. Springer Science and Business Media.

Eckert, K., Ernst, H. A., Schneider, E., Larsen, S. and Lo Leggio, L. (2003). Crystallisation and preliminary X-ray analysis of *Alicyclobacillus acidocaldarius* endoglucanase CelA. *Acta Cryst*. *D*. *59*, 139–141.

Eisenberg, D., Lüthy, R., and Bowie, J. U. (1997). [20] VERIFY3D: Assessment of protein models with three-dimensional profiles. *Methods in enzymology*. *277*, 396-404.

Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallographica Section D: Biological Crystallography*, *60*(12), 2126-2132.

Faiz, Ö. A., Colak, N. Saglam, S. Çanakçi and Beldüz, A. O. (2007). Determination and characterization of thermostable esterolytic

activity from a novel thermophilic bacterium *Anoxybacillus gonensis* A4. *J. Biochem. Mol. Biol*. *40*(4), 588-594.

Flaschel, E. and Friehs, K. (1993). Improvement of downstream processing of recombinant proteins by means of genetic engineering methods. *Biotech. Adv*. *11*, 31–78.

Frey, M. (1994). Water structure associated with proteins and its role in crystallisation. *Acta Crystallographica Section D: Biological Crystallography*. *50*(4), 663-666.

Giorgetti, A., Raimondo, D., Miele, A. E., and Tramontano, A. (2005). Evaluating the usefulness of protein structure models for molecular replacement. *Bioinformatics*, *21*(suppl_2), ii72-ii76.

Godinho, L. F., Reis, C. R, Tepper, P. G., Poelarends, G. J. and Quax, W. J. (2011). Discovery of an *Escherichia coli* esterase with high activity and enantioselectivity towards 1, 2-OIsopropylideneglycerol esters. *Appl. Environ. Microbiol. 77*(17), 6094-6099.

Grüne, T. (2013). Macromolecular Phasong: Solving the Substructure. In Read, R., Urzhumtsev, A. G., and Lunin, V. Y. (Eds.). *Advancing methods for biomolecular crystallography*. (pp. 151-157). New York: Springer.

Guss, J. M. and King, G. F. (2000). Macromolecular structure determination: comparison of crystallography and NMR. *eLS*.

Hamada, S., Hasegawa, Y., and Suzuki, Y. (2012). Identification of a GDSL-motif carboxylester hydrolase from rice bran (*Oryza sativa* L.). *Journal of Cereal Science*. *55*(2), 100-105.

Hauptman, H. A. (1991). The phase problem of x-ray crystallography. *Reports on Progress in Physics*. *54*(11), 1427.

Heikinheimo, P., Goldman, A., Jeffries, C. and Ollis, D. L. (1999). Of barn owls and bankers: a lush variety of alpha/beta hydrolases. *Structure. 7*, R141–R146.

Hendrickson, W. A. and Teeter, M. M. (1981). Structure of the hydrophobic protein crambin determined directly from the anomalous scattering of sulfur. *Nature. 290*, 107–113.

Hendrickson, W. A., Horton, J. R., and LeMaster, D. M. (1990). Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. *The EMBO journal*. *9*(5), 1665.

Holm, L., and Sander, C. (1995). Dali: a network tool for protein structure comparison. *Trends in biochemical sciences*, *20*(11), 478-480.

Hubbard, T. J., Ailey, B., Brenner, S. E., Murzin, A. G., and Chothia, C. (1998). SCOP, structural classification of proteins database: applications to evaluation of the effectiveness of sequence alignment methods and statistics of protein structural data. *Acta Crystallographica Section D: Biological Crystallography*. *54*(6), 1147-1154.

Jaeger, K. E. and Reetz, M. T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol. 16*, 396–403.

Jeffrey, G. A., and Jeffrey, G. A. (1997). *An introduction to hydrogen bonding*. (Vol. 32). New York: Oxford university press.

Jenny, R. J., Mann, K. G. and Lundblad, R. L. (2003). A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. *Protein expression and purification*. *31*(1), 1-11.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T. L. (2008). NCBI BLAST: a better web interface. *Nucleic acids research*. *36*(suppl_2), W5-W9.

Jones, T. A., and Kjeldgaard, M. (1997). [10] Electron-density map interpretation. *Methods in enzymology*, *277*, 173-208.

Joyce, M. G., Radaev, S. and Sun, P. D. (2010). A rational approach to heavy-atom derivative screening. *Acta Crystallographica Section D: Biological Crystallography*, *66*(4). 358-365.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., and Shore, V. C. (1960). Structure of myoglobin: A three-dimensional Fourier synthesis at 2 Å resolution. *Nature*. *185*(4711), 422-427.

Kikuta, Y., Ueda, H., Takahashi, M., Mitsumori, T., Yamada, G., Sakamori, K. and Matsuda, K. (2012). Identification and characterization of a GDSL lipase-like protein that catalyses the ester-forming reaction for pyrethrin biosynthesis in *Tanacetum cinerariifolium*–a new target for plant protection. *The Plant Journal. 71*(2), 183-193.

Kingsley, L. J., and Lill, M. A. (2015). Substrate tunnels in enzymes: structure–function relationships and computational methodology. *Proteins: Structure, Function, and Bioinformatics*. *83*(4), 599-611.

Koudelakova, T., Chaloupkova, R., Brezovsky, J., Prokop, Z., Sebestova, E., Hesseler, M., ... and Rezacova, P. (2013). Engineering enzyme stability and resistance to an organic cosolvent by modification of

residues in the access tunnel. *Angewandte Chemie International Edition*. *52*(7), 1959-1963.

Koshland, D. E. (1995). The key-lock theory and the induced fit theory. *Angewandte Chemie International Edition in English*. *33*(23-24), 2375-2378.

Kram, B. W., Bainbridge, E. A., Perera, M. A. D., and Carter, C. (2008). Identification, cloning and characterization of a GDSL lipase secreted into the nectar of *Jacaranda mimosifolia. Plant molecular biology. 68*(1-2), 173-183.

Krauss, R. I., Merlino, A., Vergara, A., and Sica, F. (2013). An overview of biological macromolecule crystallization. *International journal of molecular sciences*. *14*(6), 11643-11691.

Kraut, J. (1977). Serine proteases: structure and mechanism of catalysis. *Annual review of biochemistry*. *46*(1), 331-358.

Krieger, E. (2003). YASARA.

Kumar, A. (2006). *Protein Biotechnology.* Discovery Publishing House. 78-254.

Kumar, D., Kumar, L., Nagar, S., Raina, C., Parshad, R. and Gupta, V. K. (2012). Isolation, production and application of lipase/esterase from *Bacillus* sp. strain DVL43. *J. Microbiol. Biotech. Res*. *2*(4):521-528.

Kumar, S., and Nussinov, R. (2002). Relationship between ion pair geometries and electrostatic strengths in proteins. *Biophysical journal*, *83*(3), 1595-1612.

Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis 7.0 for bigger datasets. *Molecular Biology and Evolution*. *33*:1870-1874.

Ladd, M. F. C., Palmer, R. A. and Palmer, R. A. (1985). *Structure determination by X-ray crystallography* (p. 71). New York: Plenum Press.

Laemmli, U. K. (1970). Denaturing (SDS) discontinuous gel electrophoresis. *Nature*. *277*, 680-685.

Lamb, A. L., Kappock, T. J., and Silvaggi, N. R. (2015). You are lost without a map: Navigating the sea of protein structures. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. *1854*(4), 258-268.

Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of

protein structures. *Journal of applied crystallography*. *26*(2), 283-291.

Laskowski, R. A. (2001). PDBsum: summaries and analyses of PDB structures. *Nucleic acids research*. *29*(1), 221-222.

Liu, Q., Li, Z. and Li, J. (2014). Use B-factor related features for accurate classification between protein binding interfaces and crystal packing contacts. *BMC bioinformatics*. *15*(16), S3.

Lyu, P. C., Sherman, J. C., Chen, A. and Kallenbach, N. R. (1991). Alpha-helix stabilization by natural and unnatural amino acids with alkyl side chains. *Proceedings of the National Academy of Sciences*. *88*(12), 5317-5320.

Maina, C. V., Riggs, P. D., Grandea, A. G., III, Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A. and Guan, C.-D. (1988). An *Escherichia coli* vector to express and purify proteins by fusion to and separation from maltose-binding protein. *Gene*. *74*, 365–373.

Matthews, B. W. (1968). Solvent content of protein crystals. *Journal of molecular biology*, *33*(2), 491-497.

McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007). Phaser crystallographic software. *Journal of applied crystallography*, *40*(4), 658-674.

McPherson, A. (1999). *Crystallisation of biological macromolecules*.

McPherson, A. (1990). Current approaches to macromolecular crystallization. *The FEBS Journal*. *189*(1), 1-23.

McPherson, A. (1991). Review Current approaches to macromolecular crystallisation. *In EJB Reviews* 1990 (pp. 49-71). Springer Berlin Heidelberg.

Mendelsohn, L. D. (2004). ChemDraw 8 ultra, windows and macintosh versions. *Journal of Chemical Information and Computer Sciences*, *44*(6), 2225-2226.

Milner-White, E. J., and Poet, R. (1986). Four classes of β-hairpins in proteins. *Biochemical Journal*. *240*(1), 289-292.

Minor, W., Cymborowski, M., Otwinowski, Z., and Chruszcz, M. (2006). HKL-3000: the integration of data reduction and structure solution–from diffraction images to an initial model in minutes. *Acta Crystallographica Section D: Biological Crystallography*, *62*(8), 859-866.

Mølgaard, A., Kauppinen, S., and Larsen, S. (2000). Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. *Structure*, *8*(4), 373-383.

Murshudov, G. N., Vagin, A. A. and Dodson, E. J. (1997). Refinement of macromolecular structures by using maximum-likelihood method. *Acta crystallography D. 53*, 240-255.

Nardini, M. and Dijkstra B. W. (1999) Alpha/beta hydrolase fold enzymes: the family keeps growing. *Curr Opin Struct Biol.* 9, 732–737.

Nilsson, J., Ståhl, S., Lundeberg, J., Uhlén, M. and Nygren, P. Å. (1997). Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein expression and purification*. *11*(1), 1-16.

Otwinowski, Z. (1991). ML-PHARE CCP4 Proc. 80-88. *Daresbury Laboratory, Warrington, UK*.

Otwinowski, Z., and Minor, W. (1997). [20] Processing of X-ray diffraction data collected in oscillation mode. *Methods in enzymology*, *276*, 307-326.

Otwinowski, Z., and Minor, W. (2006). Denzo and scalepack. In *International Tables for Crystallography Volume F: Crystallography of Biological Macromolecules* (pp. 226-235). Springer Netherlands.

Owen, R. L. and Garman, E. (2005). A new method for predetermining the diffraction quality of protein crystals: using SOAP as a selection tool. *Acta Crystallographica Section D: Biological Crystallography*. *61*(2), 130-140.

Pace, N. C., Scholtz, J. M., and Grimsley, G. R. (2014). Forces stabilizing proteins. *FEBS letters*. *588*(14), 2177-2184.

Panda, T., and Gowrishankar, B. S. (2005). Production and applications of esterases. *Applied microbiology and biotechnology*. *67*(2), 160-169.

Pandey, A., Benjamin, S., Soccol, C. R., Nigam, P., Krieger, N., *et al*. (1999). The realm of microbial lipases in biotechnology. *Biotechnol Appl Biochem*. *29*(2), 119–131.

Parker, M. W. (2003). Protein structure from X-ray diffraction. *Journal of biological physics*. *29*(4), 341-362.

Petsko, G. A. and Ringe, D. (2004). From sequence to structure. In *Protein Structure and Function*. London: New Science Press Ltd. 2-47.

Petsko, G. A., and Ringe, D. (2008). *Protein structure and function (Primers in Biology)*. London: New Science Press Ltd. 2-47.

Pfeffer, J. M., Weadge, J. T., and Clarke, A. J. (2013). Mechanism of action of *Neisseria gonorrhoeae* O-acetylpeptidoglycan esterase, an SGNH serine esterase. *Journal of Biological Chemistry*. *288*(4), 2605-2613.

Porath, J., and Olin, B. (1983). *Biochemistry*. *22*, 1621.

Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. (1975). *Nature*. *258*, 598.

Raghunathan, K., Harris, P. T. and Arvidson, D. N. (2010). Trial by fire: Are the crystals macromolecules? *Acta Crystallographica Section F: Structural Biology and Crystallisation Communications*. *66*(5), 615–620.

Rao, L., Xue, Y., Zhou, C., Tao, J., Li, G., *et al*. (2011) A thermostable esterase from *Thermoanaerobacter tengcongensis* opening up a new family of bacterial lipolytic enzymes*. Biochim Biophys Acta. 1814*, 1695–1702.

Remington, S. J., Franken, S. M., Frolow, F., Ollis, D. L., Cheah, E., Cygler, M., ... and Schrag, J. (1992). The alpha/beta hydrolase fold. *Protein Eng*, *5*, 197-211.

Rose, P. W., Prlić, A., Altunkaya, A., Bi, C., Bradley, A. R., Christie, C. H., ... and Green, R. K. (2017). The RCSB protein data bank: integrative view of protein, gene and 3D structural information. *Nucleic Acids Research*. *45*(D1), D271-D281.

Ryu, H. S., Kim, H. K., Choi, W. C., Kim, M. H., Park, S. Y., Han, N. S., Oh, T. K. and Lee, J. K. (2006). New cold-adapted lipase from *Photobacterium lipolyticum* sp. nov. that is closely related to filamentous fungal lipases. *Appl Microbiol Biotechnol. 70*, 321-326.

Sanders, D. A. R. (2007). Protein X-ray Crystallography. In Baker, G., Dunn, S., and Holt, A. (Eds), *Handbook of Neurochemistry and Molecular Neurobiology*: *Practical Neurochemistry Methods*. (pp. 457-477). New York: Springer Science and Business Media

Sauter, A., Roosen-Runge, F., Zhang, F., Lotze, G., Jacobs, R. M. J. and Schreiber, F. (2015). Real-Time Observation of Nonclassical Protein Crystallisation Kinetics. *Journal of the American Chemical Society*. *137*(4), 1485–1491.

Sayali, K., Sadichha, P., and Surekha, S. (2013). Microbial esterase: an overview. *Int. J. Curr. Microbial. App. Sci. 2*(7), 135-146.

Schneider, T. R., and Sheldrick, G. M. (2002). Substructure solution with SHELXD. *Acta Crystallographica Section D: Biological Crystallography*, *58*(10), 1772-1779.

Selkirk, C. (2004). Ion-exchange chromatography. *Protein Purification Protocols*. 125-131.

Shakiba, M. H., Ali, M. S. M., Rahman, R. N. Z. R. A., Salleh, A. B. and Leow, T. C. (2016). Cloning, expression and characterization of a novel cold-adapted GDSL family esterase from *Photobacterium* sp. strain J15. *Extremophiles*. 20(1). 45-55.

Sheldrick, G. M. (2002). Macromolecular phasing with SHELXE. *Zeitschrift für Kristallographie-Crystalline Materials*, *217*(12), 644-650.

Shen, D., Xu, J. H., Wu, H. Y. and Liu, Y. Y. (2002). Significantly improved esterase activity of *Trichosporun brassicae* cells for ketoprofen resolution by 2-propanol treatment. *J Mol Catal B. 18*, 219–224.

Simone, D. G., Mandrich, L., Menchise, V., Giordano, V., Febbraio, F., Rossi, M and Manco, G. (2004). A Substrate-induced Switch in the Reaction Mechanism of a Thermophilic Esterase KINETIC EVIDENCES AND STRUCTURAL BASIS. *Journal of Biological Chemistry*. *279*(8), 6815-6823.

Smyth, M. S. and Martin, J. H. J. (2000). X-Ray crystallography. *Journal of Clinical Pathology*. *53*(1), 8.

Söding, J., Biegert, A., and Lupas, A. N. (2005). The HHpred interactive server for protein homology detection and structure prediction. *Nucleic acids research*, *33*(suppl_2), W244-W248.

Spencer, R. K. and Nowick, J. S. (2015). A Newcomer's Guide to Peptide Crystallography. *Israel journal of chemistry*. *55*(6-7), 698-710.

Stein, S. (ed.) (1990). *Fundamental of Protein Biotechnology.* Marcel Dekker, Inc. 9-55.

Subramaniam, S. (1998). The Biology Workbench—a seamless database and analysis environment for the biologist. *Proteins: Structure, Function, and Bioinformatics*, *32*(1), 1-2.

Talker-Huiber, D., Jose, J., Glieder, A., Pressnig, M., Stubenrauch, G. and Schwab, H. (2003). Esterase EstE from *Xanthomonas vesicatoria* (Xv_EstE) is an outer membrane protein capable of hydrolyzing long-chain polar esters. *Applied microbiology and biotechnology. 61*(5-6), 479-487.

Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied microbiology and biotechnology. 60*(5), 523-533.

Tina, K. G., Bhadra, R., and Srinivasan, N. (2007). PIC: protein interactions calculator. *Nucleic acids research*. *35*(suppl 2), W473-W476.

Upton, C., and Buckley, J. T. (1995). A new family of lipolytic enzymes?. *Trends in biochemical sciences*. *20*(5), 178-179.

Urh, M., Simpson, D. and Zhao, K. (2009). Affinity chromatography: general methods. *Methods in enzymology*. 463, 417-438.

Usón, I. and Sheldrick, G. M. (1999). Advances in direct methods for protein crystallography. *Current opinion in structural biology*. *9*(5), 643-648.

Vagin, A. and Teplyakov, A. (1997). MOLREP: an automated program for molecular replacement. *Journal of applied crystallography*. *30*(6), 1022-1025.

van den Berg (2010). Crystal structure of a full-length autotransporter. *Journal of molecular biology*. *396*(3), 627-633.

Walsh, G. (2005). *Protein: Biochemistry and Biotechnology*. John Wiley and Sons, Ltd. 1-47.

Wang, Y., Xiao, J., Suzek, T. O., Zhang, J., Wang, J., and Bryant, S. H. (2009). PubChem: a public information system for analyzing bioactivities of small molecules. *Nucleic acids research*. *37*(suppl_2), W623-W633.

Weber, P. C. (1991). Physical principles of protein crystallisation. *Advances in protein chemistry*. *41*, 1-36.

Weber, P. C. (1997). Overview of protein crystallisation methods. *Methods in Enzymology*. *276*, 13-22.

Wilkins, M. R., Gasteiger, E., Wheeler, C. H., Lindskog, I., Sanchez, J. C., Bairoch, A., ... and Hochstrasser, D. F. (1998). Multiple parameter cross-species protein identification using MultiIdent-a world-wide web accessible tool. *Electrophoresis*. *19*(18), 3199-3206.

Yaacob, M. A., Hasan, W. A., Ali, M. S., Rahman, R. N., Salleh, A. B., Basri, M., and Leow, T. C. (2014). Characterisation and molecular dynamic simulations of J15 asparaginase from *Photobacterium* sp. strain J15. *Acta Biochim Pol*. *61*(4), 745-752.

Yamaguchi, H., Maeki, M., Yamashita, K., Nakamura, H., Miyazaki, M., and Maeda, H. (2013). Controlling one protein crystal growth by droplet-based microfluidic system. *Journal of biochemistry*. *153*(4), 339-346.

Yamanaka, M., Inaka, K., Furubayashi, N., Matsushima, M., Takahashi, S., Tanaka, H., and Tanaka, T. (2011). Optimization of salt concentration in PEG-based crystallization solutions. *Journal of synchrotron radiation*. *18*(1), 84-87.

Yogavel, M., Gill, J., Mishra, P. C. and Sharma, A. (2007). SAD phasing of a structure based on cocrystallised iodides using an in-house Cu Kα X-ray source: effects of data redundancy and completeness on structure solution. *Acta Crystallographica Section D: Biological Crystallography*. *63*(8), 931-934.

Yuan, Z., Zhao, J. and Wang, Z. X. (2003). Flexibility analysis of enzyme active sites by crystallographic temperature factors. *Protein engineering*, *16*(2), 109-114.

Zheng, H., Chruszcz, M., Lasota, P., Lebioda, L., and Minor, W. (2008). Data mining of metal ion environments present in protein structures. *Journal of inorganic biochemistry*, *102*(9), 1765-1776.