CHARACTERIZATION, CRYSTALLIZATION AND STRUCTURE PREDICTION OF RECOMBINANT PROTEASE FROM *BACILLUS PUMILUS* 115B

By

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Chair : Professor Raja Noor Zaliha Raja Abd Rahman, PhD
Faculty : Biotechnology and Biomolecular Sciences

Proteases are widely used in industry as biocatalyst. Being able to synthesize peptide bonds in microaqueous environment while hydrolyse it in aqueous environment, this enzyme plays very important roles in food, pharmaceutical, detergent and leather industry. Protease from *Bacillus pumilus* 115B was capable to withstand in moderate temperature and proven to be stable in wide range of pH. To get better understanding of this enzyme as well as to enhance its productivity, molecular cloning, characterization and expression of the enzyme is compulsory. The full sequence of 1,149bp encoded a polypeptide of 383 amino acid residues from the organic solvent tolerant protease was successfully cloned into several vectors. Above all, pET 32b vector in BL21(DE3) host showed the highest expression. 115B protease was successfully purified and a single band of 33kDA mature protein was detected at the final step purification using ion exchange chromatography method. Further study on characterization, crystallization and structure prediction were carried out since the structure of this enzyme will give the new insight into organic solvent tolerant properties on a molecular level. The optimum temperature of 115B protease was found to be at 50°C and was stable in temperature range of 30°C to 45°C. The protease activity decrease rapidly at temperature higher than 55°C. 115B protease was stable in pH ranging from pH 7.0-pH 11.0 and the optimum pH was pH 8.0. The protease activity was 91% inhibited by PMSF suggesting that this protease belongs to the serine protease superfamily. Optimization on crystallization condition showed that 115B protease need the use of microseeding to kick start the nucleation process. Crystallization screening showed best crystal grew in formulation 22 from Molecular Dimension II. However, X-ray diffraction studies failed to give good diffraction spot that may result from the bad quality crystal. Homology modelling study helps to give a structural insight of the enzyme. 115B protease was modelled using crystallized

structure of subtilisin BPN complex (PDB id: 1YJB) as template for homology modelling. The predicted structure gave the overall quality Z-score of -0.305 which considered a good quality of modelled structure. The model appeared to have an Aspartic, Serine and Histidine catalytic triad, like all the subtilisin family. The predicted structure shown that 115B protease contains a calcium binding site that located at similar place in the subtilisin BPN' complex structure which is believe to attributes to the inefficient folding of subtilisin BPN' mature enzyme. Experimental result showed that expression of 115B protease was improved compared from previous recombinant 115B/pQE30 by Mahammad (2007). 115B protease was successfully crystallized with the size of 80µm, however due to the size factor, X-ray diffraction failed to give sufficient data to construct the electron density map to build the structure. Hence, homology modelling was performed and successfully predicted the structure of 115B protease. This provides preliminary overview about the structure and function of the enzyme.

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

PENCIRIAN, PENGHABLURAN DAN RAMALAN STRUKTUR REKOMBINAN PROTEASE DARI *BACILLUS PUMILUS* 115B

Oleh

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Pengerusi : Profesor Raja Noor Zaliha Raja Abd Rahman, PhD
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Protease digunakan secara meluas di dalam industri biomangkin. Kebolehan untuk mensintesis bon peptida dalam persekitaran microaqueous dan juga didalam persekitaran akueus, enzim ini memainkan peranan yang penting dalam industry makanan, farmaseutikal, bahan pencuci dan kulit. Protease daripada *Bacillus pumilus* 115B mampu bertahan pada suhu sederhana dan terbukti stabil dalam julat pH yang besar. Untuk mendapatkan pemahaman yang lebih baik daripada enzim ini dan juga untuk meningkatkan produktiviti, pengklonan molekul, pencirian dan ungkapan enzim wajib dilakukan. Urutan penuh dengan 1,149 bp polipeptida daripada 383 residu asid amino dari protease rintang pelarut organik telah berjaya diklon ke dalam beberapa vektor. pET vektor 32b di BL21 (DE3) telah menunjukkan eksperisi protin tertinggi. 115B protease telah berjaya ditulenkan dan satu jalur 33kDA protein matang dikesan di langkah terakhir penulenan menggunakan kaedah kromatografi pertukaran ion. Kajian lanjut mengenai pencirian, penghabluran dan ramalan struktur telah untuk memberikan lebih pemahaman tentang sifat-sifat enzim ini di peringkat molekul. Suhu optimum 115B protease didapati pada 50 ° C dan stabil dalam julat suhu 30 ° C hingga 45 ° C. Aktiviti protease menurun dengan cepat pada suhu yang lebih tinggi daripada 55 ° C. 115B protease stabil dalam julat pH antara pH 7.0-pH 11.0 dan pH optimum adalah pH 8.0. Aktiviti protease di rencat 91% oleh PMSF, mencadangkan bahawa protease ini berada di dalam superfamili serine protease. Pencarian kondisi optimum kristal menunjukkan 115B protease memerlukan penggunaan pembenihan mikro untuk memulakan proses penukleusan. Pemeriksaan penghabluran menunjukkan kristal terbaik berkembang dalam formulasi 22 dari Molekul Dimensi II. Walau bagaimanapun, kajian pembelauan sinar-X gagal untuk memberi keputusan pembelauan yang baik. Hal ini disebabkan kualiti kristal yang tidak memuaskan. Kajian pemodelan digunakan untuk membantu memberi gambaran struktur enzim. 115B protease telah dimodelkan menggunakan struktur kristal daripada subtilisin BPN

kompleks (PDB id: 1 1YJB) sebagai acuan untuk pemodelan homologi. Kualiti keseluruhan struktur memberikan Z-skor -0,305 yang dianggap berkualiti baik bagi struktur pemodelan. Model ini mempunyai persamaan pemangkin aktif aspartik, serine dan histidine, seperti yang terdapat pada semua keluarga subtilisin itu. Analisis struktur meramalkan menunjukkan bahawa 115B protease mengandungi sebuah laman mengikat kalsium yang terletak di tempat yang sama pada struktur kompleks subtilisin 'BPN enzim yang matang. Analisis hasil eksperimen menunjukkan bahawa ekspresi protin 115B protease telah meningkat berbanding dari rekombinan sebelumnya 115B / pQE30 oleh Mahammad (2007). Pengkristalan 115B protease telah berjaya namun disebabkan oleh faktor saiz, sinar-X pembelauan gagal memberikan data yang mencukupi untuk membina peta elektron seterusnya menentukan strukturnya. Ramalan struktur oleh pemodelan homology berjaya meramalkan struktur 115B protease dengan itu membantu dalam memberikan gambaran keseluruhan secara teori mengenai enzim tersebut.

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LIST OF ABBREVIATIONS

bp	Base pair
°C	Degree centrigade
dH_20	Distilled water
g	Gram
g/L	Gram per liter
h	Hour
kDa	Kilo Dalton
L	Liter
M	Molar
mM	Milimolar
mL	Mililiter
min	Minute
nm	Nanometer
MW	Molecular weight
ml/min	Mililiter per minute
mg/ml	Mililigram per mililiter
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
%	Percentage
µg	Microgram
µg/mL	Microgram per mililiter

µl	Microliter
µm	Micrometer
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

A protease is degradative enzyme that catalyzes hydrolysis of protein by breaking down the peptide bonds that link amino acids together in the polypeptide chain known as proteolysis. Proteases present naturally in all organisms and plays various role in physiological enzymatic reactions (Ray *et al.*, 2004).

Proteases are the most important kind of industrial enzymes (Joo *et al.*, 2002) and constitute approximately half of the total sale of industrial enzymes in the world market. Proteases were extensively use in various industrial sectors and the value in the world market is predicted to continuously grow and reached $6 billion by 2011 (Rao *et al*., 1998; David *et al*., 2009).

(Gupta *et al*., 2002) reported that detergent alkaline protease which is active and stable in the alkaline pH range dominate the enzyme market. Alkaline proteases are predominantly utilized as cleansing additive in detergents because they able to withstand the high pH conditions that occurs in washing environment. As an important industrial enzyme, alkaline protease especially subtilisin was extensively studied by scientist worldwide. Abo-Aba *et.al* (2006) reported that, there were more than half of the total industrial enzymes were extracted from genetically engineered microorganisms.

In the industrial processes, it is not practical to harvest the enzymes by isolation and purification from naturally occurring microorganism. The maintenance of wild type bacteria grow in bioprocessing fermenters is tedious and costly, as it requires special equipment suitable with its natural growth condition. The advances of molecular biology techniques have made it possible to produce enzymes in *E.coli* which is easier to maintain and handle. Genetic engineering greatly improves productivity and cost effectiveness in existing processes.

Methods for expressing large amount of protein from cloned gene introduced into new host such as *Escherichia coli* or *Bacillus* have proven invaluable in the purification, localization and also functional analysis of the proteins. For example, fusion proteins consisting of amino-terminal peptides encoded by a portion of the *E.coli* lacZ linked to eukaryotic proteins have been used to prepare polyclonal and monoclonal antibodies against these proteins. These antibodies have been used to purify proteins by immunoaffinity chromatography, in diagnostic assays to quantitate the levels of protein and to localize the proteins in organisms,tissues and individual cells by immunofluorescence.

Intact native proteins have also been produced in *E.coli* in large amount for functional studies. For example, both prokaryotic and eukaryotic DNA-binding proteins produced using *E.coli* expression vectors have been used to study the role of these proteins in gene expression.

Previously, the properties of target protein were determined by means of protein characterization. In this new era where biological system was studied at molecular level, X-ray crystallography are now used routinely by the scientist to understand functions of protein by determining their 3 dimensional structures. Protein crystallography are important in order to understand the functional inferens and the establishment of the biochemical pathway such as how a pharmaceutical drug interacts with its protein target and what changes might improve it (Scapin, 2006).

Crystal structures of proteins (which are irregular and hundreds of times larger than cholesterol) began to be solved in the late 1950s, beginning with the structure of sperm whale myoglobin by Max Perutz and Sir John Cowdery Kendrew, for which they were awarded the Nobel Prize in Chemistry in 1962 (Kendrew *et al*.,1958*).* Since that success, over 48970 X-ray crystal structures of proteins, nucleic acids and other biological molecules have been determined.

For comparison, the nearest competing method, nuclear magnetic resonance (NMR) spectroscopy has produced 7806 structures. Moreover, crystallography can solve structures of arbitrarily large molecules, whereas solution-state NMR is restricted to relatively small ones (less than 70 kDa). However, intrinsic membrane proteins remain challenging to crystallize because they require detergents or other means to solubilize them in isolation, and such detergents often interfere with crystallization. Such membrane proteins are a large component of the genome and include many proteins of great physiological importance, such as ion channels and receptors (Lundstrom K, 2006).

Protein structure prediction is divided into three parts depending on the similarity of the target to proteins of known structure. In comparative modeling, one or more template proteins of known structure with high sequence homology to the target sequence are identified. The target and template sequences are aligned, and a three-dimensional structure of the target protein is generated from the coordinates of the aligned residues of the template protein, combined with models for loop regions and other unaligned segments. Ideally, this three-dimensional model would then be refined to bring it closer to the true structure of the target protein.

Second, if no reliable template protein can be identified from sequence homology alone, the prediction problem is denoted as a fold recognition problem. Here, the primary goal is to identify one or more template protein structures that are consistent with the target sequence, that is, template folds that the target sequence might plausibly adopt. The subsequent protocol is similar to that of

comparative modeling: align the sequences and compose a three-dimensional model from the alignment.

Third, if no template structure can be identified with confidence, the target sequence may be modeled using *de novo* (or new fold) prediction methods. An outstanding question in *de novo* prediction is whether such methods can predict structures to a resolution useful for biochemical applications (Schonbrun et *al*., 2002).

This project was initiated to get better understanding of the structure and function of the enzyme thus molecular cloning, expression, characterization, crystallization and homology modelling of the enzyme is compulsory.

1.1 Problem statement

1. The extensive application of protease as industrial biocatalysts requires a further effort to understand the real potential of 115B protease.

1.2 Objective

1. To clone and express gene encoding organic solvent tolerant protease in suitable vectors
2. To purify and characterize 115B protease
3. To crystallize 115B protease using sitting drop method
4. To predict the structure of 115B protease.

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