UNIVERSITI PUTRA MALAYSIA

COMPUTATIONAL RE-DESIGN OF Streptomyces griseus CHITINASE C AND ANALYSIS OF BIOCHEMICAL, BIOPHYSICAL AND ANTIFUNGAL PROPERTIES OF DESIGNED VARIANTS

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By

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Faculty : Biotechnology and Biomolecular Sciences

Despite the importance of chitinases as potential biocontrol agents, their applications have not attracted significant attention due to the lack of stable enzyme formulations, high production costs and low yields from both wild and recombinant sources. However, through protein engineering techniques, these limitations can be overcome. Engineered mini-proteins for instance, could possess; enhanced stability, low production costs and simplified structural and functional mechanisms. Thus, this study was focused on developing miniaturized variants of *Streptomyces griseus* HUT6037 chitinase C that could serve as simpler yet potentially stable antifungal agents. Through computational techniques involving sequence analysis, docking and molecular dynamics simulation important residues and/or motifs within the catalytic cleft of SgChiC were identified as essential for recognition and binding to complex or crystalline chitin. Residues outside the catalytic clefts were thus considered targets for miniaturization. Five (5) SgChiC variants namely: M159, M140, M139, M109 and M101 were designed *in silico,* their genes were subsequently synthesised and cloned into pET-22b(+). The variants were then expressed in *Escherichia coli* BL21(DE3) and purified through on-column refolding. Biochemical assays revealed that all variants although had lower activities towards colloidal chitin when compared with the wild-type, retained the optimum temperature at 40 °C. The optimum pH for activities of variants however varied with M101 and M139 drifting towards acidic pH of 5.0 and 6.0 respectively, while M159 and M109 had optimum activities at pH 8. Interestingly, all variants retained 40-50% of the specific activity of the WT towards colloidal chitin, with M159 displaying the highest specific activity at 31.6 Umg^{-1} compared to the WT with 52.3 Umg^{-1}. Contrastingly, with chitosan, the smallest variant M101, displayed high chitosanase activity comparable with the WT with 59.6 and 61.4 Umg^{-1} respectively. M109 also displayed high chitosanase activity with a specific activity of 51.6 U/mg. Thermal denaturation studies revealed that the variants

were stable at temperatures up to 60°C. Antifungal assay towards *Fusarium oxysporum* f.sp. *cubense* (FOC) revealed that M101 and M109 had the capacity to inhibit hyphal extension against the fungus with M101 displaying comparable inhibition with the WT. M139, M140 and M159 exhibited less inhibitory effects towards the hyphal extension of FOC. Finally, a correlation between chitosanase and antifungal activities of the enzymes was observed in the study whereby only the wild type and variants with chitosanase activities clearly inhibited fungal growth. The computational approach applied in the engineering of SgChiC was therefore efficient as it yielded miniaturized variants that may be beneficial in the study of chitinases and their mechanisms. Additionally, the variants, especially M101 which exhibited some activities comparable with the wild-type can be further improved in future studies as antifungal formulation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

REKABENTUK SEMULA SECARA KOMPUTASIONAL CHITINASE C *Streptomyces griseus* DAN ANALISIS CIRI BIOKIMIA, BIOFIZIK DAN ANTIKULAT DARIPADA VARIAN YANG DIREKABENTUK

Oleh

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Walaupun kitinase penting sebagai agen biopengawalan, penggunaannya masih belum menarik perhatian signifikan kerana kekurangan formulasi enzim yang stabil, kos pengeluaran yang tinggi dan hasil yang rendah daripada keduadua sumber liar dan rekombinan. Akan tetapi, melalui teknik kejuruteraan protein, kekangan sedemikian boleh diatasi. Protein mini yang dijurutera misalnya, boleh mempunyai kestabilan yang dipertingkatkan, kos penghasilan yang rendah dan mekanisme struktur dan fungsian yang diringkaskan. Maka, kajian ini fokus kepada pembangunan varian kitinase C *Streptomyces griseus* HUT6037 yang dikecilkan yang boleh bertindak sebagai agen anti-kulat yang lebih ringkas dan stabil. Melalui teknik komputasional yang melibatkan analisis jujukan, pendokan dan simulasi dinamik molekul residu dan/atau motif penting dalam celah pemangkin SgChiC telah dikenal pasti sebagai penting untuk pengecaman dan pengikatan kepada kitin kompleks atau kristal. Residu di luar celah pemangkin oleh itu dianggap sebagai sasaran untuk pengecilan. Lima (5) varian SgChiC iaitu: M159, M140, M139, M109 dan M101 direka bentuk secara in siliko, gen-gennya kemudian disintesis dan diklonkan ke dalam pET-22b(+). Varian-varian tersebut kemudiannya dihasilkan di dalam perumah rekombinan *Escherichia coli* BL21(DE3) dan dipencilkan melalui “on-column refolding”.Ujian biokimia mendedahkan bahawa semua varian walaupun mempunyai aktiviti yang lebih rendah terhadap kitin koloid jika dibandingkan dengan jenis liar (WT), mengekalkan suhu optimum pada 40°C. pH optimum untuk aktiviti varian bagaimanapun berbeza-beza dengan M101 dan M139 yang lebih ke arah pH berasid pada 5.0 dan 6.0 masing-masing, manakala M159 dan M109 mempunyai aktiviti optimum pada pH 8. Menariknya, semua varian mengekalkan 40-50% daripada aktiviti spesifik WT terhadap kitin koloid, dengan M159 memaparkan aktiviti spesifik tertinggi pada 31.6 Umg-1 berbanding WT dengan 52.3 Umg-1. Sebaliknya, dengan kitosan, varian terkecil M101 menunjukkan aktiviti kitosanase yang tinggi setanding dengan WT dengan 59.6

dan 61.4 Umg1 masing-masing. M109 juga menunjukkan aktiviti kitosanase yang tinggi dengan aktiviti spesifik 51.6 Umg-1. Kajian denaturasi terma mendedahkan bahawa varian adalah stabil pada suhu sehingga 60°C. Ujian anti-kulat terhadap *Fusarium oxysporum* f.sp. *cubense* (FOC) mendedahkan bahawa M101 dan M109 mempunyai kapasiti untuk menghalang sambungan hifa terhadap kulat dengan M101 memaparkan perencatan yang setanding dengan WT. M139, M140 dan M159 mempamerkan kesan perencatan yang kurang terhadap lanjutan hifa FOC. Akhir sekali, korelasi antara aktiviti kitosanase dan anti-kulat enzim dapat diperhatikan dalam kajian ini di mana hanya jenis liar dan varian yang mempunyai aktiviti kitosanase jelas dapat menghalang pertumbuhan kulat. Oleh itu, pendekatan komputasional yang digunakan dalam kejuruteraan SgChiC adalah efisien kerana ia menghasilkan varian kecil yang mungkin bermanfaat dalam kajian kitinase dan mekanismenya. Selain itu, varian-varian terutamanya M101 yang mempamerkan beberapa aktiviti yang setanding dengan jenis liar boleh ditambah baik lagi dalam kajian akan datang untuk digunakan sebagai formulasi antikulat.

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LIST OF ABBREVIATIONS

CatD	Catalytic domain
CBD	Chitin binding domain
NAG/(GlcNAc)	N-acetyl-D-glucosamine
WT	Wild-type
SgChiC	*Streptomyces griseus* Chitinase C
RD	Reverse dilution
OnC	On column
BB	Binding buffer
DB	dialysis buffer
EB	elution buffer
RB	refolding buffer
WB	washing buffer
Fn3	fibronectin 3 like domain
CC	colloidal chitin
GC	glycol chitin
PDB	Protein data bank
CD	Circular dichroism
MD	Molecular dynamics
GH	Glycosyl/Glycoside hydrolase
TIM	Tophoisomerase
IPTD	Isopropyl-B-D-galactopyranoside
RMSF	Root mean square deviation
RMSD	Root mean square fluctuations

CHAPTER 1

INTRODUCTION

1.1 Introduction

Enzymes are important tools for solving global problems in health care, food processing, drug development, agriculture, environmental management and research. Their importance has influenced further studies into enzyme structure and function and triggered a ripple effect in the field of enzyme engineering through directed evolution, rational design or mutagenesis, and *de-novo* design. Generally, proteins are engineered for purposes such as elucidating the roles of specific regions of enzymes, enhancing enzymatic properties as well as for building artificial biomolecules or enzymes with novel functions. As a result, properties like substrate specificity, enantioselectivity, solvent tolerance, temperature and pH stability are common targets of most protein engineering studies for developing enzymes which are better suited for harsh industrial processes or applications. While there is still a vast area of enzymatic attributes being investigated or waiting to be unraveled, a significant number of enzymes with improved functions have been commercialized and used for various purposes. Some examples include fast and slow acting insulin, and engineered glycoproteins used for therapeutic purposes (Walsh, 2007), engineered hydrolytic enzymes such as oxidation resistant proteases (Contesini et al., 2017; Banik & Prakash, 2004), amylases (Khemakhem et al., 2009) and lipases (Druteika et al., 2020) which are used in the detergent industry (Walsh, 2007). Cellulases and chitinases are also well-known glycoside hydrolases with growing significance in industry and agriculture. Chitinases which are of interest in this study are particularly gaining widespread interests for their roles in the degradation of chitin, a highly abundant crystalline carbohydrate found in nature. They have found useful application in biotechnology for the production of chitooligosaccharides, management of chitin wastes and biological control of phytopathogenic fungi and insect pests (Swiontek et al., 2014). Chitinases (EC 3.2.1.14) are among carbohydrate binding enzymes with interesting features that can serve as targets for protein engineering studies. Their biocontrol potential is particularly of interest due to the growing need for safer alternatives for the control of fungal pathogens which are responsible for several devastating losses in agriculture. A typical destructive fungal strain known as *Fusarium oxysporum* f. sp *cubense,* is the notorious causative agent of the “Panama” disease of banana plantations (Ploetz, 2015). *F. oxysporum* sub sp. *cubense* (FOC) TR1 was responsible for the extinction of the highly desired “Gros Michel” banana cultivar in the 1950’s (Ploetz & Churchill, 2011). This species was the most widely exported banana variety at that time, before its eradication by the Panama disease. Currently, the “Cavendish” species which replaced the ‘Gros Michel’ cultivar is under threat by another race of *Foc* (Pérez-vicente et al., 2014; Ploetz & Churchill, 2011). Unfortunately, current methods used for its eradication such as utilization of chemical fungicides are not yielding significant results and mostly pose severe health and environmental risks (Nel et al., 2007). Hence, the need

for the development of alternative control measures with little or no deleterious environmental impacts is necessary.

Chitinases are potential alternatives, however, they are limited by a few factors which diminish their attractiveness as substitutes for the broad-spectrum chemical agents. These limitations include the cost of production from natural as well as recombinant sources (Sarma et al., 2013), lack of stable or appropriate formulations and narrow spectrum of antifungal activity (Sarma et al., 2013; Neeraja et al., 2010). The narrow spectrum of antifungal activity is attributed to the differences in surface microstructure and varying proportions or exposure of chitin amidst fungal cell wall proteins and glucanases (Singh et al., 2014; Yan et al., 2008). Hence, chitinases either bind poorly or have low accessibility to the chitin fibres in the cell walls of different fungal pathogens. In addition, chitinases like other glycoside hydrolases vary in their natural roles, mechanisms and structural properties which may contribute to the differences in their chitinolytic or antifungal properties. In other words, the mechanism of fungal growth inhibition by antifungal chitinases still remain unclear due to complexities arising from diverse structural properties of chitin as well as chitinases.

Some studies have clearly reported potent antifungal chitinases from plants (SierraGomez et al., 2019; Tanaka et al., 2017; Karthik et al., 2015; Kirubakaran & Sakthivel, 2007; Punja & Zhang, 1993), fungi (Deng et al., 2019) and bacteria (de la Fuente-Salcido et al., 2016; Hjort et al., 2014; Neeraja et al., 2010; Itoh et al., 2003). One particular chitinase producing group that stands out consistently for its antifungal potential is from the soil bacterium *Streptomyces sp* (Singh et al.,1999). Specifically, *Streptomyces griseus* HUT6037 ChiC (SgChiC) was the first glycosyl hydrolase (GH) family 19 chitinase identified from bacteria (Kezuka et al., 2006). In fact, after more than a decade since its first structural characterization, it has remained one of only two bacterial chitinases with known 3D structures. As such, it serves as a valuable source of structural and mechanistic information for the study of GH 19 chitinases from organisms other than plants. SgChiC is bi-modal, having a chitin binding domain (CBD) similar to the cellulose binding module (CBM) found in some bacterial enzymes, and a catalytic domain (CatD) structurally related to the GH family 19 plant chitinases (Kezuka et al., 2006; Akagi et al., 2006; Itoh et al., 2002). Its characteristic antifungal activity has been demonstrated by observing its ability to inhibit hyphal extension in *Trichoderma reesei* (Itoh et al., 2006). While the catalytic domain is known to perform most of the chitinolytic activity, studies have shown that its antifungal property is significantly lost in the absence of the CBD. Interestingly, the absence of the CBD resulted in loss of just about 50% activity towards insoluble chitin while up to 90% of its antifungal activity was abolished (Itoh et al., 2002). Despite its interesting attributes and potential importance as a bioicontrol agent, no recent study has been performed to elucidate some of its mechanisms or enhance its properties towards its development as an efficient biocontrol agent. In this study, the catalytic domain of SgChiC was redesigned by miniaturization. The variants were subsequently investigated for their biochemical, biological and biophysical properties. Prior to experimental

analyses, a computational approach was applied to identify hypothetically unimportant sites as target regions or residues on the 3D structure of SgChiC. Subsequently, the catalytic domain was redesigned by the iterative deletion of the target residues.

The design of miniature enzymes is desirable because they are generally believed to be stable and easier to study because of their small size (Starovasnik et al., 1997; Nedwidek & Hecht, 1997), they simplify complex processes and can be readily produced with higher yields. Furthermore, the miniaturization approach used is a mimic of the natural design process of chitinases whereby chitinases adapt in the environment by the loss of some loops.

1.2 Research hypothesis

Structural and functional differences among chitinases can be studied by analyzing sequence and observing the dynamics of loops, domains, motifs and residues through *in silico* techniques. Furthermore, miniaturization of the catalytic domain of SgChiC will yield chitinases with smaller domains and simplified mechanisms that (1) can be better studied for elucidating functional and structural properties and (2) can aid production and formulation of inexpensive and effective biocontrol agents.

1.3 Research questions

1. What are the key residues, structural elements and interactions that are essential for the activity and stability of SgChiC?
2. Can SgChiC be minimized by deletion of “unimportant’’ residues without the perturbation of its core structure or function?
3. What can be revealed about the biochemical and biological characteristics of SgChiC through redesigning of structural features and deletion of unimportant catalytic residues?

1.4 Problem statement

Although SgChiC is known for its antifungal potential, the complexity in structural features that define stability, antifungal activity, substrate specificity and catalytic efficiency is not well known. Therefore, the successful design of a miniature chitinase from SgChiC will simplify structural and functional complexities and yield variants that can be easily studied.

1.5 Objective

To computationally minimize SgChiC and analyse the enzymatic and antifungal properties of simplified variants towards the development of stable chitinase formulations as biocontrol agents.

1.5.1 Specific Objectives

1. To map functionally unimportant residues and motifs as targets regions to be minimized, *in silico*.
2. To miniaturize SgChiC by deleting “unimportant” target regions and analyse the properties of the designed variants *in silico*.
3. To determine the biochemical, biological and biophysical properties of the recombinant miniature variants.

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