SIGNAL PEPTIDE MODIFICATION AND SIGNAL PEPTIDE PEPTIDASE EFFECT ON CYCLODEXTRIN GLUCANOTRANSFERASE EXCRETION AND CELL LYSIS

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In memory of my *late* mother, Zabidah Binti Setafa. Al-Fatihah.

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ABSTRACT

Extracellular excretion of recombinant protein is beneficial as it can greatly reduce the cost of downstream processing and improve the product quality. However, the efforts in achieving high excretion level often leads to occurrence of cell lysis and low protein yield due to limited capacity of the transport machinery. The objectives of this study were to investigate the effect of amino acids and their locations in h-region signal peptide on cyclodextrin glucanotransferase (CGTase) excretion, and analyze the function of signal peptide peptidase (SPPase) in improving the membrane integrity of *Escherichia coli* (*E. coli*). Modification of the hydrophobic region of the N1R3 signal peptide (wild type) using site-saturation mutagenesis has improved the excretion of CGTase. The results indicated that the excretion of CGTase is highly dependent on properties of signal peptide which are hydrophobicity, secondary conformation and, the type and position of amino acids at the boundary and core segment of the h-region. Mutant signal peptides designated as M9F, V10L and A15Y enhanced the excretion of CGTase to three-fold and has demonstrated two-fold higher secretion rate than the wild type. However, high secretion rate caused nine-fold increase in cell lysis as compared to the wild type. In dual-plasmid system for co-overexpression, gene expression of CGTase fused to A15Y signal peptide and SPPase, were regulated by T7lac and P_{BAD} promoters, respectively, at induction temperature of 25ºC. It was shown that co-overexpression of SPPase and CGTase has reduced the occurrence of cell lysis that was reflected by β-galactosidase activity from 128.6 U/ml to 0.12 U/ml, which equivalent to 99% decrease when compared to the *E. coli* that expressed CGTase alone. Further improvement of CGTase excretion was obtained by co-overexpression of CGTase and SPPase with addition of glycine which has successfully maintained the low β-galactosidase level at 0.63 U/ml and increased 4.5 fold of CGTase excretion from 14.6 U/ml to 66.1 U/ml, as compared to the co-overexpression without glycine supplementation. The present results indicated that higher CGTase excretion with low cell lysis can be obtained by alteration of amino acids in the h-region signal peptide along with glycine supplementation and SPPase overexpression. This is the first report that highlights the combination of three approaches; site-saturation mutagenesis of signal peptide, SPPase overexpression and glycine supplementation in overcoming the problems of low secretion level of CGTase and high occurrences of cell lysis.

ABSTRAK

Perembesan ekstrasel protein rekombinan adalah bermanfaat kerana ia dapat mengurangkan kos pemprosesan hiliran dan meningkatkan kualiti produk. Walaubagaimanapun, usaha untuk mencapai tahap perembesan yang tinggi sering menyebabkan berlakunya lisis sel dan hasil protein yang rendah disebabkan oleh kapasiti jentera pengangkutan yang terhad. Objektif kajian ini adalah untuk mengkaji kesan asid amino dan lokasi mereka dalam segmen-h peptida isyarat dalam perembesan ekstrasel siklodekstrin glucanotransferase (CGTase), dan menganalisa fungsi peptidase peptida isyarat (SPPase) dalam meningkatkan integriti membran *Escherichia coli* (*E. coli*). Pengubahsuaian segmen hidrofobik peptida isyarat N1R3 (peptida isyarat asli) menggunakan kaedah mutasi tepu-lokasi telah meningkatkan rembesan ekstrasel CGTase. Keputusan menunjukkan tahap rembesan CGTase amat bergantung kepada sifat-sifat peptida isyarat iaitu tahap hidrofobik, konformasi sekunder dan jenis serta kedudukan asid amino di bahagian sempadan dan bahagian tengah segmen-h. Peptida isyarat mutan yang dikenali sebagai M9F, V10L dan A15Y meningkatkan rembesan CGTase kepada tiga kali ganda dan menunjukkan kadar rembesan dua kali ganda lebih tinggi daripada peptida isyarat asli. Walaubagaimanapun, kadar rembesan yang tinggi menyebabkan kenaikan sembilan kali ganda dalam lisis sel berbanding dengan peptida isyarat asli. Dalam sistem dwi-plasmid untuk ekspresi bersama, ekspresi gen CGTase bersatu dengan peptida isyarat A15Y dan SPPase yang masing-masing dikawal oleh penggalak T7lac dan P_{BAD}, pada suhu induksi 25ºC. Kajian menunjukkan bahawa ekspresi SPPase dan CGTase telah mengurangkan berlakunya lisis sel melalui penurunan aktiviti enzim β-galactosidase dari 128.6 U/ml kepada 0.12 U/ml, iaitu bersamaan dengan 99% penurunan berbanding dengan *E. coli* yang mengekspresi CGTase sahaja. Penambahbaikan perembesan CGTase diperolehi secara ekspresi bersama CGTase dan SPPase dengan tambahan glisin yang berjaya mengekalkan tahap rendah aktiviti β-galactosidase pada 0.63 U/ml dan meningkatkan 4.5 kali ganda rembesan CGTase dari 14.6 U/ml kepada 66.1 U/ml berbanding dengan ekspresi bersama tanpa penambahan glisin. Keputusan ini menunjukkan bahawa rembesan CGTase yang lebih tinggi dengan kadar lisis yang rendah boleh diperoleh dengan mengubah asid amino di segmen-h peptida isyarat bersama-sama dengan penambahan glisin serta ekspresi SPPase. Ini merupakan laporan pertama yang menggabungkan tiga strategi; mutasi tepu-lokasi terhadap peptida isyarat, ekspresi SPPase dan penambahan glisin dalam menyelesaikan masalah tahap rembesan CGTase yang rendah dan lisis sel yang tinggi.

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cm	-	centimeter
g	-	gram
h	-	hour
L	-	liter
M	-	Molar
ml	-	milliliter
mM	-	milimolar
ng	-	nanogram
nm	-	nanometer
rpm	-	revolutions per minute
%	-	percentage
°C	-	degree celcius
µg	-	microgram
µl	-	microliter
µm	-	micromolar

LIST OF ABBREVIATIONS

ABC transporter	-	ATP-binding cassette transporter
Ala	-	alanine
ATP	-	adenosine-5-triphosphate
ara	-	arabinose
B. lehensis	-	*Bacillus lehensis*
bp	-	base pair
BSA	-	bovine serum albumin
$CaCl_2$	-	calcium chloride
Ca^{2+}	-	calcium ion
CD	-	cyclodextrin
CGTase	-	cyclodextrin glucanotransferase
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleotide triphosphate
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenedianetetra-acetate
G1		signal peptide of cyclodextrin glucanotransferase
GSP	-	general secretory pathway
HCl	-	hydrochloric
Hly	-	Hemolysin
IM	-	inner membrane
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
Da, kDa	-	Dalton, kilo Dalton
DNAse	-	deoxyribonuclease
Dsb	-	Disulfide binding

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lac	-	lactose
LB	-	Luria-Bertani
LPS	-	lipopolysaccharides
min	-	minute
Na^+	-	sodium ion
NEB	-	New England Biolabs
NPN	-	1-*N*-phenylnaphthylamine
OD_{600}	-	optical density at wavelength 600 nm
OM	-	outer membrane
OFAT	-	one factor at one time
OMP	-	outer membrane protein
ONPG	-	Ortho-nitrophenyl-β-galactosidase
PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
pmf	-	proton motive force
RNA	-	ribonucleic acid
RBS	-	ribosome binding site
SDS	-	sodium dodecyl sulfate
Sec	-	secretory
SP	-	signal peptide
sp.	-	species
SPase	-	signal peptidase
SPPase	-	signal peptide peptidase
SRP	-	signal recognition particle
T2SS	-	type two secretion system
TAE	-	Tris-acetic acid-EDTA
Tat	-	Twin-arginine translocase

TCA	-	Trichloroacetic acid
TE	-	Tris-EDTA
TEMED	-	N, N, N', N'-tetramethylethylenediamine
U	-	unit of enzyme activity
v/v	-	volume per volume
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

1.1   Background of Study

All recent progress in genetic engineering and protein engineering has led to the more economical production of recombinant protein or enzyme mainly due to the simpler and faster cultivation process and the discovery of suitable organism which could be engineered to produce high volume of desired protein with higher standard and quality. Over the years, development in industrial biotechnology has focused more in finding the effective and efficient protein production system to meet the huge demands of the industry. The gram negative bacterium *Escherichia coli* (*E. coli*) is one of the most frequently used host for industrial recombinant protein production compared with other established expression hosts available due to the ability to grow rapidly at high cell density on cheaper substrates, very well-characterized genetically and the availability of many tools that have been developed including cloning vectors and mutant host for gene cloning and expression (Baneyx and Mujacic, 2004).

However, *E. coli* is incapable in producing some proteins that require post-translational modification for activity. Overexpression of intracellular recombinant proteins in *E. coli* has commonly failed to reach a correct conformation and often resulted in the formation of insoluble aggregates known as inclusion bodies. The protein can only be recovered by expensive and complicated denaturation and refolding processes which usually give low yield of soluble refolded protein. Besides the expensive downstream processing, proteins expressed intracellularly are also prone to protease degradation.

One approach to tackle these issues is to have the recombinant proteins secreted into the periplasm or released into the growth medium (Choi and Lee,

2004). Targeting the proteins into more oxidizing environment of periplasm and culture medium effectively improve the protein quality such as biological activity, stability and solubility (Jong *et al*., 2010) due to correct protein folding and less proteolysis activity. In addition, the recovery of secreted proteins from the culture medium rather than from cytoplasm greatly reduces the downstream processing costs and simplified the separation process because there is no cell disruption needed and thus, less contamination with the endogenous protein (Yoon et al., 2010).

Protein secretion is a complex, multi-step reaction involving many export components. For the protein to be recognized by transport components, all secreted proteins are synthesized with an amino-terminal signal peptide (Pugsley, 1993) that acts as a targeting and recognition signal (Mergulhão *et al*., 2005). The signal peptide contains the information needed to direct the newly synthesized export-competent protein to the translocation pathway. The signal peptide is removed by signal peptidase to release only the mature region of the protein to the periplasm for complete folding and localization (De Bona *et al*., 2012). Then, the remnants of cleaved signal peptide within inner membrane were further degraded by membrane bound protease known as signal peptide peptidase (SPPase) (Ichihara *et al*., 1984).

Although there are significant improvements and a wide range of systems for heterologous protein excretion using *E. coli* have been developed, low secretion level and high occurrences of cell lysis due to ruptured of inner and outer membrane during the expression period remains a considerable bottleneck, which resulted in low protein yield (Fu *et al*., 2005). Occasionally, hypersecretion of recombinant proteins resulted in the formation of periplasmic inclusion bodies which further caused the burden to the host cell (Tesfai *et al*., 2012). This limitation often related to the inefficient and limited capacity of secretion machineries (Mergulhão *et al*., 2005; Rosenberg, 1998) and the demand for protein translocation had exceeding the capacity, arising from the high rate of protein synthesis (Simmons and Yansura, 1996; Mergulhão *et al.*, 2004).

Previously, a bacteria *Bacillus* sp. G1 was isolated and found capable of secreting enzyme cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) which

catalyses the conversion of starch to produces 89% β-CD and 11% γ-CD with no detection of α-CD (Sian et al., 2005). In this study, CGTase will be used as a reported protein to measure the performace of mutated signal peptide in protein secretion process. The CGTase is becoming significant interest due to the high commercial value of cyclodextrins (CDs) which extensively used in industries such as toiletries, cosmetic, pharmaceuticals, agriculture, chemical and food. Previous reports showed that the overproduction of CGTase using *E. coli* often problematic owing to the intracellular localization and the formation of inactive inclusion bodies (IBs) that lead to low yield (Yang et al., 2014). Secretion of CGTase is highly desirable compared to intracellular expression as it could leads to easier purification steps and improves product quality. Scientific advancements have been carried out to genetically engineer the signal peptide to facilitate high secretion of CGTase into the extracellular medium. Previous reports on employed signal peptide modification strategies to improve CGTase secretion in *E. coli* are to increase the net charge of n-region (Ismail et al., 2011) or introduce helix-breaker in h-region (Jonet et al., 2012). Recently, used of homologous DacD signal peptide derived from *E. coli* strongly promoted the extracellular secretion of CGTase up to 3.3 fold compared to native signal peptide (Sonnendecker et al., 2017).

Although the signal peptides can export the proteins out of the membrane, the efficiency of transport was very limited and most of proteins were still remain in the cytoplasm or periplasm. Furthermore, low secretion level and high occurrences of cell lysis still remains a considerable obstacle (Jonet et al., 2012; Tesfai et al., 2012). To achieve a high level of CGTase secretion, the choice of optimal signal peptide remains the first and most important hurdle to be overcome.

It is interesting to note that, a recent review by Low *et al.* (2013) concluded that the hydrophobic core is a key feature for signal peptides to functioned properly like a security pass for a secreted protein to be exported through SecYEG translocon. Both *in vivo* and *in vitro* experiments have revealed that a minimum level of total hydrophobicity is critical to complete the processing of signal peptide and protein translocation prior to cleavage event (Chou and Kendall, 1990; Mori *et al.*, 1997; Rusch *et al.*, 1994). Either deletion or substitution of hydrophobic residues, or

varying the hydrophobic values below the acceptable threshold could ruin the protein transport process (Doud *et al.*, 1993; Izard *et al.*, 1996; Kendall *et al.*, 1986; Rusch *et al.*, 2002). Although the importance of core hydrophobic region of signal peptide in protein translocation has been widely studied, a significant conclusion on the role of amino acid in this region is rather complicated since there is heterogeneity in amino acid composition. Therefore, in this study, in order to overcome the first problem of low secretion, site-saturation mutagenesis approach is employed to rule out the ideal amino acid at specific position in the h-region signal peptide and subsequently identify the optimal mutant for improved production of excreted recombinant CGTase.

Protein that has been successfully transported into the periplasmic compartment will need to pass through the outer membrane barrier in order to be excreted outside the cell. In this study, further improvement in CGTase excretion from periplasmic to the extracellular medium in the aspect of minimizing the occurrences of cell lysis is achieved by synergistic promoting effect of signal peptide peptidase overexpression and supplementation of permeabilizing agents. Firstly, SPPase overexpression facilitated fast clearance of remnant signal peptide within the membrane, thus avoiding the signal peptide from hindering the translocation channel. Secondly, the supplementation of permeabilizing agents enhance the permeability of outer membrane without compromising the membrane integrity, enabling the maximum released of CGTase in periplasmic space into the extracellular medium without cell lysis.

1.2   Problem Statement and Novelties of Study

Targeting the synthesized protein into extracellular culture medium offers several advantages such as correct protein folding due to more oxidative environment, minimizes formation of inclusion bodies, providing protection from cellular protease, yield minimum contamination with host's protein and simplified downstream processing. Although significant improvements and a wide range of

systems for heterologous protein excretion using *E. coli* have been developed, low secretion level and high the occurrences of cell lysis due to ruptured of inner and outer membrane during the expression period still remains a considerable obstacle. This limitation often related to the inefficient and limited capacity of secretion machineries and the demand for protein translocation had exceeding the capacity, arising from the high rate of protein synthesis.

This report is the first to describe the combination of approaches to overcome the problems of low secretion level of CGTase and high occurrences of cell lysis. Firstly, the present study demonstrate the site-saturation mutagenesis approach is employed to rule out the ideal amino acid at specific position in the h-region signal peptide and subsequently identify the optimal mutant for improved CGTase secretion from cytoplasm to the periplasm and subsequently to the extracellular medium. Secondly, further improvement in the aspect of minimizing the occurrences of cell lysis is achieved by co-promoting effect of SPPase overexpression and supplementation of permeabilizing agents. SPPase overexpression facilitated fast clearance of remnant signal peptide within the membrane, thus avoiding the signal peptide from hindering the translocation channel. While the supplementation of permeabilizing agents enhance the permeability of outer membrane without compromising the membrane integrity, enabling the maximum released of CGTase in periplasmic space into the extracellular medium without cell lysis.

1.3   Objective

In order to achieve high extracellular excretion of recombinant protein CGTase with low occurrence of cell lysis during the expression, the objectives of this study focuses on:

1. To study the effect of h region amino acids modification of signal peptide on secretion efficiency in *E. coli.*

2. To study and analyse the effect of SPPase overexpression and permeabilizing agents' supplementation on cell membrane integrity.

1.4 Scopes of Study

This study focuses on the optimization of secretion capacity by appropriate strategies to alleviate several bottlenecks that limit the production of recombinant proteins in the *E. coli* host.

i. Cloning, construction, and evaluation of secretory expression system using CGTase as a reporter protein in *E. coli*.

ii. Improvement of CGTase excretion via modification of h-region signal peptide by site-saturation mutagenesis.

iii. Investigation on the effect of mutation at h-region signal peptide towards recombinant CGTase excretion and cell lysis.

iv. Study on the effect of SPPase overexpression towards recombinant CGTase excretion and cell lysis.

v. Analysing of the effect of several permeabilizing agents' supplementation (e.g.: glycine, Tween 20, Triton X-100 and chitosan) to the recombinant CGTase excretion and cell lysis.

vi. Optimization of culturing conditions including inducers concentrations (Arabinose: 0 – 2 %; IPTG: 0 – 1 mM) and post-induction temperature (20 – 37 ℃) on recombinant CGTase excretion and cell lysis.

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