EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF MALTOGENIC AMYLASE FROM *Bacillus lehensis* G1

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ABSTRACT

This study was carried out for the expression and characterization of maltogenic amylase (MAG1) from *Bacillus lehensis* G1. Amplification of 1741 base pair gene fragments encoding MAG1 and expression in *Escherichia coli* have been successful. The expression of MAG1 was optimized at 30°C and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for a period of 12 hour post-induction time. Purification of the crude enzyme was done using the ACTAprime System which uses the concept of affinity chromatography. The optimum temperature and pH of the purified MAG1 were 40 °C and pH 7.0 respectively. The enzyme did not show potent thermostability and was stable at pH ranging from 7.0 to 9.0. The purified MAG1 has a preference towards cyclodextrin (CD) specifically β-CD. Starches were least favored by MAG1 followed by pullulan. Only the metal ion Mn^{2+} increased the activity of MAG1 while K^{2+}, Li^{2+} and Mg^{2+} slightly affect its activity. The metal ion Ca^{2+} significantly reduced MAG1 activity while Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Ni^{2+} drastically reduced the activity of MAG1. In terms of additives, only 2-mercaptoethanol (2-ME) managed to enhance the activity of MAG1 while ethylenediaminetetraacetic acid (EDTA) and Tween20 did not affect its activity. Drastic reduction of MAG1 activity was caused by phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), methanol and ethanol. The hydrolysis pattern of MAG1 was studied using CDs and maltooligosaccharides. The hydrolysis of CDs resulted in the formation of maltose but no glucose was detected. MAG1 was also able to linearize the CDs. MAG1 did not react with glucose, maltose and maltotriose but did react with maltotetraose, maltopentaose, maltohexaose and maltoheptaose to produce mainly maltose and maltotriose. The K_m and V_{max} of MAG1 towards β-CD were 6.358 mg/mL and 91.63 µmol/min respectively.

ABSTRAK

Penyelidikan ini telah dijalankan untuk ekspresi dan mencirikan enzim maltogenic amylase (MAG1) yang diperolehi daripada *Bacillus lehensis* G1. Amplifikasi sebanyak 1741 pasang bes fragmen yang mengkodkan MAG1 dan ekspresi dalam *Escherichia coli* telah berjaya. Ekspresi MAG1 adalah optimum pada suhu 30°C dengan induksi 0.5 mM isopropil β-D-1-tiogalaktopiranosid (IPTG) selama 12 jam. Penulenan enzim telah dijalankan menggunakan sistem ACTAprime yang menggunakan konsep kromatografi afiniti. Suhu dan pH optimum bagi enzim yang telah ditulenkan adalah pada 40 °C dan pH 7.0. Enzim ini tidak menunjukkan kestabilan yang positif pada suhu tinggi dan stabil pada pH dari 7.0 ke 9.0. MAG1 yang telah ditulenkan lebih cenderung kepada siklodekstrin (CD) terutamanya β-CD. Kanji kurang digemari oleh MAG1 diikuti oleh pullulan. Aktiviti MAG1 telah dipertingkatkan oleh Mn^{2+} manakala K^{2+}, Li^{2+} dan Mg^{2+} tidak terlalu memberi kesan kepada aktiviti MAG1. Aktiviti MAG1 telah direncat oleh Ca^{2+} dan direncat dengan mendadak oleh Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Ni^{2+}. Selain daripada itu, 2-merkaptoetanol (2-ME) juga meningkatkan aktiviti MAG1 tetapi aktivitinya tidak dipengaruhi oleh asid atelindiamintetraasetik (EDTA) dan Tween20. Perencatan yang mendadak pada aktiviti MAG1 adalah disebabkan oleh fenilmetilsulfonil florida (PMSF), natrium dodecil sulfat (SDS), metanol dan etanol. Hasil hidrolisis oleh MAG1 dikaji dengan menggunakan CD dan maltooligosakarida. Hidrolisis CD menghasilkan maltosa sebagai produk utamanya tetapi tiada penghasilan glukosa dapat dikesan. MAG1 juga mampu menukar CD kepada bentuk linearnya. MAG1 tidak bertindak balas dengan glukosa, maltosa dan maltotriosa tetapi bertindak balas dengan maltotetraosa, maltopentosa, maltoheksosa dan maltoheptosa untuk menghasilkan maltosa dan maltotriosa. Nilai K_m dan V_{max} bagi MAG1 terhadap β-CD adalah 6.358 mg/mL dan 91.63 μmol/min.

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LIST OF SYMBOLS/ABBREVIATION

Asn	-	Asparagine
Asp	-	Aspartic acid
BCIP	-	5-Bromo-4-Chloro-3-Indolyl Phosphate
Blast	-	Basic Local Alignment Search Tool
Bp	-	base pair
DNA	-	Deoxyribonucleic acid
EC	-	Enzyme Commision
EDTA	-	ethylene diamine tetraacetic acid
eg.	-	example
et al.	-	and frinds
EtOH	-	Ethanol
g/L	-	gram per litter
Glu	-	Glutamate
His	-	Histidine
HPAEC	-	High Performance Anion Exchange Chromatography
HPLC	-	High Performance Liquid Chromatography
IPTG	-	IsoPropyl β-D-1-ThioGalactopyranoside
kb	-	kilo base
KCl	-	potassium chloride
KI	-	potassium iodide
LMW	-	Low Molecular Weight
MeOH	-	Methanol
mg/mL	-	milligram per milliliter

mM	-	millimolar
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
NBT	-	Nitro Blue Tetrazolium chloride
nm	-	nano meter
PAGE	-	polyacarylamide gel electrophoresis
PMSF	-	phenylmethanesulfonyl fluoride
rpm	-	revolutions per minute
SDS	-	sodium dedocyl sulphate
Ser	-	serine
TBE	-	Tris/Borate/EDTA
TBS	-	Tris Buffered Saline
TBSTT	-	Tris Buffered Saline Tween-20 Triton
TE	-	Tris/EDTA
TLC	-	Thin Layer Chromatography
TSS	-	Transformation and Storage Solution
U/mL	-	unit per milliliter
UV	-	ultraviolet
UV-Vis	-	ultraviolet visible
v/v	-	volume per volume
w/v	-	weight per volume
α	-	alpha
β	-	beta
γ	-	gamma
µL	-	micro litter
%	-	percentage
℃	-	degree Celsius
Ca^{2+}	-	calcium ion
Co^{2+}	-	cobalt ion
Cu^{2+}	-	copper ion
Fe^{2+}	-	iron ion
K^{+}	-	potassium ion
Li^{2+}	-	lithium ion
Mg^{2+}	-	magnesium ion

Mn^{2+}	-	manganese ion
Ni^{2+}	-	nickel ion
Pb^{2+}	-	lead ion
Zn^{2+}	-	zinc ion
H_2SO_4	-	sulfuric acid
KH_2PO_4	-	monopotassium phosphate
$MgSO_4$	-	magnesium sulfate
T_m	-	melting temperature

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Alpha-amylase (EC 3.2.1.1.) is one of the enzymes that have been investigated most thoroughly and used most widely in the starch hydrolytic industry. These amylase are endo-acting amylases which means the enzyme hydrolyze α-1,4-glycosidic linkage of internal starch polymer randomly. This in turn resulted in oligosaccharides with varying lengths and α-dextrins that retains its α-1,6-glycosidic linkages. There are various amylolytic enzymes such as pullulanase (EC 3.2.1.41; Kuriki *et al*., 1988), cyclodextrin glucanotransferase (EC 2.4.1.19; Sakai *et al*,. 1987), cyclodextrinase (EC 3.2.1.54; Kitahata *et al*, 1983), and maltogenic amylase (EC 3.2.1.133; MAase; Kim *et al*,. 1992) in which these enzymes exhibits novel enzymatic properties that clearly differentiate it from those of α-amylase, being capable of hydrolyzing poly- and/or maltooligosaccarides such as pullulan and cyclodextrins (CDs) as well as its extensive transglycosylation activity.

Maltogenic amylases which belong to the glycoside hydrolase family 13 (GH13) are very unique where these enzymes have multi-substrate specificity towards starch, pullulan, and CDs, which is modulated by dimerization of the

enzyme. Other than its typical-α-amylase hydrolytic activity, maltogenic amylase is also able to perform a transglycosylation activity via formation of various glycosidic linkages such as α-1,3- and α-1,6-linkages to produce branched oligosaccharides from substrate such as liquefied starch (Park *et al.,* 2000). This enzyme is named maltogenic amylase because in general it will hydrolyze CD into mainly maltose and glucose.

The enzyme activity was found to be cell-bounded which means that the enzyme is intracellular (Kolcuoğlu *et al.,* 2010). It was proven by the predicted amino acid sequence with no sign of signal sequence necessary for secretion of the enzyme outside the cell. In order to exploit its benefits, maltogenic amylase has long been discovered and isolated such as from *B. licheniformis* (Kim *et al*., 1992), *B. stearothermophilus* (Cha *et al*., 1998), and a *Thermus* species (Kim *et al.*, 1999).

From previous researches on maltogenic amylase, it has been proven that maltogenic amylase is able to produce branched side chains when reacted with amylopectin cluster (Kim *et al.*, 2006). Moreover, this enzyme hydrolyzes acarbose, a potent α-amylase inhibitor (Park *et al*., 1998). The combined activities of maltogenic amylase which are hydrolysis and transglycosylation have been utilized in the production of branched oligosaccharides and various modified sugars (Kim *et al*., 1994; Lee *et al*., 1995). Maltogenic amylase is also a highly effective anti-staling agent used for bread making in industrial bakeries.

Different source of enzymes exhibits their own unique characteristic even though they have some similarities in terms of function, substrate they act upon or their structure. Finding the enzymes of highly useful characteristic is very important as it can contribute in variety of industries. This is where maltogenic amylase from *Bacillus lehensis* G1 becomes an interest. Maltogenic amylase has very useful industrial applications and exploring another source of this enzyme can give us more option to consider.

Amount of this enzyme that can be obtained from the wild microorganism is normally limited and their characteristics are unknown. Therefore, their applicability to meet with the conditions of a reaction criteria such as high temperature is still unclear. We intend to clone and over-expressed the maltogenic amylase in expression system such as *Escherichia coli* (*E. coli*) and characterize the recombinant enzyme. This will contribute in research to improve the characteristic of the enzyme such as thermostability by protein engineering. It is an important characteristic for industrial application where a thermostable enzyme is preferred. Other than that, an efficient way to produce this enzyme is crucial in order to obtain a low cost production.

1.2 Objectives of Research

The objectives of this research are to clone and express maltogenic amylase from *Bacillus lehensis* G1 (MAG1) in *E. coli* and to characterize the purified recombinant maltogenic amylase.

1.3 Scopes of Research

1.3.1 Amplification of maltogenic amylase gene using polymerase chain reaction (PCR) method

1.3.2 Cloning and expression of maltogenic amylase gene in *E. coli* expression system

1.3.3 Purification and characterization of recombinant maltogenic amylase

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