GLYCATION OF INDUSTRIAL α-AMYLASE AND GLUCOAMYLASE UNDER HIGH REDUCING SUGAR CONDITIONS

PORNPONG SUTTHIRAK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
(BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005

ISBN 974-04-6588-9

Thesis
Entitled

GLYCATION OF INDUSTRIAL α-AMYLASE AND GLUCOAMYLASE UNDER HIGH REDUCING SUGAR CONDITIONS

.....

Mr. Pornpong Sutthirak
Candidate

.....

Asst. Prof. Sittiwat Lertsiri, Ph.D.
Major-Advisor

.....

Assoc. Prof. Manop Suphantharika, Ph.D.
Co-Advisor

.....

Asst. Prof. Apinya Assavanig, Ph.D.
Co-Advisor

.....

Asst. Prof. Pimchai Chaiyen, Ph.D.
Co-Advisor

.....

Asst. Prof. Salee Kiewkarnka, Ph.D
Acting Dean
Faculty of Graduate Studies

.....

Assoc. Prof. Manop Suphantharika, Ph.D.
Chair
Doctor of Philosophy Programme in
Biotechnology
Faculty of Science

Thesis
Entitled

GLYCATION OF INDUSTRIAL α-AMYLASE AND GLUCOAMYLASE UNDER HIGH REDUCING SUGAR CONDITIONS

was submitted to the Faculty of Graduate studies, Mahidol University
for the degree of Doctor of Philosophy (Biotecnology)
on
6 October, 2005

.....

Mr. Pornpong Sutthirak
Candidate

.....

Asst. Prof. Sittiwat Lertsiri, Ph.D.
Chair

.....

Assoc. Prof. Manop Suphantharika, Ph.D.
Member

.....

Asst. Prof. Apinya Assavanig, Ph.D.
Member

.....

Asst. Prof. Pimchai Chaiyen, Ph.D.
Member

.....

Mrs. Pongsuda Pongtanya, Ph.D.
Member

.....

Asst. Prof. Salee Kiewkarnka, Ph.D
Acting Dean
Faculty of Graduate Studies
Mahidol University

.....

Prof. Amaret Bhumiratana, Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENT

I would like to express my deepest and sincere gratitude to my advisor, Asst. Prof. Dr. Sittiwat Lertsiri, for his kind supervision, continuous guidance and encouragement throughout the course of this study which enable me to attain thesis successfully.

I am grateful and appreciation to the member of advisory committee, Asst. Prof. Dr Saovanee Dharmsthiti, Asst. Prof. Dr. Manop Suphantharika, Asst. Prof. Dr. Apinya Assavanig and Asst. Prof. Dr. Pimchai Chainyen for their valuable comments, suggestions and encouragement for the completion of this study. I am very grateful to Dr. Pongsuda Pongtanya for her discussion, comments and suggestions.

This thesis was partially supported by a scholarship from "the Ministry of Education, Commission of Higher Education, subproject; Graduate Study and Research in Agricultural Biotechnology".

I am thankful to Department of Biotechnology, Faculty of science, Mahidol University and Department of Bioindustry, Faculty of Technology and Management, Prince of Songkla University for giving me an opportunity to study Doctoral degree.

I wish to thank Cinnamon Company, Ltd. (Thailand) and The East Asiatic (Thailand) Public Company, Ltd. for kindly support the enzyme.

Special thanks are given to all members in Food Biotechnology Laboratory and Food Flavor Research Unit (BT101 and BT115), for their help with sincerity, kindness, encouragement, friendship and enjoyment. I also thank Mrs. Daungnatre Issarangkul Na Ayudhaya, Miss Sudaporn Luechai, all members in Center of Biotechnology, Institute for Research and Development in Science and Technology, Mahidol University.

Finally, my sincere profound gratitude is extended to Mrs. Siriporn Kongwattananonta, Miss Sirusa Kritsanapan, Miss Piyarat Nakawirote, Mrs. Mookkarin Unjitwattana and her family, and especially my parents for their infinite love, understanding and full moral support.

Pornpong Sutthirak

GLYCATION OF INDUSTRIAL α-AMYLASE AND GLUCOAMYLASE UNDER HIGH REDUCING SUGAR CONDITIONS

PORNPONG SUTTHIRAK 4338100 SCBT/D

Ph.D. (BIOTEHNOLOGY)

THESIS ADVISORS: SITTIWAT LERTSIRI, Ph.D., MANOP SUPHANTHARIKA, Ph.D., APINYA ASSAVANIG, Ph.D., PIMCHAI CHAIYEN, Ph.D.

ABSTRACT

Starch hydrolysis by α-amylase and glucoamylase is employed under high reducing sugar and high temperature conditions. Under such conditions, the enzymes can readily undergo glycation leading to the alteration of enzyme properties and functions. Thermostable α-amylase (KLE), mesophilic α-amylase (BAN) and purified glucoamylase (PG) were glycated by saccharides under high reducing sugar and high temperature conditions. The extension of glycation was monitored by the increase in amount of 5-(hydroxymethyl)-2-furfuraldehyde (HMF) released from the glycated protein. The glycation progressed in both soluble and aggregate enzymes with increasing incubation period. The increases in molecular weight (MW), different migration patterns of protein bands on Native-PAGE and the alteration of pI value were observed in glycated KLE. However, the glycation of PG could not be monitored by SDS-PAGE, Native-PAGE and pI value due to the low degree of glycation of this enzyme. The glycation led to the greater thermal stability and wider range of pH tolerance of KLE and PG. This was due to the formation of new interactions (*i.e.* hydrogen bond, electrostatic interactions and hydrophobic interaction) between saccharide chains and the enzyme molecule. The saccharide chains also limited unfolded space of protein structure resulting in the resistance to thermal treatment. Meanwhile, the glycated BAN showed the contrary effects due to the difference in progressive level of glycation and position of amino acid residues that glycation occurred. The glycation of KLE and BAN did not affect the kinetic parameters. The glycation only led to lower capacity to convert gelatinized cornstarch of BAN. In case of PG, the glycation did not affect the kinetic parameters when maltose was used as substrate. However, the glycation lowered the affinity to bind substrate maltodextrin. The elevation of the rate of maltodextrin hydrolysis and the greater efficiency of glucoamylase to convert the substrate maltodextrin into glucose was observed when the enzyme was glycated.

KEY WORDS: GLYCATION/ THERMOSTABLE α-AMYLASE/ MESOPHILIC α-AMYLASE/GLUCOAMYLASE/ 5-(HYDROXYMETHYL-2-FURFURALDEHYDE)/ THERMOSTABILITY/ pH STABILITY/ KINETIC PARAMETERS/

132 P. ISBN 974-04-6588-9

การเกิดไกลเคชันของเอนไซม์อัลฟา-อะมัยเลสและกลูโคอะมัยเลสที่ใช้ในอุตสาหกรรมภายใต้ภาวะน้ำตาลรีดิวซ์สูง (GLYCATION OF INDUSTRIAL α-AMYLASE AND GLUCOAMYLASE UNDER HIGH REDUCING SUGAR CONDITIONS)

พรพงษ์ สุทธิรักษ์ 4338100 SCBT/D

ปร.ด. (เทคโนโลยีชีวภาพ)

คณะกรรมการควบคุมวิทยานิพนธ์ : สิทธิวัฒน์ เลิศศิริ, Ph.D., มานพ สุพรรณธริกา, Ph.D., อภิญญา อัศวนิก, Ph.D., พิมพ์ใจ ใจเย็น, Ph.D.

บทคัดย่อ

การย่อยสลายแป้ง (สตาร์ช) โดยใช้เอนไซม์อัลฟา-อะมัยเลสและกลูโคอะมัยเลสภายใต้ภาวะการมีน้ำตาลรีดิวซ์และอุณหภูมิสูง ทำให้เอนไซม์เกิดไกลเคชันขึ้น นำไปสู่การเปลี่ยนแปลงคุณสมบัติและมีผลกระทบต่อหน้าที่ของเอนไซม์ การศึกษาการเกิดไกลเคชันของเอนไซม์พบว่า ในภาวะการย่อยสลายแป้งในอุตสาหกรรม อัลฟา-อะมัยเลสที่ทนร้อน (KLE) และที่ชอบอุณหภูมิปานกลาง (BAN) รวมทั้งกลูโคอะมัยเลส (PG) เกิดไกลเคชันขึ้น โดยสังเกตจากการเพิ่มปริมาณของ 5-(hydroxymethyl)-2-furfuraldehyde (HMF) ตลอดช่วงระยะเวลาการบ่มเอนไซม์กับน้ำตาลรีดิวซ์ นอกจากนี้ยังพบการเพิ่มขึ้นของน้ำหนักโมเลกุล การเปลี่ยนรูปแบบการเคลื่อนที่ของแถบโปรตีนบน Native-PAGE และการเปลี่ยนแปลงค่า pI ของ KLE ที่เกิดไกลเคชันกับมอลโทเด็กซ์ทริน ในขณะที่การเกิดไกลเคชันของ PG อยู่ในระดับต่ำจนไม่สามารถตรวจพบด้วยวิธีดังกล่าว การเกิดไกลเคชันยังเพิ่มความสามารถในการทนอุณหภูมิสูงและทนต่อการเปลี่ยนแปลง pH ของ KLE และ PG เนื่องจากการเกิดพันธะหรือแรงอื่นๆ เช่น พันธะไฮโดรเจน แรง electrostatic และ แรง hydrophobic ระหว่างโมเลกุลของเอนไซม์และน้ำตาลรีดิวซ์ นอกจากนี้สายของโมเลกุลน้ำตาลที่เกาะติดกับเอนไซม์ยังจำกัดการคลายตัวของเอนไซม์ทำให้เพิ่มความสามารถในการทนอุณหภูมิสูง ในขณะที่ BAN ให้ผลในทางตรงกันข้าม ทั้งนี้อาจเนื่องมาจากระดับของการเกิดไกลเคชันรวมทั้งตำแหน่งของกรดอะมิโนที่เกิดไกลเคชันแตกต่างกัน การเกิดไกลเคชันไม่มีผลต่อค่าทางจลนพลศาสตร์ของ KLE และ BAN อย่างไรก็ตามการเกิดไกลเคชันของ BAN ทำให้ความสามารถในการย่อยสลายแป้งลดลง การเกิดไกลเคชันของ PG ไม่มีผลต่อค่าทางจลนพลศาสตร์เมื่อใช้น้ำตาลมอลโตสเป็นสารตั้งต้น ในกรณีที่ใช้มอลโทเด็กซ์ทรินเป็นสารตั้งต้นพบว่า การเกิดไกลเคชันทำให้ความสามารถในการจับกับสารตั้งต้นลดลง นอกจากนี้ยังพบว่า อัตราการย่อยสลายมอลโทเด็กซ์ทรินและความสามารถในการเปลี่ยนให้เป็นกลูโคสเพิ่มสูงขึ้นเนื่องมาจากการเกิดไกลเคชันของเอนไซม์

132 หน้า ISBN 974-04-6588-9

CONTENTS

CONTENTS

(continued)

CONTENTS

(continued)

CONTENTS

(continued)

CONTENTS

(continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

(continued)

FIGURE **Page**

LIST OF FIGURES

(continued)

FIGURE **Page**

LIST OF FIGURES

(continued)

FIGURE **Page**

LIST OF ABBREVIATIONS

%	Percent
^{o}C	Degree Celsius
et al.	*Et all* (Latin), and others
i.e.	*Id est* (Latin), that is
DW	Distilled water
g	Gram
mg	Milligram
µg	Microgram
L	Liter
ml	Milliliter
µl	Microliter
M	Molar
mM	Millimolar
h	Hour
min	Minute
N	Normal
V	Volt
OD	Optical density
rpm	Revolution per min
KLE	Thermostable α-amylase
BAN	Mesophilic α-amylase
PG	Purified glucoamylase
gKLE	Glycated thermostable α-amylase
gBAN	Glycated mesophilic α-amylase
gPG	Glycated purified glucoamylase

CHAPTER I
INTRODUCTION

In aqueous solution, protein is a complex and hierarchically organized molecule with three-dimensional conformation. This structure exhibits a protein stability. The environmental conditions such as temperature, hydration, salinity and pressure affect the stability of the protein and its function. Post-translational modification such as glycation possibly leads to alteration of the three-dimensional structure of protein. The glycation naturally occurs in food and biological systems whenever reducing sugar is present together with proteins. This nonenzymatic reaction is initiated by the condensation between carbonyl group from reducing sugar and amino group on amino acid residues such as N-terminal amino acid, Lys, Arg, and His residues of the protein molecule. The glycated protein in the form of Amadori products is then further dehydrated, leading to intramolecular cross-linking of the protein molecule (Belitz and Grosch, 1999; Namiki, 1988; Yeboah *et al*., 1999). The glycation occurring in various kinds of enzyme results in the alteration of enzyme properties and functions.

The major application of starch hydrolysis is for the production of glucose, which is subsequently used to produce crystalline dextrose, dextrose syrup, and high-fructose syrup. Glucose, the main product from starch hydrolysis process, is further fermented to produce other products such as ethanol, amino acid and organic acid. In the past, the conversion of starch to oligosaccharides and dextrose was based on acid hydrolysis. The starch hydrolysis process is now replaced by enzymatic process since the acid hydrolysis requires the use of corrosive resistant material and causes rise of color and salt content (Gerhartz, 1990). The commercial enzymatic process of starch hydrolysis is divided into two major processes *i.e.* liquefaction and saccharification. Firstly, the gelatinized starch molecules are liquefied by the action α-amylase to yield maltodextrin. This process is called liquefaction. Maltodextrin is further hydrolyzed by the action of glucoamylase in saccharification process to give glucose as the final

product (Knorr, 1987; Pomeranz, 1991; Crabb and Mitchinson, 1997). In liquefaction process, 30-40% granular starch is added into cold water (pH 6.0-6.5) containing 20-80 ppm Ca^{2+} and the enzyme is added. Because starch is generally insoluble in water at room temperature and starch granules are quite resistant to penetration of both water and amylolytic enzymes. It is necessary to gelatinize starch granules by heating at about 95-105^{o}C in order to make starch absorb water and swell, and to be easily hydrolyzed by thermostable α-amylase. Enzymatic hydrolysis is completed for 1-2 h to obtain the required dextrose equivalent (D.E.) around 8 to 12. After that, the resulting maltodextrin is further saccharified by the glucoamylase. Saccharification process is carried out at 55 to 60^{o}C for 48-92 h and yields glucose syrup with dextrose equivalence (D.E.) of 97 to 98 as a product (Knorr, 1987; Gerhartz, 1990; Pomeranz, 1991, Wong, 1995).

Since both the industrial α-amylase and glucoamylase are employed in starch hydrolysis process under high reducing sugar and high temperature conditions, the enzymes were assumed to undergo the glycation under such conditions. The glycation occurred on amylolytic enzymes might involve in the alteration of its properties, functions, and stability. However, most research has been performed regarding to enzymes glycated in biological systems and physiological models (Arai *et al.*, 1987; Baldwin *et al*., 1995; Chiou *et al*., 1999; Seidler and Seibel, 2000). In this thesis, the alteration of enzyme properties and kinetic parameters due to the glycation of industrial amylolytic enzyme was investigated. Enzyme activity and degree of the glycation was determined to explain the effect of the glycation on enzyme functions including kinetic parameters.

CHAPTER II
OBJECTIVES

1. To investigate the glycation of industrial amylolytic enzymes under high temperature and high reducing sugar conditions.
2. To study the effects of glycation on properties and kinetic parameters amylolytic enzymes.

Overall plan

1. Purification of amylolytic enzymes
2. Study of the progress in glycation during incubation of amylolytic enzymes and saccharides
3. Study of the thermostability and pH stability of glycated amylolytic enzymes comparing to the intact form
4. Study the effect of glycation on kinetic parameters of glycated amylolytic enzyme comparing to the intact form
5. Monitor the degree of glycation changes during hydrolysis of gelatinized cornstarch by thermostable α-amylase

CHAPTER III
LITERATURE REVIEW

1. The Glycation

The interaction between amino and carbonyl compounds resulting in a complex changes in biological and food systems, called the "amino-carbonyl reaction" has exerted a strong influence on existence throughout the aging, and the browning and flavor changes associated with processing of foods. This reaction was first discovered in 1912 by Louis-Camille Maillard. Since that time this reaction, so-called the Maillard reaction, continued to being significant focus of attention in the chemistry of foods. Later, studies on the effect of the reactions on nutritional and physiological properties, as well as changes in the physiochemical properties of protein and antioxidant activity, began to increase in number. Not only the Maillard reaction has been studied in food systems, but also the influence of this reaction on enzyme properties and functions under physiological conditions have been studied (Wong, 1986; Namiki, 1988; Belitz and Grosch, 1999).

In 1953, Hodge divided the chemical scheme of the Maillard reaction into three stages. An initial stage involves the formation of glucosyl-amino products following by Amadori rearrangement to form an Amadori product. This stage is called "glycation" when the amino compound is protein. An intermediate stage involves dehydration and fragmentation of sugar and Amadori product, amino acid dehydration, etc. A final stage involves protein crosslinking, as well as aldol condensation, polymerization and formation of heterocyclic compounds and colored product, known as melaniodin (Figure 1).

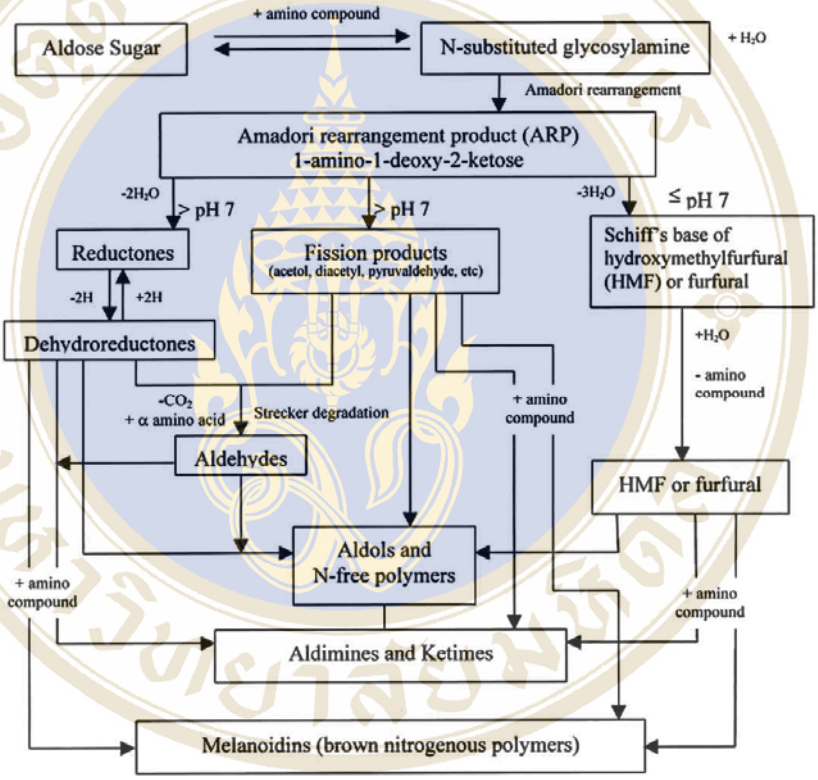


Figure 1 The Maillard reaction pathways adapted from Hodge (Martins *et al.*, 2001)

1.1 Mechanism of the glycation (the initial stage of the Maillard reaction)

It is well known that the rate of glycation is strongly dependent on the nature of reactants and the reaction conditions, especially pH and temperature. The reaction rates of aldoses in general are higher than those of ketoses, those pentoses are higher than those of hexose. Basic amino acids generally undergo glycation more rapidly than acidic amino acids (Namiki, 1988).

The glycation is initiated by the simple condensation between the carbonyl group (the aldehyde from the reducing sugar) and the free amino group on protein molecule, to give an *N*-subtituted glycosylamino compound following by the reversible formation of the Schiff base derivatives. This condensation reaction initiated by the attached of nucleophilic nitrogen, with an unshared electron pairs, on the carbonyl carbon. The reaction usually requires an acidic catalyst. Protonation of the carbonyl group should enhance the reactivity to the nucleophilic reagent, while the protonation of the nitrogen of the amino group inhibits the attack on the carbonyl carbon (Figure 2a and 2b).

The formation of the *N*-substituted glycosylamine is usually very fast and the product is unstable; especially in the aqueous system, it is susceptible to reverse hydrolysis as well as to additional irreversible reaction. It is difficult to isolate glycosylamine because they are immediately converted into the Amadori products, *N*-substituted 1-amino-1-deoxy-2-ketoses. This Amadori reaction is normally catalyzed by weak acids. Because the amino acids serve as their own acid catalysts, so the reaction is rapid even in the absence of acid addition. It should be noted that the glycation are all favored by acidic conditions (Namiki, 1988).

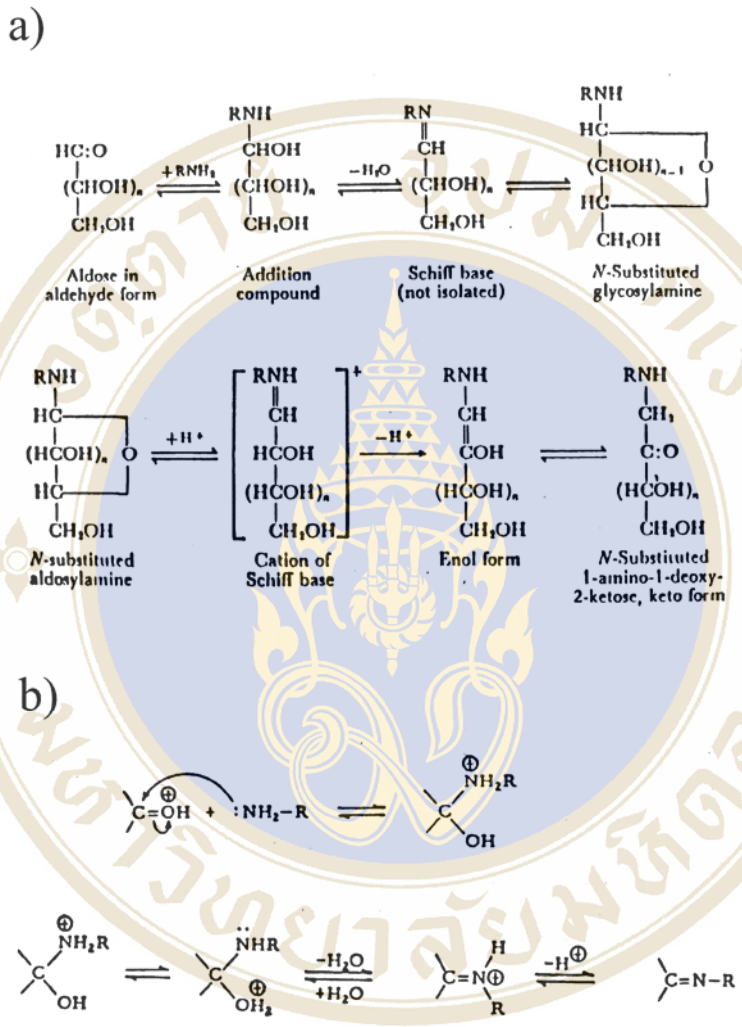


Figure 2 a) The reaction between carbonyl group and amino group to yield the Amadori product (the glycation reaction);
b) The condensation of carbonyl compounds with amino compound (Namiki, 1988).

1.2 Sugar fragmentation during the glycation

The Amadori compounds in the glycation reaction mixture could exist as enaminol structure, a very reactive compound for the glycation. Hodge (1953) proposed the formation of pyruvaldehyde and other carbonyl compounds from 1-deoxyglucosone, the product (through 2,3-enolization) of the Amadori compound fragmentation. In addition, the cleavage would occur at the C-2/C-3 position of the sugar to give two-carbon carbonyl products by a reverse aldol mechanism is also postulated.

1.3 Formation of three carbon and other sugar fragmentary

The sugar fragmentation produces the C2 and C3 carbonyl compounds or their imine derivatives, following by the fragmentation of these compounds to yield glycolaldehyde, glyoxal, glyceraldehydes, methylglyoxal, etc., or their imine derivatives (Namiki, 1988).

1.4 The Amadori rearrangement products

The Amadori products, a key intermediate in the glycation, are obtained from glycosylamino products. Many researchers isolate the Amadori products from food system. Recently, due to their important effects on the physical, nutritive and physiological properties of proteins, many works have been done on the Amadori product formation by sugar protein reactions in both of food and biological systems.

Analytical techniques have produced more detail information on the nature of the Amadori products. High-performance liquid chromatography (HPLC) becomes a popular tool for analyzing the Amadori products. Structure analyzes by high-resolution NMR have shown that the Amadori rearrangement products exist mainly in the β-pyranosyl form at pH greater than 7 and favor β-furanosyl form at pH 3.

The Amadori products further undergo degradation to give various intermediates as follows: formation of labile enolized intermediates, 1,2-enolization followed by elimination of the hydroxy group at C-3 and deamination at C-1 giving 3-deoxyhexosone, the reactive dicarbonyl product itself, and later furfural as well as the reactive carbonyl compound (Namiki, 1988).

On the other hand, 2,3-enolization of the Amadori products followed by elimination of the amino group from C-1 yields the 1-deoxydicarbonyl intermediate, which further reacts to produce reactive fission carbonyl products such as methylglyoxal, diacetyl and others. However, several researchers reported that the Amadori products yielded colored products without undergoing conversion to deoxyosones or other compounds (Namiki, 1988).

1.5 Enolization and degradation of the Amadori products

In intermediate stage, 1,2-enolization is considered to be favored under acidic conditions and 3-deoxysones, furfural and hydroxymethylfurfural are the main products which have been isolated and identified form acidic sugar-amino acid reaction mixture as well as in actual food systems. On the contrary, no furfural and only methylfuranone production was observed at pH 7 in all the Amadori derivatives. These indicate that the formation of furfural is favored by acidic condition (Namiki, 1988).

1.6 Protein cross-linking

The role of glucose as the participating sugar in the cross-linking reaction has been studied (Figure 3). Dicarbonyl compounds such as methylglyoxal and glyoxal have been found to be active crosslinkers both *in vitro* and *in vivo*. The mechanisms of glyoxal and methylglyoxal generation *in vivo* have been determined, in an attempt to understand the progression of the glycation reaction relevant to hyperglycemic complication. Number of works has confirmed the generation of dicarbonyls via glucose autooxidation (Woolf and Dean, 1987; Wells-Knecht *et al.*, 1995). It appears that dicarbonyls can be generated on degradation of the Amadori products, a key intermediate in the the glycation reaction. Researchers have suggested that methylglyoxal and glyoxal can be generated from the degradation of the Schiff base adduct initially formed on reaction of glucose with an amine (Glomb and Monnier, 1995; Thonalley *et al.*, 1999; Miller *et al.*, 2003).

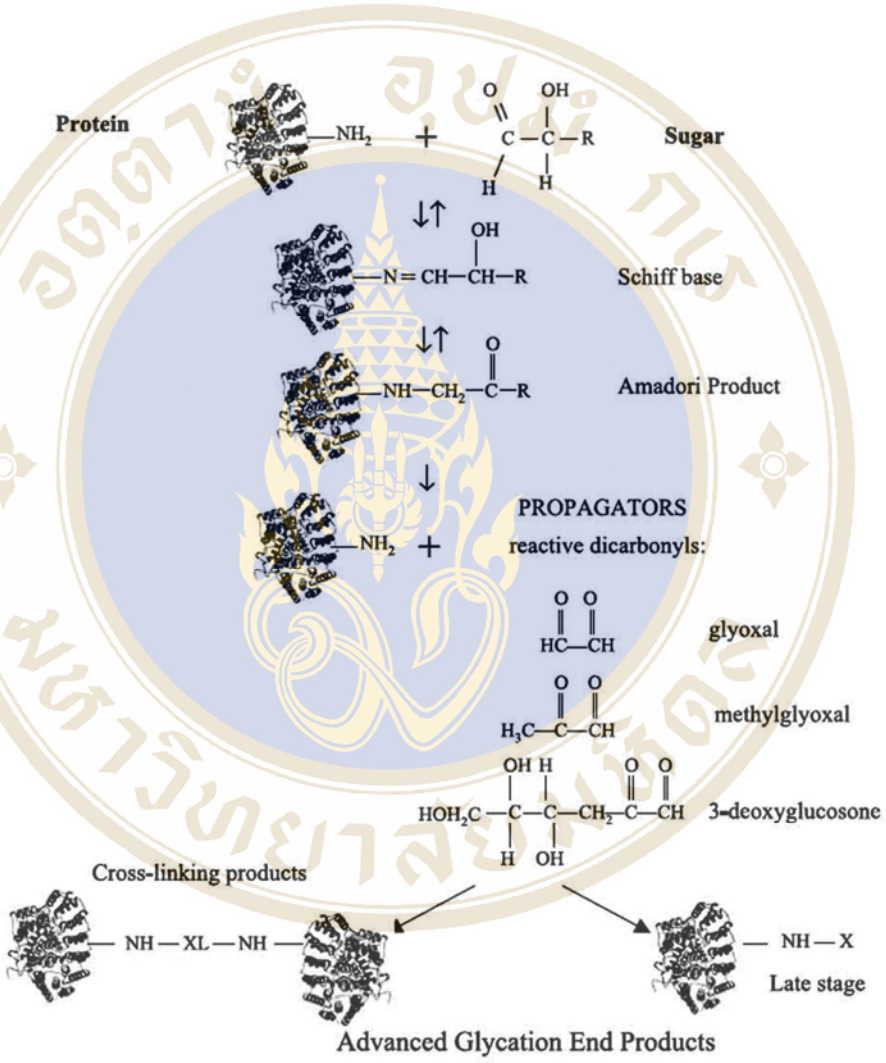


Figure 3 The protein cross-linking due to the glycation (Lapolla *et al.*, 2005)

1.7 Parameters influencing the glycation reaction

The glycation reaction is strongly affected by intrinsic and extrinsic factors such as temperature and duration of heating, pH, type of reactant, amino group to sugar ratio water content and oxygen.

1.7.1 Reactant type

For sugars, the rate of reaction depends on the rate at which the sugar cyclic structure opens to the reducible, acyclic form and this increases with increasing pH. The reactivity order of hexoses is D-galactose>D-manose>D-glucose. Reducing disaccharides are considerably less reactivity than their corresponding monomers. The rate of the glycation is also dependent on type and amino acid composition of protein. However, most research has been done on the relative reactivity of free amino acid. Lysine appears to be the most reactive amino acid due to the fact that it has two available amino groups (Ashor and Zent, 1984; O'Brien and Massey, 1989). However, the reactivity of lysine is dependent on the conditions of the reaction being studied. Several works are carried out on protein, especially the enzyme. The extent of the glycation also seems to vary according to the sugar and amine ratio. The initial formation step of the Schiff base is dependent on the concentration of both sugar and amino acid.

1.7.2 Temperature and pH

The effect of temperature and duration time of heating on the glycation was studied by Maillard, who reported that the rate of reaction increases with temperature. The Strecker degradation is favored at high temperature. The duration time of heating is also important for the reaction. The pH has a significant effect in the glycation. The reaction rate generally has a minimum at the pH of 3 and increases with increasing pH. The reactivity of the sugar and amino group is also highly influenced by the pH. The acyclic form of the sugar and the unprotonated form of the amino group, considered to be the reactive forms, are favoured at higher pH. The following equilibrium can be written for the amino group:

$$R\text{-}NH_2 + H^+ \leftrightarrows R\text{-}NH_3^+$$

The lower the pH, the more protonated amino group is present in the equilibrium, therefore, less reactive with the sugar. This equilibrium is dependent on pH and pK_a of amino acid (Martins *et al*., 2001).

1.8 The protein denaturation

Protein is a complex and hierarchically organized structure. In aqueous solvent, a protein polypeptide chain generally folds into three-dimensional conformation. This conformation exhibits a marginal stability. It is well-known that environmental conditions such as temperature, hydration, salinity and pressure affect on the stability of protein, as well as on the kinetics of enzymatic reaction. The major changes in the secondary, tertiary and quaternary structures without cleavage of backbone peptide bonds are regarded as denaturation. However, denaturation also involves loss of ordered structure. Depending on the denaturation conditions, protein is assumed undergo several denatured states differing slightly in free energy. Some denatured states possess more folded structure than other and the globular proteins resemble a random coil when fully denatured (Damodaran, 1996).

The effect of glycation on proteins may be beneficial or detrimental, depending on the extent of the glycation. Some of the beneficial effects of protein glycation are improvement of emulsifying properties, gelation, water holding capacity, and forming properties in food application. The glycation is also associated with the loss of nutrition in food protein due to the unavailability of lysine residues blocked and the damage of other essential amino acids (Aoki *et al.*, 1999; Yeboah *et al.*, 1999 ; Takahashi *et al.*, 2000 ; Yeboah *et al.*, 2000). The glycation also occurs in various kinds of proteins under physiological conditions resulting in the inactivation of the protein. Arai *et al.* (1987) studied the glycation of Cu-Zn-superoxide dismutase occurring when incubated with D-glucose. Lysine residues were glycated, resulting in the inactivation of the enzyme, especially via the glycation of Lys122 and Lys128 which locate in the active site. However, the other lysine residues were found to be related to the inactivation of enzyme as well.

Baldwin *et al.* (1995) reported that the glycation of glutathione peroxidase increases K_m by approximately 3 folds in comparison to nonglycated form. The increasing in K_m is related to the glycation at ε-amino group of Lys110 which lies on the surface of enzyme ≈15 Å away from the active site.

1.9 Determination of the glycated protein

Several methods for the analysis of the glycated protein include electrophoresis or chromatographic techniques such as high performance liquid chromatography/mass spectrometry (HPLC/MS) and HPLC for hydrolysis products of the glycated protein.

Under mildly acidic conditions, the glycated protein is degraded to 5-(hydroxymethyl)-2-furfuraldehyde (HMF). The method for the evaluation of the lysine modification caused by the glycation reaction was improved by Molales and Jimenez-Perez (1998). It is based on the indirect determination of the Amadori products as bound-HMF by reverse-phase HPLC. The best digestion conditions have been settled at 2 mg for freeze-dried protein/ml of 0.3 N oxalic acid. A digestion time of 120 min was selected as a good compromise between duration of analysis and rate of degradation of the glycated protein to free HMF.

Humany *et al.* (2002) studied the glycation between glucose and lysozyme. They applied peptide mapping by matrix-assisted laser desorption/ ionization time-offlight mass spectrometry (MALDI-TOF MS) to investigate the formation of structurally specific Amadori products on the glycated lysozyme, produced upon incubation with glucose. They were able to detect specific modifications as the Amadori products on glycated lysozyme. The glycation on human serum albumin derived from different concentrations of glucose, methyl glyoxal and glyoxylic acid were also studied by MALDI-TOF MS. The mass data were compared in terms of the degree of lysine and arginine side chain modification. The molecular masses were found to gradually increase with increasing concentrations of the individual modifier without reaching a plateau (Schmitt *et al*., 2005).

2. Starch Hydrolysis Process

Starch is known as the major part of the human diet, as well as it is the important raw material in various industries. It is synthesized naturally in a variety of plants. Starch molecules are glucose polymers linked together with the α-1,4 and α-1,6 glucosidic bonds. Because of the existence of two types of linkages, different structures are possible for starch molecules. An unbranched, single chain polymer of 500 to 2000 glucose subunits with only the α-1,4 glucosidic bonds is called amylose (Figure 4a). On the other hand, the present of α-1,6 glucosidic linkages results in a branched glucose polymer called amylopectin (Figure 4b). The degree of branching in amylopectin is approximately one per twenty-five glucose units in the branched segments (Pomeranz, 1991; Fennema, 1996; Belitz and Grosch, 1999).

In addition to amylose and amylopectin, starch usually contains small amounts of proteins and lipids. On the basis of X-ray diffraction, starch granules are a semicrystalline character, indicating a high degree of orientation of the glucan molecules. About 70% of starch granules are regarded as amorphous and 30% as crystalline. The amorphous regions consist of not only a main amount of amylose, but also a considerable part of the amylopectin. The crystalline regions contain primarily amylopectin. A model shown in figure 5 is under discussion for crystalline regions of the starch granule. It is composed of double helixes of amylopectin and mixed amylose/amylopectin double helixes, helixes of amylose with enclosed lipids, free amylose and free lipids (Figure 5) (Belitz and Grosch, 1999).

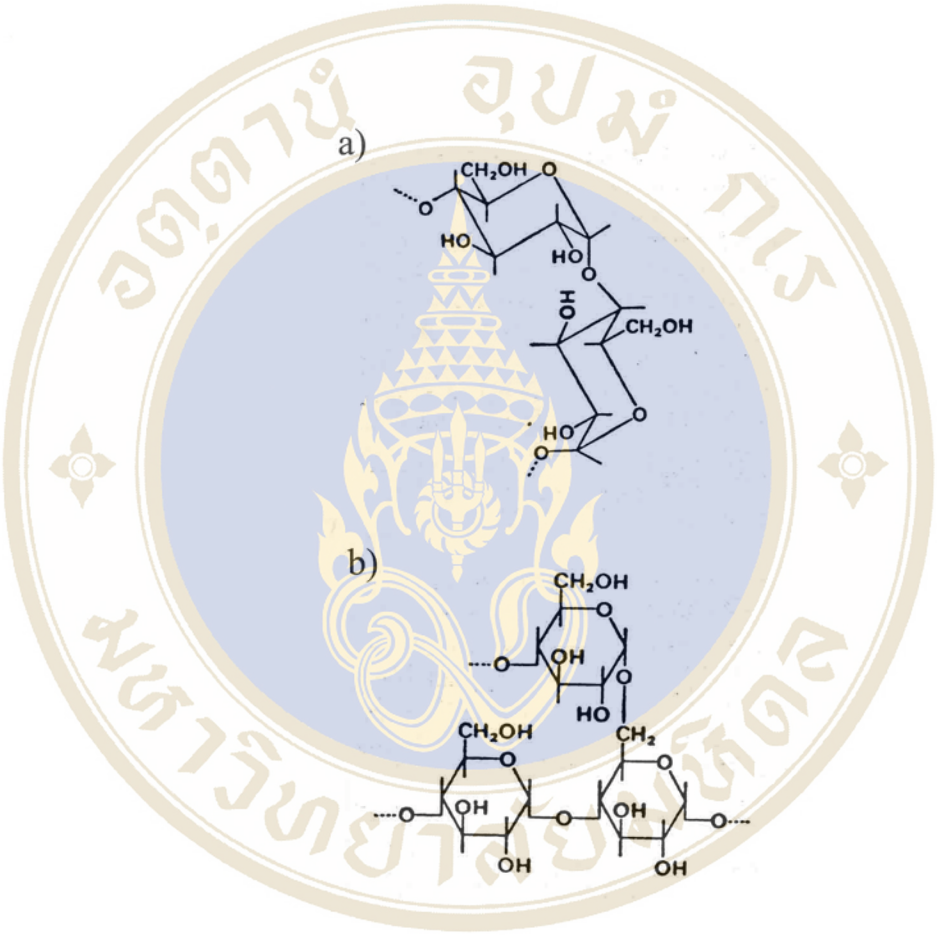


Figure 4 a) α-1,4 glucosidic bonds in amylose; b) α-1,6 glucosidic bonds in amylopectin (Belitz and Grosch, 1999)

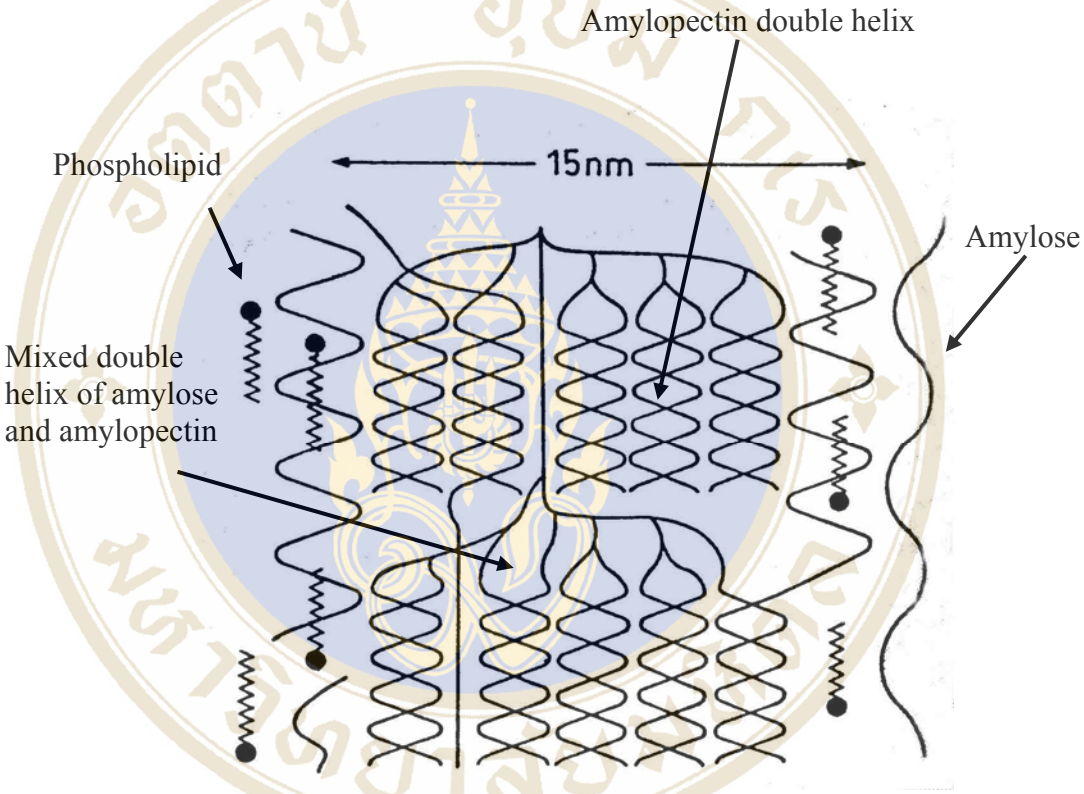


Figure 5 Model of a crystalline region in a starch granule (Belitz and Grosch, 1999)

Starch is generally insoluble in water at room temperature since it exists in the form of crystalline micelle. Its granules are resistant to penetration of both water and amylolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules. However, when an aqueous suspension of starch is heated, the hydrogen bonds are weakened, starch granules can absorb water and swell. This process is called gelatinization (Pomeranz, 1991).

In the past, acid hydrolysis of starch was widely used. As the acid hydrolysis requires the use of corrosive resistant material and causes rise of color and salt content, it is now replaced by enzymatic process. The commercial enzymatic process to convert starch into mono- and oligosaccharides can be divided into 2 major processes (liquefaction and saccharification) (Figure 6). The breakdown of large molecules drastically reduces the viscosity of gelatinized starch solution. This process is called liquefaction. The final stages of depolymerization are mainly the formation of mono, di, and tri-saccharides. This process is called saccharification. Most hydrolytic enzymes such as α-amylase in liquefaction process are specific at α-1,4-glucosidic links, while the α-1,6-glucosidic links can be cleaved for complete hydrolysis of amylopectin to glucose by glucoamylase in saccharification process (Knorr, 1987; Pomeranz, 1991; Crabb and Mitchinson, 1997). Glucose, the final product from saccharification process, is further converted to high fructose syrup by the action of glucose isomerase. In conclusion, a three-enzyme series of α-amylase, glucoamylase and glucose isomerase converts starch to maltodextrin, then to glucose and finally to a mixture of glucose and fructose. The starch hydrolysis products are widely used in industry such as glucose, glucose syrup and high fructose syrup. Glucose is further converted to ethanol, amino acids or organic acids (Crabb and Mitchinson, 1997).

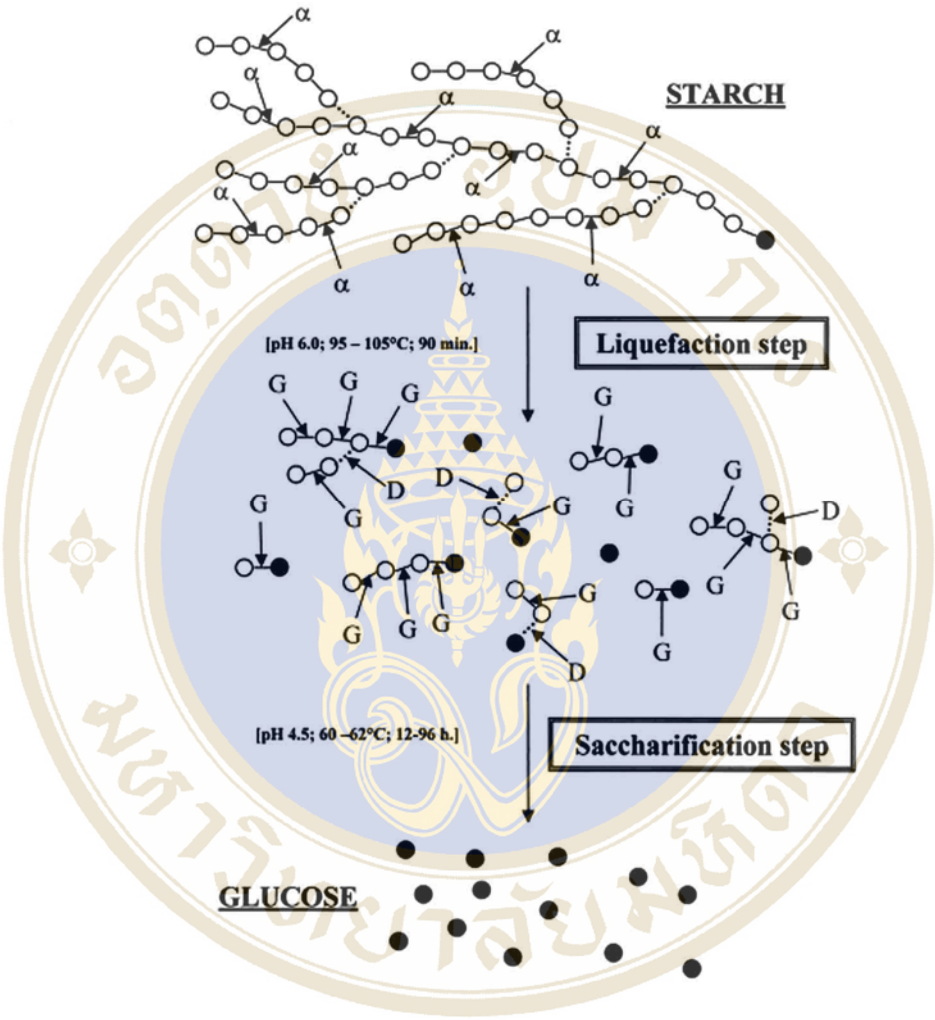


Figure 6 Industrial enzymatic hydrolysis of starch into glucose and pattern of action of amylolytic enzymes. α; α-amylase, G; glucoamylase, D; debranching enzyme (pullulanase), o; non-reducing glucose unit, •; reducing end glucose unit.

2.1 Liquefaction process

The objective of starch liquefaction process is to convert a concentrated suspension of starch granule into a short chain polysaccharide or malodextrin. Manufacturers use various methods to conduct starch liquefaction including application of α-amylase with the same principle. The starch slurry is adjusted to approximately 30-40% (w/w), at pH 6.0-6.5, containing 20-80 ppm Ca^{2+} and the enzyme is added. After that, the slurry is heated to reach to gelatinization temperature. In order to assure the removal of all lipid-amylose complexes, the temperature preferred for gelatinization is above 100°C. The starch granules begin to swell irreversibly and the amylose in the granules becomes soluble and is released. The viscosity of the solution increases dramatically (Knorr, 1987; Gerhartz, 1990; Pomeranz, 1991; Crabb and Mitchinson, 1997; Lamdubwong and Seib, 2001). Because of the high temperature to gelatinize starch granules, this has driven the replacement of α-amylase from *Bacillus amyloliquefaciens* with the more thermostable enzymes from *B. sterothermophilus*, *B. licheniformis*, and *B. subtilis*. Although these enzymes are able to operate briefly at temperature as high as 105°C, the process cannot be performed below pH 5.9 as α-amylase thermostability decreases at lower pHs. The α-amylase is usually supplied at high activities so that the enzyme dose is 0.5-0.6 kg enzyme/ton substrate. Hydrolysis process to obtain dextrose equivalence (D.E.) required is completed at 90-100°C for 1-2 h. D.E. is defined as the number-average degree of polymerization compare to 100 of total glucose equivalence. The maximum D.E. obtainable using bacterial α-amylase is around 40 but prolonged treatment leads to the formation of maltulose, which is resistant to the further hydrolysis by glucoamylase. D.E. 8-12 are required in commercial processes hydrolyzed by α-amylase prior to saccharification process. This process requires pH adjustment back down to pH 4.2-4.5. These adjustments increase the chemical costs and require additional ion-exchange refining of the final product for salt removal (Knorr, 1987; Gerhartz, 1990; Pomeranz, 1991; Crabb and Mitchinson, 1997; Lamdubwong and Seib, 2001).

α-Amylase being able to operate at lower pH can reduce these costs, simplify the process and reduce high-pH by product formation in the liquefaction. Amylases from extremophiles, especially thermo-acidophile, have promising properties, but none has been produced at commercially available levels. The liquefied starch is then usually saccharified but comparatively small amounts of the product is spray-dried and commercialized as maltodextrin for food industries, e.g. bulking agents and baby food (Knorr, 1987; Gerhartz, 1990; Pomeranz, 1991; Crabb and Mitchinson, 1997; Lamdubwong and Seib, 2001).

2.2 Saccharification process

The maltodextrins formed by α-amylase hydrolysis of starch are then further saccharified by action of the glucoamylase. This process removes single glucose residues from non-reducing end of maltodextrin until all of the saccharide is degraded to glucose. The enzyme is produced industrially from *Aspergillus niger* or a closely related species, for example, *A. awamori*. Glucoamylase produced by *A. niger* with a pH optimum near 4.5, is stable at 60^{o}C.

For saccharification, the concentration of maltodextrin solution is diluted to 30-32% and pH is adjusted to 4.2-4.5. At this pH, it is the optimum for glucoamylase and the action of α-Amylase is terminated to maintain an average chain length optimal for saccharification. The saccharification process is usually carried out in a stirring tank using soluble enzyme at 55-60^{o}C between 24-96 h. The final product of glucoamylase action is a glucose syrup with D.E. of 97-98. This consists of 95-97% glucose and 3-5% higher oligosaccharides. After the process is finished, enzyme is removed by heat denaturation or ion exchange chromatography. There are some practical difficulties in this process; for example, the substrate is a mixture of both amylose and amylopectin. Glucoamylase is efficient to cleave the α-1,4 linkage, but it reaches a branch point (α-1,6 linkage), the enzyme is slower to hydrolyze the bond and isomaltose is formed. To solve this problem, glucoamylase is blended together with pullulanase, which has ability to break down the α-1,6 linkage. The key of success is the pullulanase enzyme which has the same optimum conditions as glucoamylase (Knorr, 1987; Gerhartz, 1990; Pomeranz, 1991; Crabb and Mitchinson, 1997).

A second practical problem is that the process conditions require a high maltodextrin concentration to be economical, resulting in a high glucose concentration. Under high glucose condition, glucoamylase tends to convert the action giving maltose as a product. The solution is to balance the dosage of the enzyme, the temperature of the reaction, and the reaction time that leads to thermal inactivation of the glucoamylase at the later stage. These result in a less enzyme activity remained, leading to less reversion of glucose to maltose. One goal in saccharification is to improve the thermostability of glucoamylase, allowing the process to be run at higher temperature and dry-solid levels for shorter time periods (Crabb and Mitchinson, 1997).

3. α-Amylase

3.1 General characteristic of α-amylase

α-Amylase (EC 3.2.1.1; 1,4-D-glucan glucanohydrolase) is an endoglucosidase that hydrolyzes the α-1,4-glucosidic bond in starch, glycogen and various oligosaccharides at internal position, releasing dextrins and oligosaccharides. This enzyme has been studied extensively in various aspects: structure and function, secretion, and industrial application. The three-dimensional X-ray structures of α-amylase from various sources have been reported by several researchers (Swift *et al*., 1991; Brady *et al*., 1991; Machius *et al*., 1995; Fujimoto *et al*., 1998). These studies provide overall folding of α-amylase. Despite differences in their amino acid sequences, they have similar three-dimensional structures with three domains: domain A consisting of a central $(\beta/\alpha)_8$-barrel flanking the active site, domain B overlaying the active site from one site and domain C consisting of a β-structure with a Greek-key motif (Figure 7) (Fujimoto *et al*., 1998). The molecular weight of this enzyme is in the range of 50 kDa and requires Ca^{2+} to maintain stability and activity. The optimum pH varies depending on enzyme source and temperature (Wong, 1995). α-Amylase is obviously synthesized in a wide variety of organisms. Commercial enzymes in the industrial hydrolysis of starch are produced by *Bacillus amyloliquefaciens, B. licheniformis* and *B. subtilis.* They differ principally in their tolerance to high temperature (Knorr, 1987; Gerhartz, 1990).

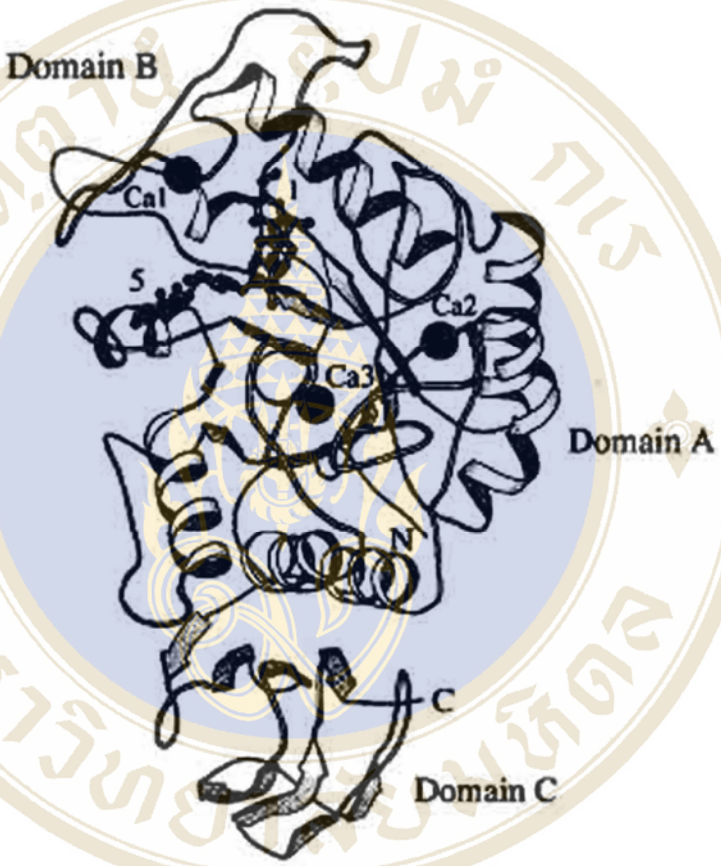


Figure 7 The ribbon model of α-amylase structure from *B. subtilis* (BSUA-EQ208/G5) generated with the program MOLSCRIPT. The G5 molecule is indicated as a ball-and-stick plot and both end residues are numbers (Kraulis, 1991)

3.2 Three-dimensional structure of α-amylase produce by *B. subtilis*

3.2.1 Overall structure

In 1998, Fujimoto *et al.* studied the crystal structure of mutant α-amylase from *B. subtilis* (BSUA-EQ208) complexed with maltopentaose (G5). The final model of BSUA-EQ208/G5 is made up of 425 amino acid residues and one G5 molecule. The total 438 amino acid residues deduced from the DNA sequence, N-terminal residues –2 and –1 and C-terminal residues 426 to 436 are excluded from the model. BSUA consists of a single polypeptide chain with approximately 26% α-helix and 22% β-sheet has dimension of approximately 35x40x70Å. The central part (domain A, Leu1 to Ile100 and Thr152 to Gly347) comprises of $(\beta/\alpha)_8$-barrel. The long active site cleft is located on the C-terminal side of the central β-barrel of domain A is overlaid by the prominent excursion part (domain B, Asn101 to Asn151) from one side. The C-terminal region (domain C, Gln384 to Asp425) comprises of eight β-strands containing a Greek key motif. All most all β-strands and α-helixes of the $(\beta/\alpha)_8$-barrel in domain A and Greek key motif in domain C are conserved for α-amylase from *B. subtilis*, *B. licheniformis*, *A. oryzae* and pig pancreatic. Domain B is the most variable region, as only β-strands at both end and one α-helix are conserved. BSUA has a shorter length of domain B, while another bacterial α-amylase form *B. licheniformis* has a unique structure of domain B in that it is exceptionally long and does not posses the conserved α-helix Bα1 (Machius *et al.*, 1995; Fujimoto *et al.*, 1998).

3.2.2 The active site and substrate binding site

The five glucose units of G5 are numbered as Glc1 to Glc5 from the non-reducing end toward the reducing end. Glc1 is positioned in a hydrophobic pocket formed by the three aromatic rings of Tyr59, Trp60 and Phr105 and two side-chains of Leu142 and Leu144. A water molecule is positioned in a pocket formed by the main-chain atoms of Gln63, Phe105 and Lue144 and mediates hydrogen bonds between NE2 of Gln63 and the oxygen atom 5 (O5) of Glc1. Oxygen atom 2 (O2) of Glc1 is involved in an internal hydrogen bond with the O3 of Glc2 of the adjacent glucose. Glc1 has no direct hydrogen bond with the protein atom in the complex, while an intermolecular hydrogen bond is observed between O2 of Glc1 and the side-chain of Lys27 of the symmetry-related protein molecule. Glc2 participates in three hydrogen bonds with three protein residues: Try59 and Gln63 are at the bottom of the cleft and Asn273 is near the molecular surface. Aromatic ring of Trp58 and Tyr59 make a hydrophobic wall on one side of Glc2 and Leu142 on the other side (Fujimoto *et al*., 1998).

Glc3 is buried deeply at the bottom of the cleft, and tightly bound by seven hydrogen bonds and a stacking interaction with Tyr62. The hydrogen bonding residues His102, Arg174 and Asp176 are at the bottom of the cleft and Asp269 is near the molecular surface. The O2 and O3 of Glc3 hydroxyl groups do not participate in the internal O2-O3’ hydrogen bonds, instead they contribute to two bifurcated hydrogen bonds between OD1 of Asp269 and NE2 of His268 and between OD2 of Asp269 and NE2 of His268 (Figure 8) (Fujimoto *et al*., 1998).

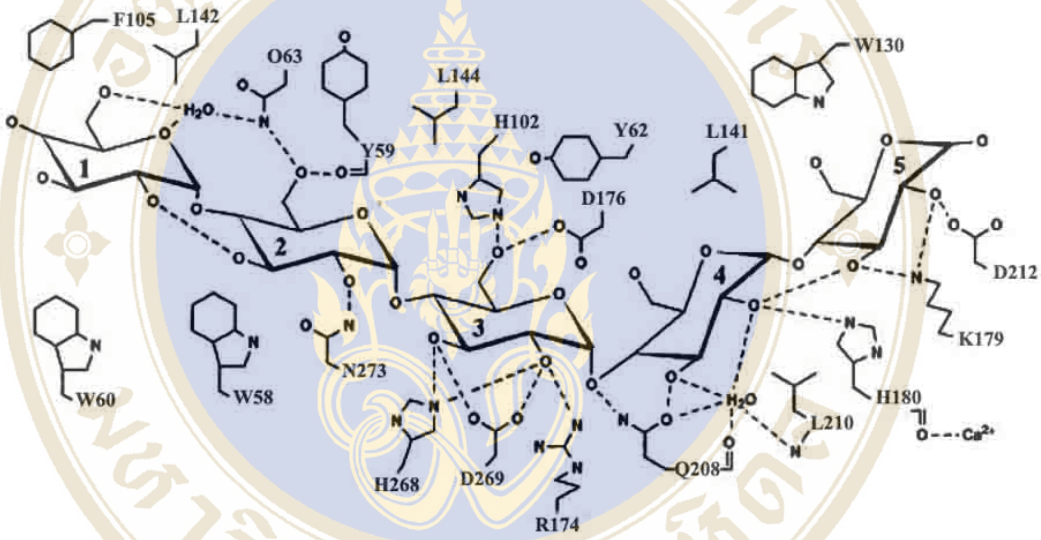


Figure 8 Schematic drawing of the hydrogen-bonding network between BSUA-EQ208 and G5. Two water molecules mediating hydrogen bonds and hydrophobic residues interacting with G5 are also indicated (Fujimoto *et al.*, 1998).

The catalytic reaction is considered to occur at the α-1,4-glucosidic bond between Glc3 and Glc4, releasing Glc4 and Glc5as a maltose molecule (Robyt and French, 1970). The three catalytic site residues Asp176, Gln208 and Asp269 form triangle around the cleavage point. The residue Gln208 forms two hydrogen bonds with oxygen atom of Glc4. A water molecule buried in the vicinity and mediates interaction between Glc4 and the protein by five hydrogen bonds. This water molecule is likely to exist as bound water for assisting stabilization of a local conformation of the protein and for binding the substrate. The O2 of Glc4 hydroxyl group forms a hydrogen bond with the side-chain of His180, which is involved in calcium binding through its carbonyl oxygen. Glc4 has hydrophobic interactions with Leu141 on one side and with Lue210 on the other side. Glc5 makes three hydrogen bonds with side-chains of Lys179 and Asp212, and a stacking interaction with Trp130 on one side. The other side of Glc5 is exposed to solvent (Fujimoto *et al*., 1998).

3.2.3 The calcium binding site

Calcium binding site is generally observed in mammalian, barley and fungal α-amylases. The structure if Ca-1 binding site is strictly conserved among α-amylases except the enzyme produced from *B. licheniformis*, where the calcium ion is not present. Two carbonyl oxygen atoms of Thr137 and His180 and three side-chain oxygen atoms of Asn101 and Asp146 in a bidentate mode are the ligands, forming a pentagon ring. These ligand residues are conserved except Thr137. In α-amylase structure produced from *B. subtilis*, one water molecule occupies one side of an apical position of the pentagon and is anchored by the hydrogen bond with Thr149 and Glu182. His180 in domain A interacts with substrate. Thus, Ca-1 could contribute to stabilization of the structure of the active site. Although the effect of calcium on the activity is not prominent in *B. subtilis* α-amylase, calcium protects the enzyme from protease degradation (Takase *et al.*, 1988).

Ca-2 is located at the N-terminal side of $(\beta/\alpha)_8$-barrel and before Aβ4. Ca-3 is an intermolecular calcium ion which is located between the loop before Aα7 and the loop before Aβ3 of the symmetry-related molecule. This Ca ion may be important for the crystal packing formation because no crystals were obtained without calcium ion (Fujimoto *et al*., 1998).

3.3 Catalytic mechanism of α-amylase

According to the active site and substrate binding site from three-dimensional structure of α-amylase from *B. subtilis*, the results suggest that Glu208 acts as a general acid (proton donor), and Asp176 and Asp269 as a general base (catalytic nucleophile). α-Amylase cleaves the α-1,4-glucosidic linkage with the configuration around the anomeric carbon atom of the polysaccharide chain, the reducing end formed upon cleavage of the substrate is released in the α-configuration. The proposed catalytic mechanism consists of three steps. Firstly, the glycosidic oxygen is protonated by the proton donor, following by a nucleophilic attack on the C1 of the sugar residue in the –1 subsite by the catalytic nucleophile. After the glycone part of the substrate leaves, a water molecule is activated, presumably by the new deprotonated of proton donor. This water molecule hydrolyzes the covalent bond between the oxygen of the nucleophile and the C1 of the sugar residue, thus complete the catalytic cycle (Figure 9). The third conserved acid in the active site, As269, plays no direct role in this catalytic mechanism, nevertheless is known to be important for catalysis. The carboxyl group of Asp269 participates in hydrogen bonds to the O2 and O3 hydroxyl groups of Glc3, showing a role as substrate binding (Fujimoto *et al.*, 1998; Nielsen and Borchert, 2000).

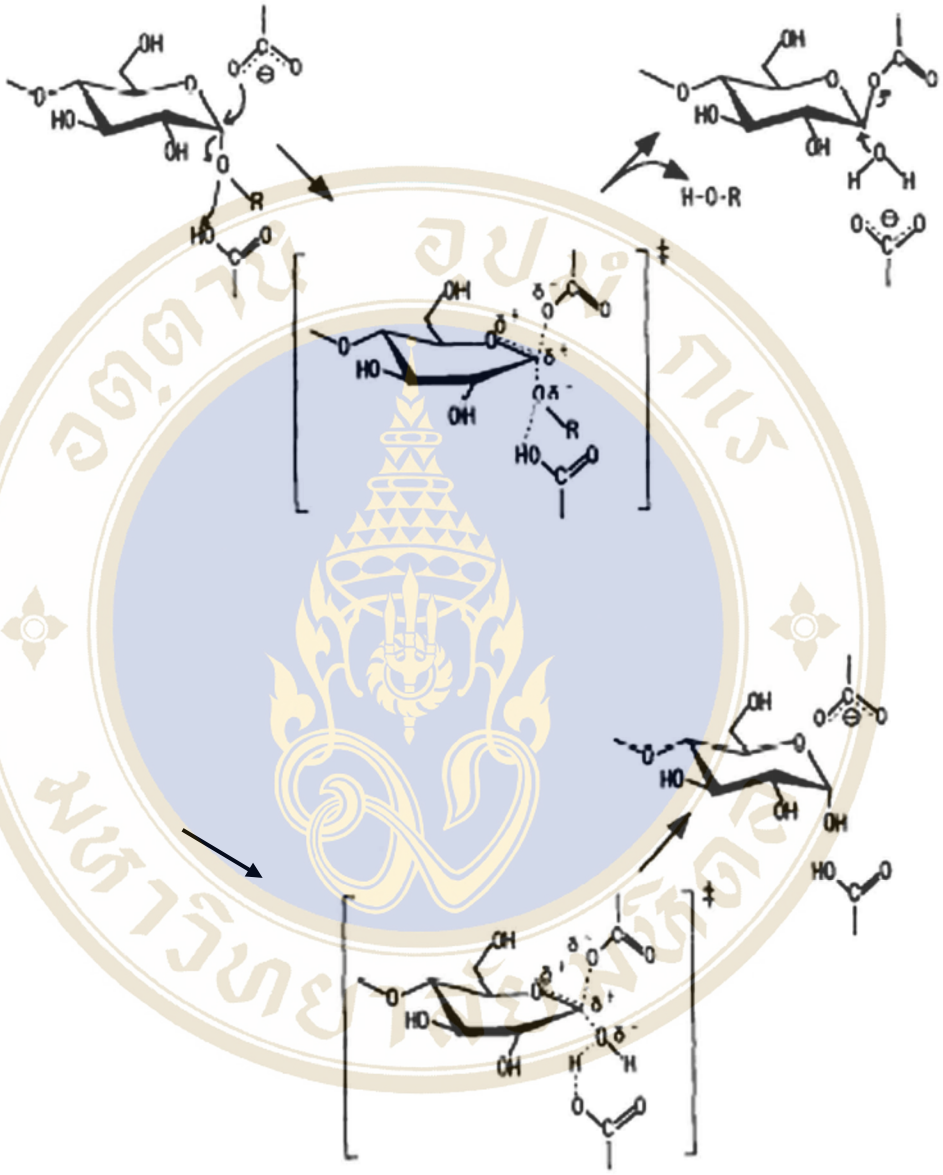


Figure 9 Catalytic step in glycosidic linkage cleavage of α-amylase. The proton donor protonates the glycosidic oxygen and the catalytic nucleophile attacks at C1 leading to the formation of the first transition state. The catalytic base promotes the attack of the incoming water molecule on the formation of the covalent intermediate resulting in a second transition state, leading to hydrolysis (Kuriki and Imanaka, 1999).

4. Glucoamylase

4.1 General characteristic of glucoamylase

Glucoamylase (EC 3.2.1.3; 1,4-α-D-glucan glucohydrolase) is an exoglucosidase that catalyzes both of α-1,4 and α-1,6-glucosidic linkages, releasing β-D-glucose from the non-reducing end of the starch and related poly- and oligosaccharide. Fungal glucoamylase is widely used in the manufacture of glucose and fructose syrup. Although activity toward the α-1,6 linkage is only 0.2% of that for the α-1,4 linkage, this suffices to adversely affect the yield in industrial saccharification. The three-dimensional structure of the catalytic domain form *A. awamori* var. *X100* has been described in detail for native and ligand-complex forms. Furthermore, preliminary structure determination was made of wild-type and mutants of *A. niger* glucoamylase which has 94% sequence identity to glucoamylase from *A. awamori* var. *X100*. The optimum pH is 4.0-4.4, with stability over a pH range of 3.5-5.5. The optimum temperature is in the range of 58 to 65°C. Glucoamylase contains 1-20% carbohydrate, mainly mannose, glucose, galactose and glucosamine (Wong, 1995).

4.2 Structure of glucoamylase

Glucoamylase is a multidomain enzyme consisting of a catalytic domain connected to a starch-binding domain by an *O*-glycosylated linker domain. The catalytic domain folds as a twisted $(\alpha/\alpha)_6$-barrel with a central funnel-shaped active site, while the starch binding domain folds as an antiparallel β-barrel and has two binding sites for starch.

4.2.1 Catalytic domain

Catalytic domain of glucoamylases from *A. awamori* var. *X100, A. niger* and *Saccharomycopsis fibuligera* share a very similar fold. It consists of 13 α-helices of which 12 α-helices form an $(\alpha/\alpha)_6$-barrel. In this fold, six outer and six inner α-helixes surrounding the funnel-shaped active site, are constituted by the six highly conserved α-α segments, that connects the N-terminal of the inner with the C-terminal of the outer helixes. The catalytic site includes the general acid and base catalysts Glu179 and Glu400 situated at the bottom of the pocket (Figure 10) (Sauer *et al.*, 2000).

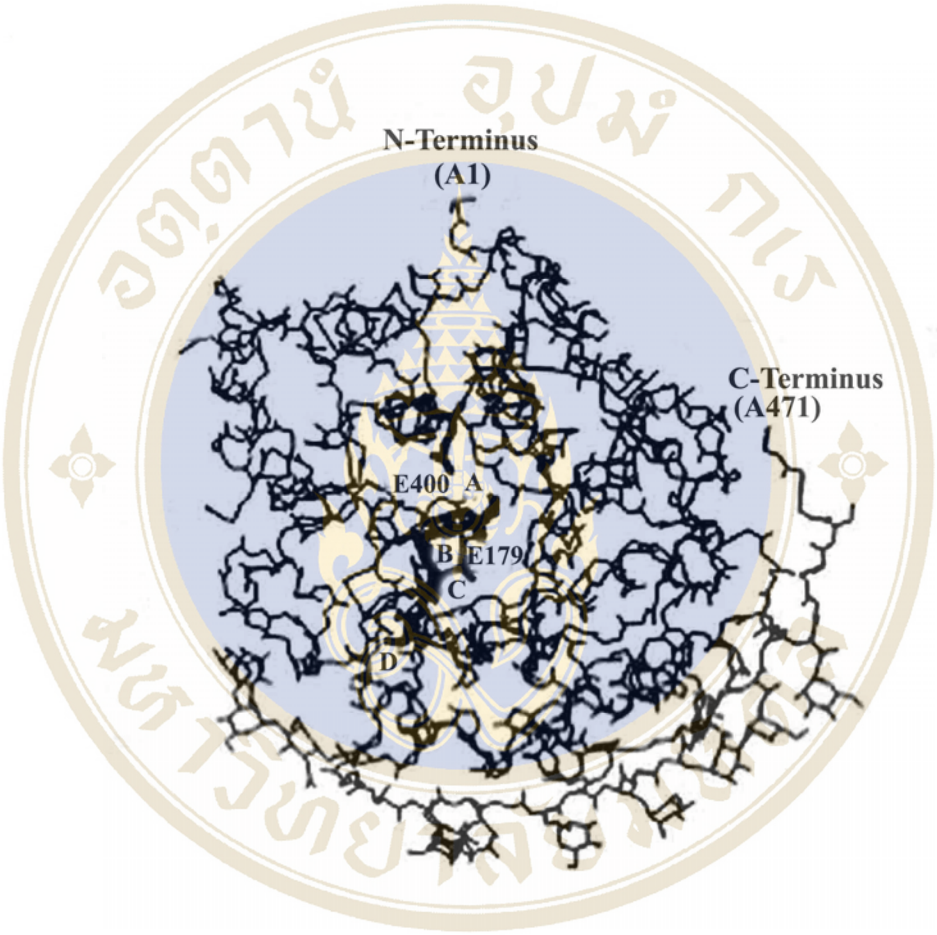


Figure 10 Structure of the catalytic domain (amino acid 1-471) of *A. awamori* var. X100 GA complexed with the pseudotetrasaccharide acarbose. The C- and N-terminal are indicated together with the side chains of the two catalytic residues E179 and E400 (Sauer *et al*., 2000).

4.2.2 Starch-binding domain (SBD)

The C-terminal starch binding domain (SBD) of *A. niger* consists of eight β-strands organized in two β-sheets forming a twisted β-barrel structure. Two starch-binding sites are located on opposite sides of the top of the domain, away from the linker attachment point as shown in Figure 11 (Sauer *et al*., 2000).

4.2.3 Linker region

The serine- and threonine-rich *O*-glycosylated region of *A. niger* glucoamylase (amino acid 440-508) contained a very highly *O*-glycosylated C-terminal segment of about 30 amino acids connects with SBD. This particular part of the linker has been attributed roles in stability, secretion and digestion of raw starch. Mass spectrometric analysis of the peptide Asn430-Phe519 shows a high degree of heterogeneity in the amount of attached sugar, approximately 63 moles of hexose are attached to the peptide. The first part (amino acid 440-471) of the *O*-glycosylated region carries about 10 exposed single mannosyl residues that together with the two *N*-glycosidically linked units at Asp171 and Asp395 form a belt of carbohydrate around the globular catalytic domain (Sauer *et al*., 2000).

4.2.4 Overall structure

Fungus glucoamylase produces two forms. The complete form requires SBD for degradation of insoluble starch. The lacking of SBD form has very low activity on insoluble starch. The complete three-dimensional structure of the intact glucoamylase comprising of catalytic domain, the linker region and SBD, is not well elucidated (Sauer *et al*., 2000).

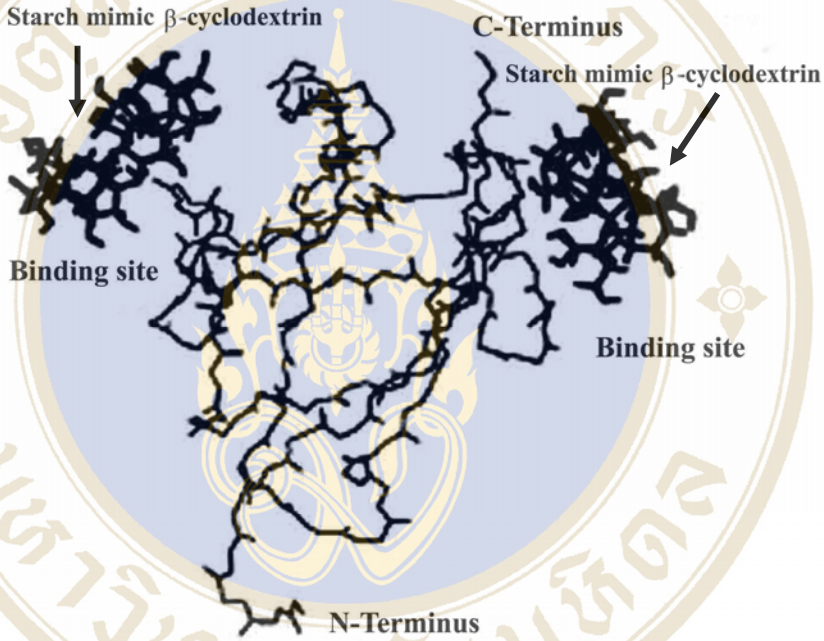


Figure 11 Structure of the starch binding domain (SBD) from *A. niger* glucoamylase complexed with the starch mimic β-cyclodextrin at the two binding sites (Sauer *et al*., 2000).

4.3 Catalytic mechanism of glucoamylase

The mechanism of hydrolysis involves proton transfer to the glycosidic oxygen of the scissile bond from a general acid catalyst; formation of an oxocarbonium ion; and a nucleophilic attack of water assisted by a general base catalyst. Glu179 and Glu400 in glucoamylase from *A. niger* have been identified as the general acid and the general base catalyst, respectively. The pH-dependencies of steady-state kinetic parameters are in accordance with a rate determining hydrolysis step involving these two catalytic residues (Figure 12).

The glucoamylase catalysis occurs with inversion of the anomeric configuration in a single displacement mechanism and the gap between the catalytic acids. In this mechanism the proposed covalent bond between substrate and protein has the consequence that high precision of the spatial positioning of the two catalytic groups in necessary for the nucleophilic attack on the glycosidic bond. For binding mechanism, conserved tryptophan residues are involved in interactions of the glucoamylase with substrate (Sauer *et al*., 2000).

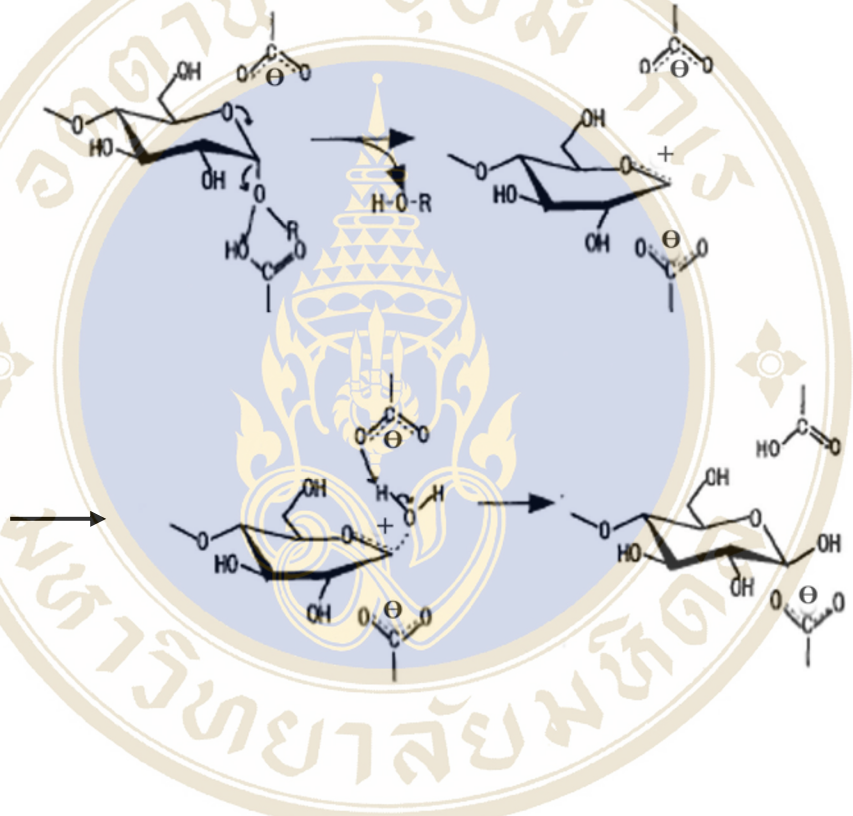


Figure 12 The catalytic mechanism of glucoamylase illustrating the action of the catalytic base Glu400 (top) and acid Glu179 (bottom) in the water-assisted hydrolysis of substrate involving inversion of the configuration of the anomeric carbon (Kuriki and Imanaka, 1999).

5. Inactivation of the Amylolytic Enzymes

In 1994, Ray *et al.* reported that the industrial utilization of enzymes often encounters the problem of thermal inactivation of the enzyme. Since an increase in temperature causes an increment in vibrating energy of the molecule and collision energy between molecules, resulting in denaturation of the protein. At high temperature, the enzymes undergo partial unfolding by heat-induced disruption of non-covalent interaction. The mechanism of thermal inactivation of β-amylase was determined by Ray *et al.* (1994). The circular dichroism (CD) spectrum of β-amylase indicated that thermal inactivation is associated with extensive denaturation and unfolding of the protein molecule. Kondo and Urabe (1995) studied the effect of temperature on the extent of activity reductions and conformational changes in α-amylases with different temperature. α-Amylases from *B. subtilis* and *B. licheniformis*, which has high thermostability, were adsorbed on ultrafine silica particles at various temperatures. Since these enzymes contain large amount of α-helix, the conformational changes can be measured by α-helix content from circular dicroism spectra. The extent of activity reduction upon adsorption of α-amylase form *B. subtilis* on ultrafine silica particles was closely correlated with that of conformational changes, and both of them were increased by raising temperature. On the other hand, the extent of activity reduction of α-amylase from *B. licheniformis* was much smaller than α-amylase from *B. subtilis*. Both activities of α-amylases were significantly increased by raising temperature from 4 to 40^{o}C. Suzuki *et al*. (1989) studied the effect of amino acid residues on the thermostability of α-amylase. It is concluded that the alteration of amino acid sequence enhanced the thermostability. The deletion of Arg^{176} leads to very low activity. The decrease in positively charged residues resulted in the enhancement of thermostability. The effect of operating conditions on enzyme hydrolysis of wheat starch by commercial α-amylase was studied by Ozbek and Yuceer (2001). They reported that the inactivation mechanisms of α-amylase are different for each set of process conditions such as enzyme concentration, viscosity, the amount of hydrolysate and processing time. Fitter *et al*. (2001) found that the folding state shows higher structural flexibility for the thermophilic protein as compared to the mesophilic homologue. The inactivation of

α-amylase by temperature in stirred batch reactor was studied by Apar and Ozbek (2004). They concluded that both temperature and processing time are involved in the inactivation of α-amylase after the starch hydrolysis process. The inactivation mechanisms are different and specific to the enzyme depending on the sources and hydrolysis conditions of the enzyme.

It is known that the glycation of enzymes leads to the alteration of enzyme activity and kinetic parameters. Most of the research has been performed relevantly to the enzyme glycation occurring in biological systems under physiological conditions. However, there are no data concerning on the glycation of amylolytic enzymes in starch hydrolysis. These enzymes are applied under high temperature and high reducing sugar conditions in the industry. This research is the first work to investigate the effect of glycation in such enzymes on the alteration of their properties.

CHAPTER IV
MATERIALS AND METHODS

Materials

1. Enzymes

1.1 Thermostable α-amylase (KLEISTASE T10S; Daiwa Kasei K.K., Japan; KLE), produced by *Bacillus subtilis*, was a gift from Cinnamon Company, Ltd. (Thailand). The optimum temperature was in the range of 95 to 105°C.

1.2 α-Amylase (BAN® 480L, Novozymes, Denmark; BAN), produced by *B. amyloliquefaciens*, was a gift from The East Asiatic (Thailand) Public Company Limited. The optimum temperature was in the range of 70 to 90°C.

1.3 Glucoamylase (DAIZYME; Daiwa Kasei K.K., Japan), produced by *Aspergillus niger*, was obtained from Cinnamon Company, Ltd. (Thailand). The optimum temperature was 60°C.

2. Chemicals

All chemicals were of the best grade available supplied from Merck (Darmstadt, Germany) unless otherwise stated.

2.1 Food grade maltodextrin (MORREX, D.E. = 15-19) (Stamford Food Industries, Malaysia).

2.2 Cornstarch (CALBIOCHEM, Darmstadt, Germany).

2.3 5-(Hydroxymethyl)-2-furfuraldehyde (HMF) (Fluka, Buchs, Switzerland).

2.4 D-glucose (BDH, England).

2.5 Bovine serum albumin (Fraction V) (Sigma, MO, USA).

2.6 HPLC grade acetonitrile (Lab Scan, Thailand).

Methods

1. Purification of α-amylase and glucoamylase

The purity of enzymes was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). KLE and BAN showed the single protein band on SDS-PAGE. These two enzymes were dialyzed against 20 mM phosphate buffer solution (PBS, pH 6.5) to get rid of low molecular weight compound and salt which might be used as the enzyme stabilizer. The dialyzed KLE and BAN were used in the further experiment. Glucoamylase showed three protein bands on SDS-PAGE. It was necessary to perform the purification prior to using this enzyme in the further experiment.

Glucoamylase was purified by a modified method of Odibo and Ulbrich-Hofmann (2001). One milliliter of the crude enzyme containing 74.91 mg of protein was applied to a Q-sepharose Fast Flow column (1.6 cm x 40 cm, Amersham Pharmacia Biotech, Ltd., UK) previously equilibrated with 20 mM acetate buffer (pH 4.5). Elution was performed with a 400 ml linear gradient (0.3 to 0.5 M) of NaCl at a flow rate of 1 ml/min. Three milliliters of each fraction were collected using a fraction collector (Model 2110; Bio-Rad; CA, USA). The enzyme activity, optical density (OD) at 280 nm and total soluble protein content were determined. The active fractions were pooled and concentrated by ultrafiltration (VEVACELL 250; Vivascience, Germany).

The partially purified enzyme was further purified by a Phenyl-Sepharose CL-4B column (2 cm x 40 cm, Sigma; MO, USA). Before loading the enzyme on the column, ammonium sulfate was added to final concentration of 1.0 M. After equilibrated the column with 20 mM acetate buffer (pH 4.5, containing 1.0 M ammonium sulfate), the enzyme concentrated was applied to the column. The protein was then eluted with a 250 ml linear gradient from 1 to 0 M ammonium sulfate in the same buffer. Three milliliters of each fraction were collected, and the enzyme activity, OD_{280} and total soluble protein content were then determined. The active fractions were pooled and concentrated by VEVACELL 250 (Vivascience, Germany). The purity of the enzyme was assessed by SDS-PAGE.

2. Glycation between amylolytic enzymes and reducing sugar

Maltodextrin, the final products of liquefaction process, was used to conduct the glycation. Reaction mixtures (20 ml) were prepared to contain KLE (0.6 mg protein/ml) and maltodextrin or glucose (350 mg/ml) in 20 mM PBS, pH 6.5. The concentration of maltodextrin based on the initial concentration of starch used in the liquefaction process. The reaction mixtures were incubated in a shaking water bath at 150 rpm, 95^{o}C. The samples were withdrawn at specific time intervals (*i.e.* 0, 30, 60, 120, and 180 min) and rapidly cooled in an ice-bath to terminate the enzyme activity. After that, the reaction mixtures were centrifuged (4500x*g*, 45 min, 4^{o}C) to remove protein aggregate. Total soluble protein, reducing power and degree of glycation were then determined. The remaining samples were dialyzed against 20 mM PBS (pH 6.5) at 4^{o}C to exclude sugars. The dialyzed samples were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native gel electrophoresis (Native-PAGE). After that, the dialyzed samples were concentrated by ultrafiltration (Ultrafree-15; Millipore, USA), and determination for pI value. Glucose, the more active glycating sugar comparing to maltodextrin, was also used to conduct the glycation with KLE by the same procedure.

The reactions between BAN and maltodextrin were prepared following the method described above. The incubation temperature was in the range of optimum temperature (70 and 90^{o}C), and samples were withdrawn at 0, 6, 12, 24, 48, 72 h and 0, 1, 2, 3, 4 h, respectively. Total soluble protein, reducing power and degree of glycation were then determined. The remaining samples were dialyzed against 20 mM PBS (pH 6.5) at 4^{o}C to exclude sugars. The dialyzed samples were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native gel electrophoresis (Native-PAGE).

In case of purified glucoamylase (PG), maltodextrin which is the substrate in saccharification process was used to conduct the glycation. Reaction mixtures (15 ml) were prepared to contain glucoamylase (0.1 mg protein/ml) and maltodextrin (350 mg/ml) in 20 mM acetate buffer (pH 4.5) under aseptic conditions (to prevent the thermophilic microorganism growth). The reaction mixtures were incubated in a shaking water bath at 150 rpm, 60^{o}C. The sample was withdrawn at specific time intervals (*i.e.* 0, 12, 24, 48, and 72 h). The enzyme activity was terminated by rapidly cooled to 4 ^{o}C. The sample aliquot (250 µl) was supplemented with 4% perchloric acid (250 µl) and kept at -20^{o}C for determination of reducing power and glucose content. The remaining samples were centrifuged (4500x*g*, 45 min, 4^{o}C) to exclude aggregate prior to protein assay. The portions were dialyzed against 20 mM acetate buffer (pH 4.5, 4^{o}C) to exclude the maltodextrin. Then, it was further concentrated by ultrafiltration (Ultrafree-15; Millipore, USA), prior to the measurement of the degree of glycation and pI value including the protein SDS-PAGE and Native-PAGE. The sugar attached on enzyme molecule on SDS-PAGE was stained by toluidine blue.

3. Relationship between total sugar and HMF released from glycated bovine serum albumin (BSA)

Reaction mixtures were prepared to contain BSA (10 mg protein/ml) and maltodextrin (18 mg/ml) in 20 mM PBS (pH 6.5), under aseptic condition. After that, the reaction mixtures were incubated in an incubator at 50^{o}C. The samples were withdrawn at specific time intervals (*i.e.* 0, 0.5, 1, 2, 3, 5 and 8 days). Total soluble protein, degree of glycation and total sugar content were determined.

4. Preparation of glycated amylolytic enzymes

The glycated amylolytic enzymes were prepared in order to the studied the effect of glycation on enzyme stabilities and kinetic parameters comparing to the intact enzymes. The glycated KLE (gKLE) was prepared by incubating the reaction mixture (100 ml) of the enzyme (0.6 mg protein/ml) with 350 mg/ml maltodextrin solution (20 mM PBS, pH 6.5) at 95^{o}C, 120 min. The reaction mixture was then rapidly cooled in an ice-bath and centrifuged at 4^{o}C (4500x*g*, 45 min). After the supernatant was dialyzed against 20 mM PBS (pH 6.5), the solution was further concentrated by ultrafiltration (Ultrafree-15; Millipore, USA). The glycated BAN (gBAN) was prepared by the same method as gKLE except that the incubation temperature and duration were set at 70 and 90^{o}C for 48 h and 2 h, respectively.

In case of glycated PG (gPG), One-hundred milliliter of reaction mixture was prepared by mixing the purified enzyme (0.1 mg protein/ml) with 350 mg/ml maltodextrin solution (20 mM acetate buffer, pH 4.5) and incubated at 60^{o}C for 24 h. The reaction mixture was then centrifuged at 4^{o}C (4500x*g*, 45 min) to remove protein precipitate. After the supernatant was dialyzed against 20 mM acetate buffer (pH 4.5), the solution was further concentrated by ultrafiltration (Ultrafree-15; Millipore, USA). This glycated enzyme (gPG) was used to study the enzyme stability and kinetic parameters comparing with the purified glucoamylase (PG).

5. Stability study of glycated amylolytic enzymes

5.1 Thermal stability study of glycated amylolytic enzymes

Thermal stability of gKLE was studied by incubating two milliliters of gKLE (0.6 mg protein/ml) in 20 mM PBS (pH 6.5) at 95^{o}C. Samples were taken at specific time intervals (*i.e.* 0, 5, 10, 20, and 30 min) and assayed for total soluble protein and enzyme activity. KLE was used as a control experiment. In case of gBAN, the solution contained gBAN (glycated at 70 and 90^{o}C) and BAN (control experiment) were incubated in 20 mM PBS (pH 6.5) at 70 and 90^{o}C, respectively. Samples were withdrawn at 0, 5, 10, 20 and 30 min, total soluble protein and enzyme activity were then determined.

One milliliter of gPG (0.1 mg protein/ml) was incubated in 20 mM acetate buffer (pH 4.5) at 60°C. Samples were taken at specific time intervals (*i.e.* 0, 2, 4, and 6 h) and assayed for total soluble protein and enzyme activity. PG was used as a control experiment. The glucoamylase glycated with glucose was prepared as described above to compare the heat stability.

5.2 pH stability study of glycated amylolytic enzymes

Two milliliters of the enzyme (KLE and gKLE, 0.6 mg protein/ml) were incubated in 20 mM of various buffer solution, acetate buffer (pH 4.5), PBS (pH 5.5-7.5) and tris-HCl buffer (pH 8.5) at 95°C for 5 min. Total soluble protein and enzyme activity were then determined. The pH stability study of glycated BAN was performed by the same procedure as pH stability study of glycated KLE. The temperatures were set at 70 and 90°C for BAN glycated at 70 and 90°C, respectively. Total soluble protein and enzyme activity were then determined comparing with the intact form (BAN).

In case of gPG, one milliliter of the enzyme (gPG and PG, 0.1 mg protein/ml) was incubated in 20 mM acetate buffer (pH 3.5, 4.5, 5.5) and 20 mM phosphate buffer (pH 6.5) at 60°C for 1 h. Total soluble protein and enzyme activity were then determined.

6. Effect of glycation on enzyme kinetic parameters

The kinetic parameters of gKLE were determined at optimum conditions (pH 6.5, 95°C). The reaction was conducted by mixing a fixed activity of enzyme (8.50 U/ml working volume) with different cornstarch concentrations (5 to 40 mg/ml). The mixture was withdrawn at specific time intervals to analyze reducing power. The kinetic parameters were determined by fitting the initial rate data to the Michaelis-Menten equation by non-linear regression using the SigmaPlot software (SPSS Inc., USA). The values of K_m and maximal velocity (V_{max}) were calculated based on Lineweaver-Burk plot comparing with the intact form.

The kinetic parameters of both BAN glycated by maltodextrin at 70 and 90°C were determined at 70°C (in 20 mM PBS, pH 6.5). The reaction was performed by mixing a fixed activity of enzyme (26.23 U/ml substrate solution) with different cornstarch concentrations (10 to 60 mg/ml). The mixture was withdrawn at specific time intervals to analyze reducing power. The kinetic parameters were determined by fitting the initial rate data to the Michaelis-Menten equation by non-linear regression using the SigmaPlot software (SPSS Inc., USA). The values of K_m and maximal velocity (V_{max}) were calculated based on Lineweaver-Burk plot.

In case of gPG the kinetic parameters were also determined at optimum conditions (pH 4.5, 60°C). The reaction was conducted by mixing a fixed activity of enzyme (0.73 U/ml substrate solution) with different substrate concentrations (maltose and maltodextrin). The mixture was withdrawn at specific time intervals to analyze glucose concentration by HPLC. The kinetic parameters were determined by fitting the initial rate data to the Michaelis-Menten equation by non-linear regression using the SigmaPlot software (SPSS Inc., USA). The values of K_m and maximal velocity (V_{max}) were calculated based on Lineweaver-Burk plot.

7. Hydrolysis of cornstarch by thermostable α-amylase (KLE) in 4-L scale

Since the glycation of KLE had been done before using maltodextrin as the glycating sugar. However, the glycation might occur during the hydrolysis process of cornstarch in liquefaction process. Hence, the hydrolysis of cornstarch by KLE was performed in order to monitor the progress of the glycation under the liquefaction process conditions. The reaction mixtures (4 L) contained α-amylase (0.09 mg protein/ml) in 35% cornstarch in 20 mM PBS (pH 6.5) were incubated in a water bath (95°C) and stirred with a stirrer (Model RW20.n; Kika Labortechnik, Malaysia), 250 rpm. Samples (50 ml) were withdrawn at specific time intervals (0, 30, 60, 90, 120 and 180 min) and 1 ml of sample aliquot was added with 4% perchloric acid (1 ml) to determine the reducing power. The enzyme activity in the remaining samples was terminated by rapidly cooled to 4°C. After that, the samples were centrifuged (4500x*g*, 4°C, 45 min) and dialyzed against 20 mM PBS (pH 6.5, 4°C). Enzyme activity and degree of glycation were determined.

8. Analytical procedure

8.1 α-Amylase activity

The enzyme activity was measured by adding 100 µl of appropriately diluted enzyme solution in 1.9 ml of 1% pregelatinized cornstarch in 20 mM PBS pH 6.5. The substrate and enzyme solution were preincubated at 60°C for 5 min before mixed together. The reaction was carried out at 60°C for 10 min, and was stopped by adding 2 ml of 3,5-Dinitrosalicylic acid solution (DNS). DNS solution was prepared by dissolving 1 g of DNS and 300 g of sodium potassium tartrate in 200 ml of 2 M NaOH and diluting to 1000 ml with distilled water (DW). The reaction mixture was heated in the boiling water bath for 10 min and rapidly cooled in ice-bath. OD570 were then monitored to determine the reducing power released from the enzyme action. α-Amylase activity was defined as the amount of enzyme necessary to produce 1 µmol of glucose per min at 60°C (Swamy and Seenayya, 1996).

8.2 Glucoamylase activity

The enzyme activity was measured by adding 200 µl of appropriately diluted enzyme solution in 600 µl of 20 mg/ml maltose in 20 mM acetate buffer pH 4.5. The substrate and enzyme solution were preincubated at 60°C for 5 min before mixed together. The reaction was carried out at 60°C for 30 min, and was stopped by adding 800 µl of 4% perchloric acid. Glucose concentration was determined by the modified HPLC conditions of Shaw *et al.* (2003). An HPLC column (MetaCarb 87H column, 300 mm x 7.8 mm, MetaChem Technologies Inc., U.S.A.) was equipped in Shimazu 6A HPLC set (Shimazu, Japan). The column temperature was 40°C. The mobile phase was 0.048 N H_2SO_4 in deionized water with the flow rate of 0.6 ml/min. Refractive index change was measured by refractoMonitor @ IV (LDC Analytical, USA). One unit of glucoamylase activity was defined as the amount of enzyme necessary to produce 1 µmol of glucose per min at 60°C.

8.3 Degree of glycation

The extension of the glycation was assayed based on 5-(hydroxymethyl)-2-furfuraldehyde (HMF) released from acid hydrolysis of glycated protein (Morales and Jimenez-Perez, 1998). The aggregate protein was washed with DW (3 times) and freeze-dried prior to the addition of 0.3 M oxalic acid solution (500 µl). The mixture was then hydrolyzed in boiling water bath for 2 h. After neutralized with 1 N NaOH, the solution was filtered through 0.45 µm cellulose acetate membrane filter. To determine HMF released, the 50-µl portion of neutralized sample was submitted onto HPLC equipped with Synergi 4u Fusion-RP 80 column (4.60 mm x 250 mm, Phenomenex, USA). The column temperature was 40^{o}C. The mobile phase was 5% acetonitrile in 0.2% phosphoric acid with the flow rate of 1 ml/min. Absorbance was monitored at 280 nm (Lertsiri *et al*., 2001). To assess the HMF released from soluble protein, the protein was firstly precipitated by trichloroacetic acid solution (5% final concentration) prior to hydrolysis with oxalic acid solution.

8.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protocols for SDS-PAGE were described by Laemmli (1970). Briefly, a vertical slab gel apparatus (Atto, Japan) was used with 10% separating and 4% stacking polyacrylamide gel. The samples (200 µl) were dissolved in loading buffer (containing 2 µl of 2-mercaptoethanol, 20 µl of 10% SDS solution and 100 µl of 0.5 M Tris buffer pH 6.8) and heated in a boiling water-bath for 2 min before loading into each well. Electrophoretic separation was carried out with a constant voltage of 50 V for 1 h in the beginning and following with 100 V until the Bromophenol Blue tracking dye reached the bottom of the separating gel. The gel was stained with Coomessie Brilliant Blue R-250 by transferring the gel into Coomessie Brilliant Blue solution for 1 h. The staining solution was discarded and about 100 ml of destaining solution was added and the process was repeated until appeared clear background. For estimation of relative molecular weight, prestained SDS-PAGE standard broad range (Bio-Rad; CA, USA) was run along with the protein sample. The enzyme molecular weight (MW) was calculated base on a standard calibration curve of MW marker between log MW and relative mobility (Rf). The detection of sugar on protein

molecule was performed by immersing the gel in toluidine blue solution (2 g/L in 0.1 M acetic acid) for 20 min (Carney, 1994). The dye was removed and the gel was destained in 3% acetic acid for 90 min with several changes of reagent. After that, the gel was washed with distilled water until clear. The protein band appears as purple bands against a faint blue background.

8.5 Native-Polyacrylamide Gel Electrophoresis (Native-PAGE)

Native-PAGE was performed following the protocols described in 8.4 with the absence of sodium dodesyl sulfate and no 2-mercaptoethanol reduction. A vertical slab gel apparatus (Atto, Japan) was used with 10% separating and 4% stacking polyacrylamide gel. The samples (200 µl) were dissolved in loading buffer (using 22 µl instead of 2 µl of 2-mercaptoethanol, 20 µl of 10% SDS solution and 100 µl of 0.5 M Tris buffer pH 6.8, without SDS addition) and heated in a boiling water-bath for 2 min before loading into each well. Electrophoretic separation was carried out with a constant voltage of 50 V for 1 h in the beginning and following with 100 V until the Bromophenol Blue tracking dye reached the bottom of the separating gel. The gel was stained with Coomessie Brilliant Blue R-250 as describe in 8.4.

8.6 Isoelectric Focusing Gel Electrophoresis

Isoelectric focusing was done on the Phastsystem assembly using PhastGel IEF in the pH range 3-9 (Amersham Pharmacia Biotech, UK). The procedures were followed the method according to the manufacture's instruction.

8.7 Determination of sugar by HPLC

The sample was deproteinized by 4% perchloric acid and centrifuged at 10,000 rpm (Model 202M; Sigma, Germany) for 10 min. The supernatant was passed thought 0.45 µ cellulose acetate membrane prior to inject onto HPLC. Sugar concentration was determined by the modified HPLC conditions of Shaw *et al*. (2003). An HPLC column (MetaCarb 87H column, 300 mm x 7.8 mm, MetaChem Technologies Inc., U.S.A.) was equipped in Shimazu 6A HPLC set (Shimazu, Japan). The column temperature was 40^{o}C. The mobile phase was 0.048 N H_2SO_4 in deionized water with the flow rate of 0.6 ml/min. Refractive index change was measured by refractoMonitor @ IV (LDC Analytical, USA).

8.8 Determination of reducing power by dinitrosalicylic acid assay

Reducing power was measured following the method of Benfeld (1955). The 3,5 dinitrosalicylic acid (DNS) was prepared by dissolving 1 g of DNS and 300 g of sodium potassium tartrate in 200 ml of 2 M NaOH and diluting to 1000 ml with distilled water (DW). Sample solution (200µl), appropriately diluted with DW, was added with 2 ml DNS solution. The reaction mixture was heated at 100^{o}C for 10 min, then rapidly cooled to room temperature and determined the reducing power at optical density of 570 nm. The reducing power was calculated based on the standard curve of D-glucose.

8.9 Total sugar by phenol-sulfuric acid method

The protein was firstly precipitated by trichloroacetic acid solution (5% final concentration) and centrifuged at 10,000 rpm (Model 202M; Sigma, Germany) for 10 min. The precipitate protein was washed with 5% trichloroacetic acid solution (3 times) prior to addition with 400 µl of DW. Total sugar was determined following the method of Dubois and coworker (1956). The mixture was added with 400 µl of 5% phenol reagent (5 g phenol in 100 ml DW) and rapidly added with 2 ml of concentrated sulfuric acid (38% H_2SO_4) by directly to the solution surface without allowing it to touch the side of the tube. The solution was left at room temperature for 10 min, then shaken vigorously and determined the absorbance at 490 nm after 30 min. Glucose was used as standard.

8.10 Total soluble protein concentration

Total soluble protein concentration was assessed following the Bradford's method (Bradford, 1976). The dye reagent was prepared by dissolving 0.1 g of Coomassie Brilliant blue G-250 in 50 ml of 95% of ethanol. The dye solution was added with 100 ml of H_3PO_4 before diluted to 1000 ml with DW. A glass tube containing 200 µl of appropriately diluted sample was added with 2 ml of dye reagent. The reaction mixture was left at room temperature (20 min) for color development. The developed color was then determined at optical density 595 nm. The amount of protein was calculated based on a standard curved of bovine serum albumin solution.

9. Data analysis

Data were analyzed using SPSS software, San Rafael, CA. Analysis of Variance (ANOVA), Tukey, and paired t-test were used to describe the significance of the effect of incubation time, glycation, enzyme activity and chemical changes. Each value was an average from three separate experiments.

CHAPTER V
RESULTS

1. Purification of commercial glucoamylase from *Aspergillus niger*

The crude glucoamylase showed three protein bands on a SDS-PAGE (Figure 13). Purification of crude glucoamylase was carried out by a Q-sepharose Fast Flow column and a Phenyl-Sepharose CL-4B column in succession. In the Q-sepharose Fast Flow anion-exchange step, two peaks of enzyme activity were observed in a chromatogram (Figure 14). Figure 15 showed the protein band on SDS-PAGE after the enzyme was applied onto the Q-sepharose Fast Flow column. The fractions collected from the first peak in the chromatogram (fraction number 11 to 35) showed the single band on SDS-PAGE. These active fractions were pooled, concentrated and further purified by a hydrophobic interaction, the Phenyl-Sepharose CL-4B column. The active fractions showed a single peak after passed through a column (Figure 16). The purity of glucoamylase was assessed by a SDS-PAGE. The pooled fractions from Phenyl-Sepharose CL-4B column showed a single protein band on SDS-PAGE with a molecular weight of 77.6 kDa (Figure 14). Table 1 shows the purification recovery of glucoamylase yielding about 12.28% with the purification fold of 1.1.

Figure 13 SDS-PAGE (10% gel) analysis of purified glucoamylase produced by *A. niger*. Lane 1, MW marker; Lane 2, crude enzyme (6 μg); Lane 3, crude enzyme after dialysis (6 μg); Lane 4, partially purified glucoamylase after the crude enzyme was applied onto Q-sepharose Fast Flow column (3 μg); Lane 5, purified glucoamylase after the partially purified enzyme was applied onto Phenyl-Sepharose CL-4B column (3 μg).

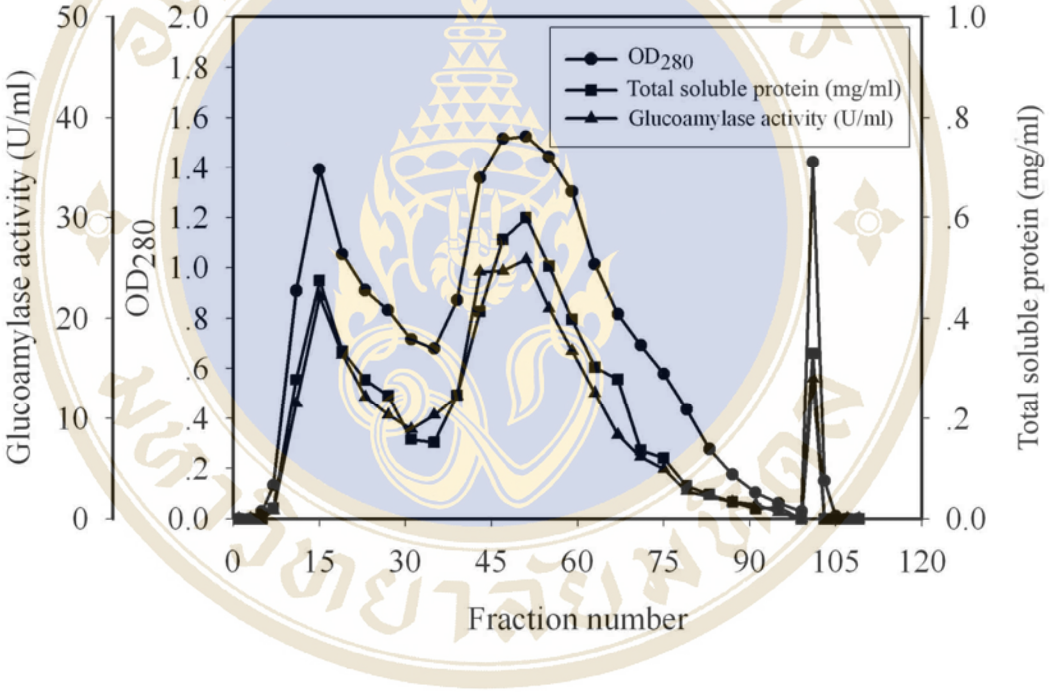


Figure 14 Q-sepharose Fast Flow column chromatography of glucoamylase. The column was equilibrated with 20 mM acetate buffer (pH 4.5). Elution was done by linear gradient of 0.3-0.5 M NaCl at a flow rate of 1 ml/min.

Figure 15 SDS-PAGE (10% gel) analysis of crude glucoamylase after the enzyme was passed through Q-sepharose Fast Flow column. Lane 1, MW marker; Lane 2-10, fraction number 11, 15, 23, 35, 39, 43, 51, 63, 67 and 101, respectively.

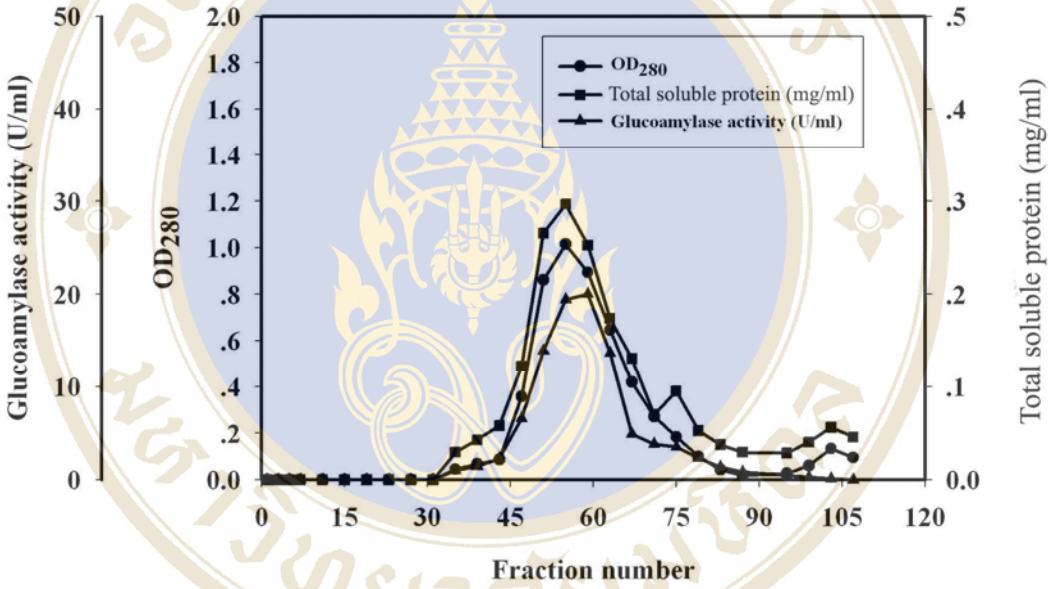


Figure 16 Phenyl-Sepharose CL-4B column chromatography of glucoamylase. The column was equilibrated with with 20 mM acetate buffer (pH 4.5) containing 1.0 M ammonium sulfate. Elution was done by linear gradient of 1 to 0 M ammonium sulfate at a flow rate of 1 ml/min.

Table 1 Summary of the purification recovery of glucoamylase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (activity) (%)	Purification fold
1. Crude enzyme	1111.74	63260	56.90	100	1
2. Q Sepharose	161.25	10805	67.01	17.08	1.18
3. Phenyl-sepharose	117.16	7771	66.33	12.28	1.17

2. Reaction between protein and reducing sugar

2.1 Total soluble protein and reducing power during incubation of amylolytic enzyme with reducing sugar

2.1.1 KLE incubated with maltodextrin or glucose

Maltodextrin or glucose solution (350 mg/ml) was incubated with KLE (0.6 mg/ml) at 95^{o}C for 180 min. Total soluble protein and reducing power were shown in Figures 17a and 17b. In case of maltodextrin addition, the soluble protein was found to gradually aggregate during incubation (0.51 to 0.42 mg/ml). On the other hand, the rapid aggregation was observed in control experiments (KLE only). However, soluble protein in control experiments slightly increased after 30 min of incubation. In case of glucose addition, soluble protein rapidly aggregated. Nevertheless, the soluble protein (KLE+glucose) was higher than the control experiment, $p<0.05$.

The reducing power was increased rapidly during 60 min and becoming constant after prolonged incubation of maltodextrin and KLE (Figure 17b). In case of glucose addition, the reducing power was constant (330-350 mg glucose/ml).

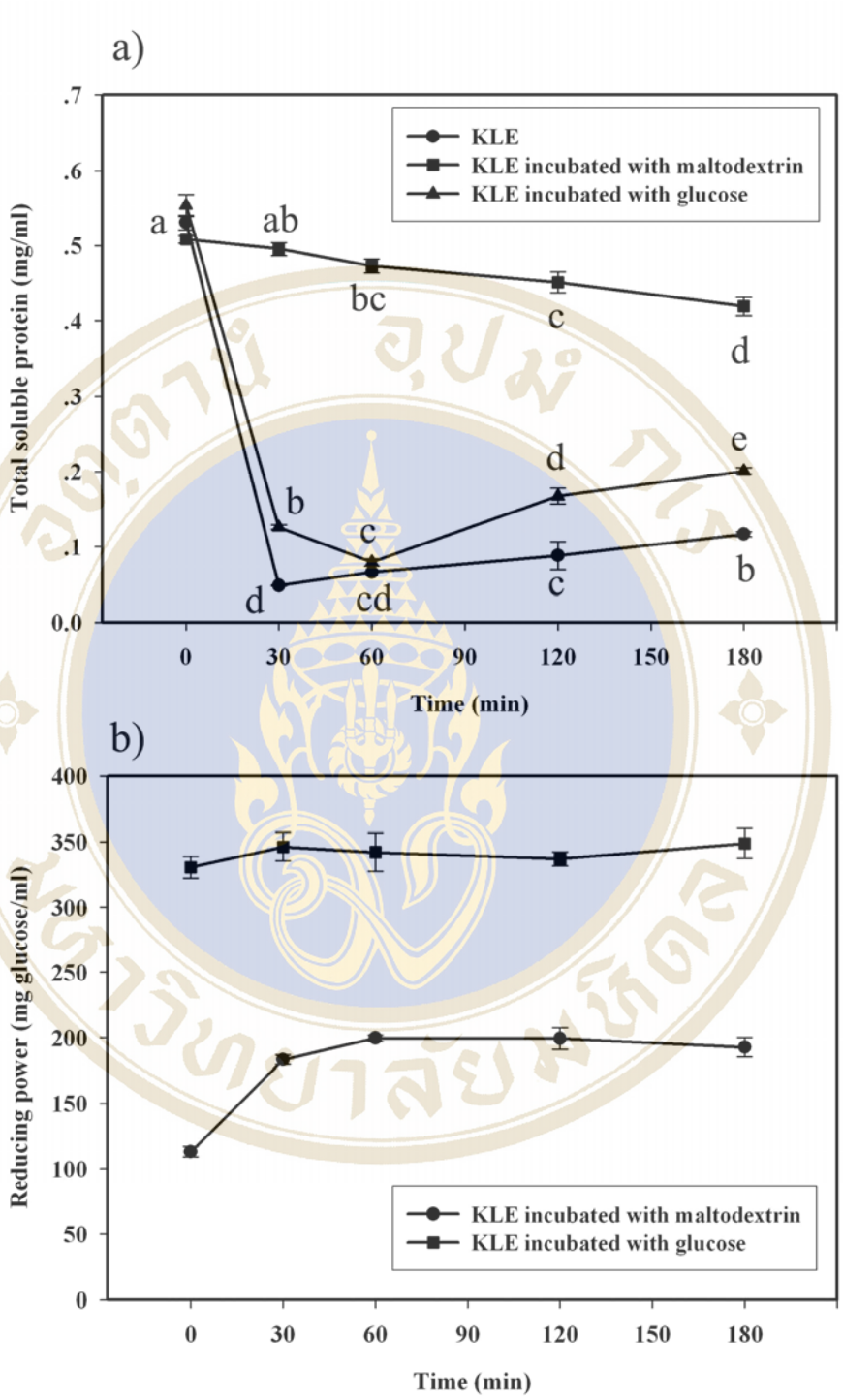


Figure 17 a) Total soluble protein; and b) Reducing power, changed during incubation of KLE (0.6 mg/ml) with maltodextrin or glucose (350 mg/ml) at 95^{o}C. Different small letters show the significantly difference ($p<0.05$) between time interval of incubation in each experiment.

2.1.2 BAN incubated with maltodextrin

Reaction mixture of BAN (0.6 mg protein/ml) and maltodextrin (350 mg/ml) was prepared and incubated at 70 and 90^{o}C for 72 and 4 h, respectively. Incubation of BAN without maltodextrin addition was used as the control experiment. Total soluble protein and reducing power were illustrated in Figure 18. The soluble protein gradually decreased (0.58 to 0.23 mg/ml) during incubation at 70^{o}C with maltodextrin addition. The rapid aggregation was observed in control experiments (BAN only) during 6 h (0.59 to 0.32 mg/ml) and becoming constant after prolonged incubation (Figure 18a). These results were similar to those at 90^{o}C (Figure 18b). The soluble protein was rapidly aggregated in control experiment within 2 h incubation period while the gradually decreased was observed with maltodextrin addition. The reducing power of maltodextrin was increased rapidly during 6 h incubation and slightly increased after prolonged incubation at 70^{o}C. In case of incubation at 90^{o}C, the reducing power slightly increased within 2 h incubation period. The reducing power reached the maxima at 260 and 192 mg/ml for 70 and 90^{o}C, respectively.

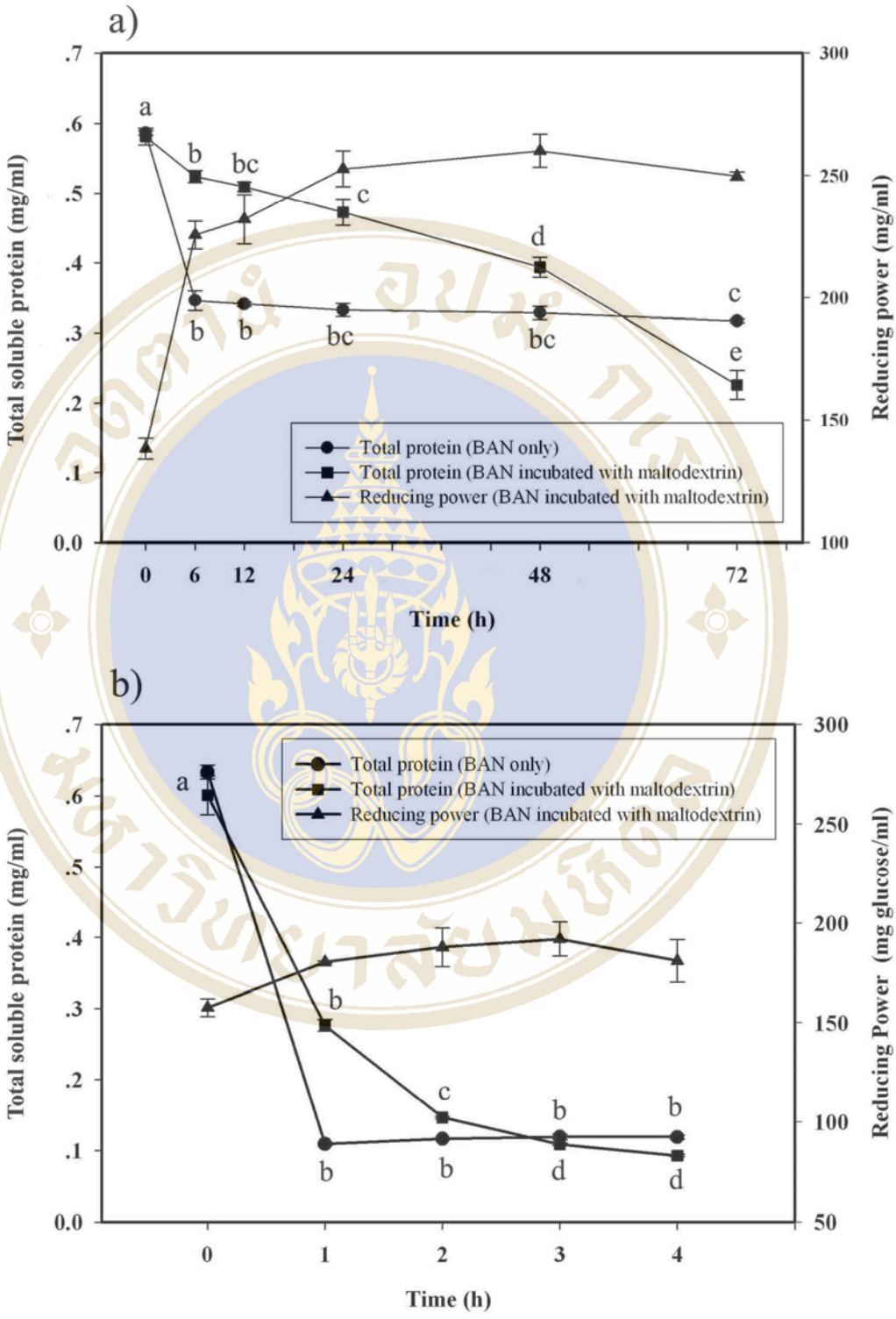


Figure 18 Total soluble protein and reducing power during incubation BAN (0.6 mg/ml) with maltodextrin (350 mg/ml), a) at 70°C; and b) at 90°C. Different small letters show the significantly difference ($p<0.05$) between time interval of incubation in each experiment.

2.1.3 PG incubated with maltodextrin

Maltodextrin solution (350 mg/ml) was treated with glucoamylase (0.1 mg protein/ml) at 60^{o}C for 72 h. As a result, the reducing power and glucose content increased to maxima within 12 h of incubation and were constant when prolonged the incubation. After 12 h, the glucose concentration reached 350 mg/ml (Figure 19). The soluble protein was found to aggregate during the incubation. When the glucoamylase was incubated at 60^{o}C without the substrate in the control experiment, soluble protein completely aggregated within 12 h. On the other hand, when the glucoamylase was incubated with maltodextrin as a substrate, the soluble protein gradually decreased to 0.04 mg protein/ml within 72 h.

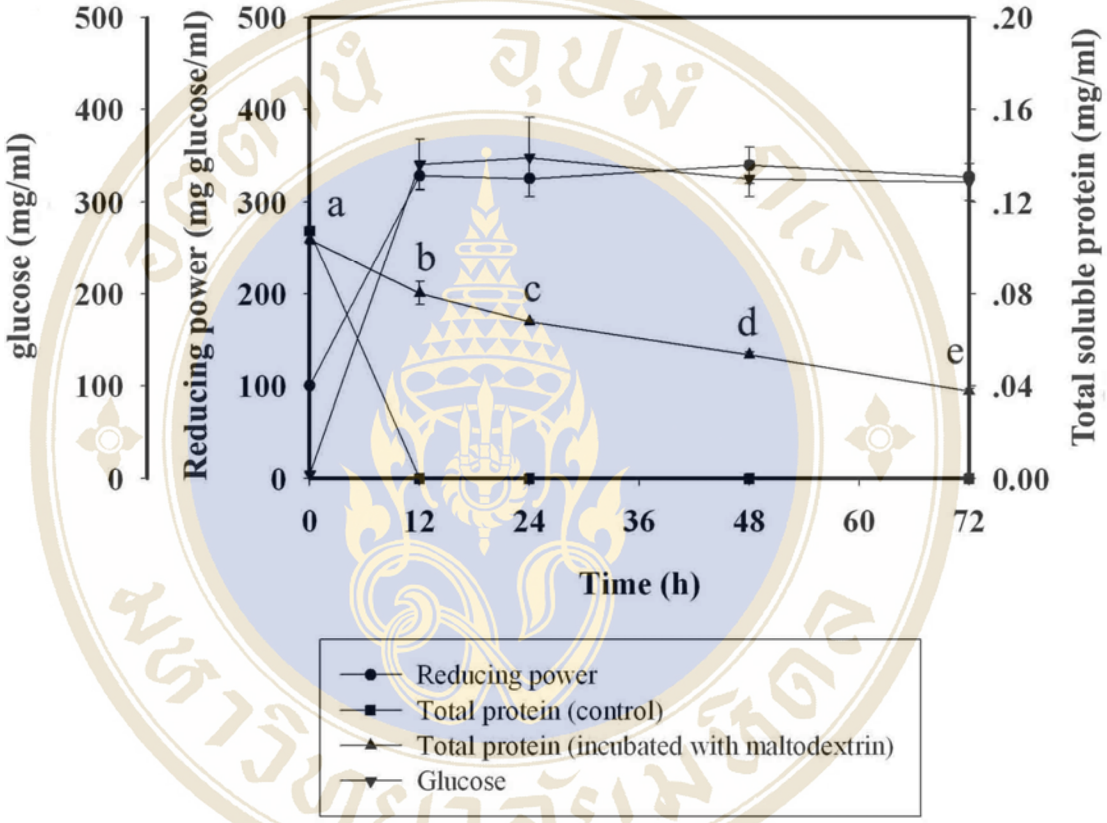


Figure 19 Total soluble protein, reducing power and glucose content during incubation PG (0.1 mg/ml) with maltodextrin (350 mg/ml) at 60°C. Different small letters show the significantly difference ($p<0.05$) between time interval of incubation in each experiment.

2.2 Total sugar and degree of glycation during incubation of bovine serum albumin (BSA) with maltodextrin

BSA was incubated with maltodextrin at 50^{o}C. Total sugar and degree of glycation were determined. The standard curve was plotted to describe the relationship between HMF released form glycated protein and total sugar content (Figure 20). The linear regression was obtained (r^2 = 0.9975) with linear equation of y = 6.9045x + ($2x10^{-5}$), where y and x stand for total sugar content (mole/g protein) and HMF released (mole/ g protein), respectively.

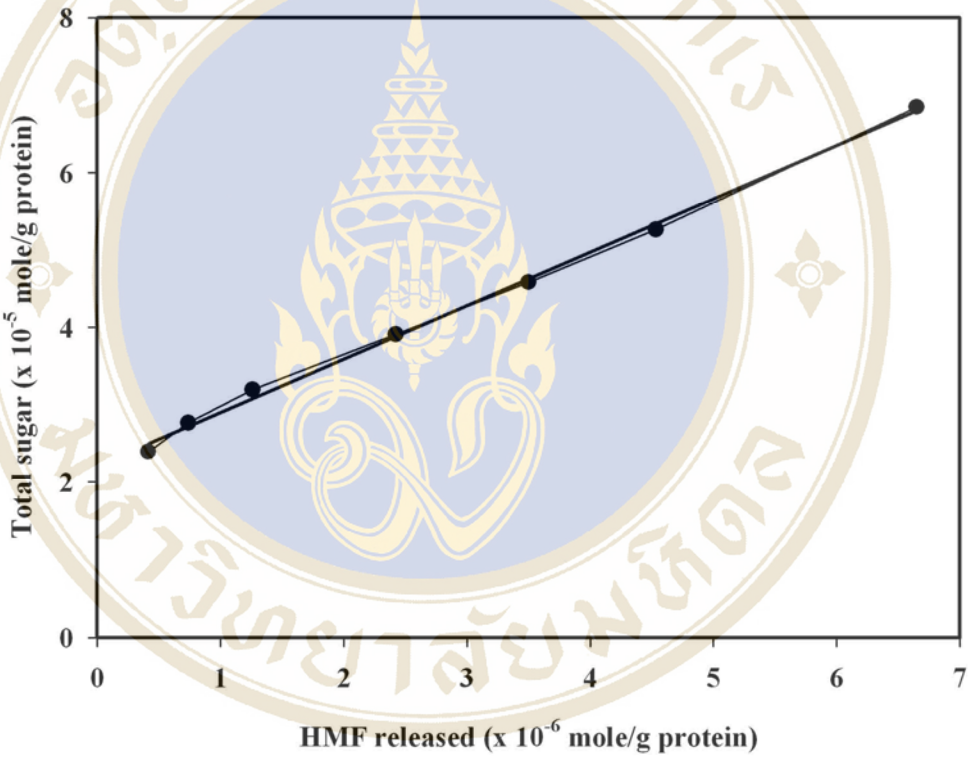


Figure 20 Relationship between total sugar and HMF released from glycated BSA.

2.3 Degree of glycation during incubation of amylolytic enzyme with reducing sugar

2.3.1 Reaction between KLE and maltodextrin or glucose

The results showed that HMF released from the soluble protein (KLE + maltodextrin) significantly increased from 0.06 to 0.76 µg/mg protein during incubation (Figure 21a), $p<0.05$. These results corresponded to the results of HMF released from aggregate protein (Figure 21b). Moreover, HMF released from the aggregate protein (KLE + maltodextrin) was higher than the control experiment ($p<0.05$). The HMF released from both soluble and aggregate protein was significantly progressed during prolonged incubation ($p<0.05$). However, the HMF released from soluble protein was higher than that of the aggregate protein in all experiments ($p<0.05$).

In case of incubation with glucose, the HMF released from the soluble protein (Figure 22a) significantly increased form 0.09 to 1.59 µg/mg protein after prolonged incubation period, $p<0.05$. The HMF released from aggregate protein (Figure 22b) increased from 1.14 to 2.23 µg/mg protein within 60 min of incubation and remained constant until 180 min of incubation. The results also showed that the HMF released from the aggregate was significantly higher comparing to the soluble protein, $p<0.05$.

2.3.2 Reaction between BAN and maltodextrin

At 70^{o}C, the HMF released from soluble protein (BAN with maltodextrin addition) significantly increased from 0.19 to 0.72 µg/mg protein with increases in incubation time and slightly decreased after 48 h incubation, $p<0.05$ (Figure 23a). In contrary to control experiment (BAN only), the amount of HMF decreased during prolonged incubation. The HMF released from aggregate protein (BAN with maltodextrin addition) was significantly progressed during prolong incubation, $p<0.05$ (Figure 23b). These results were similar to the HMF released from soluble protein incubated at 90^{o}C. The HMF significantly increased during prolonged incubation and reached the maximum (1.46 µg/mg protein) at 4 h incubation period. However, the HMF released from aggregate protein was constant and tended to decrease after 3 h incubation. In the experiment with maltodextrin addition, the HMF released from

soluble protein incubated at 70 and 90^{o}C was significantly higher than control experiments as well.

2.3.3 Reaction between PG and maltodextrin

It was found that the HMF released from the soluble protein taken from the hydrolysis mixture (PG+maltodextrin) was significantly increased from 0.35 to 0.69 μg/mg protein within 24 h incubation, $p<0.05$ (Table 2). After that, the degree of glycation could not be monitored. This was due to the less amount of soluble protein remained in the reaction mixture. The degree of glycation also could not be monitored in control experiment due to the complete aggregation of soluble protein within 12 h incubation period. The HMF released from the aggregate protein (glucoamylase incubated with maltodextrin) was significantly higher than the control experiment (glucoamylase incubated without maltodextrin), $p<0.05$ (Table 3). The HMF seemed to be constant after 12-h incubation.

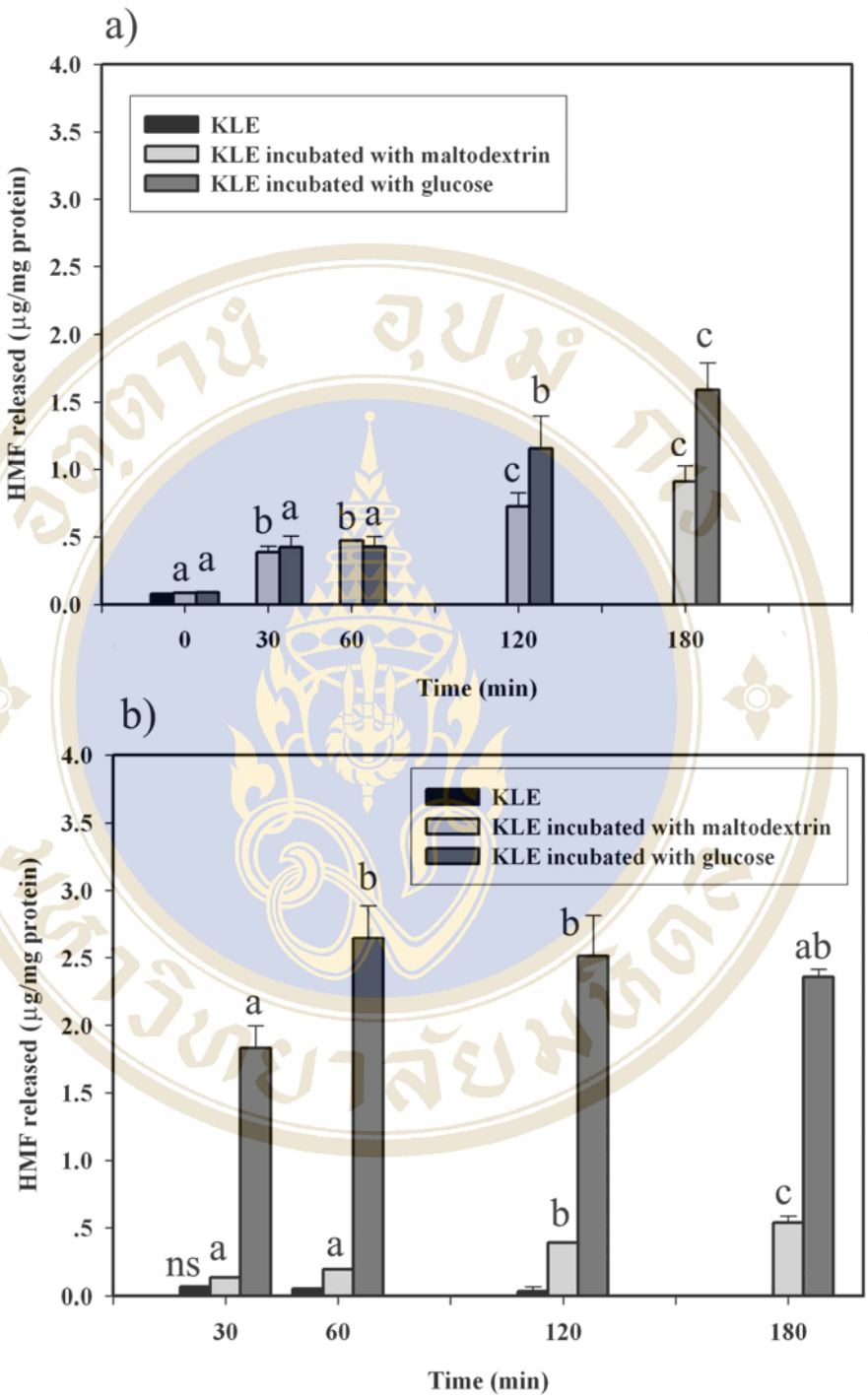


Figure 21 HMF released during incubation KLE (0.6 mg/ml) and maltodextrin or glucose (350 mg/ml) at 95°C; a) soluble protein; b) aggregate protein. Different small letters show the significantly difference ($p<0.05$) between time interval of incubation in each experiment. The "ns" stands for "not significant difference" ($p>0.05$).

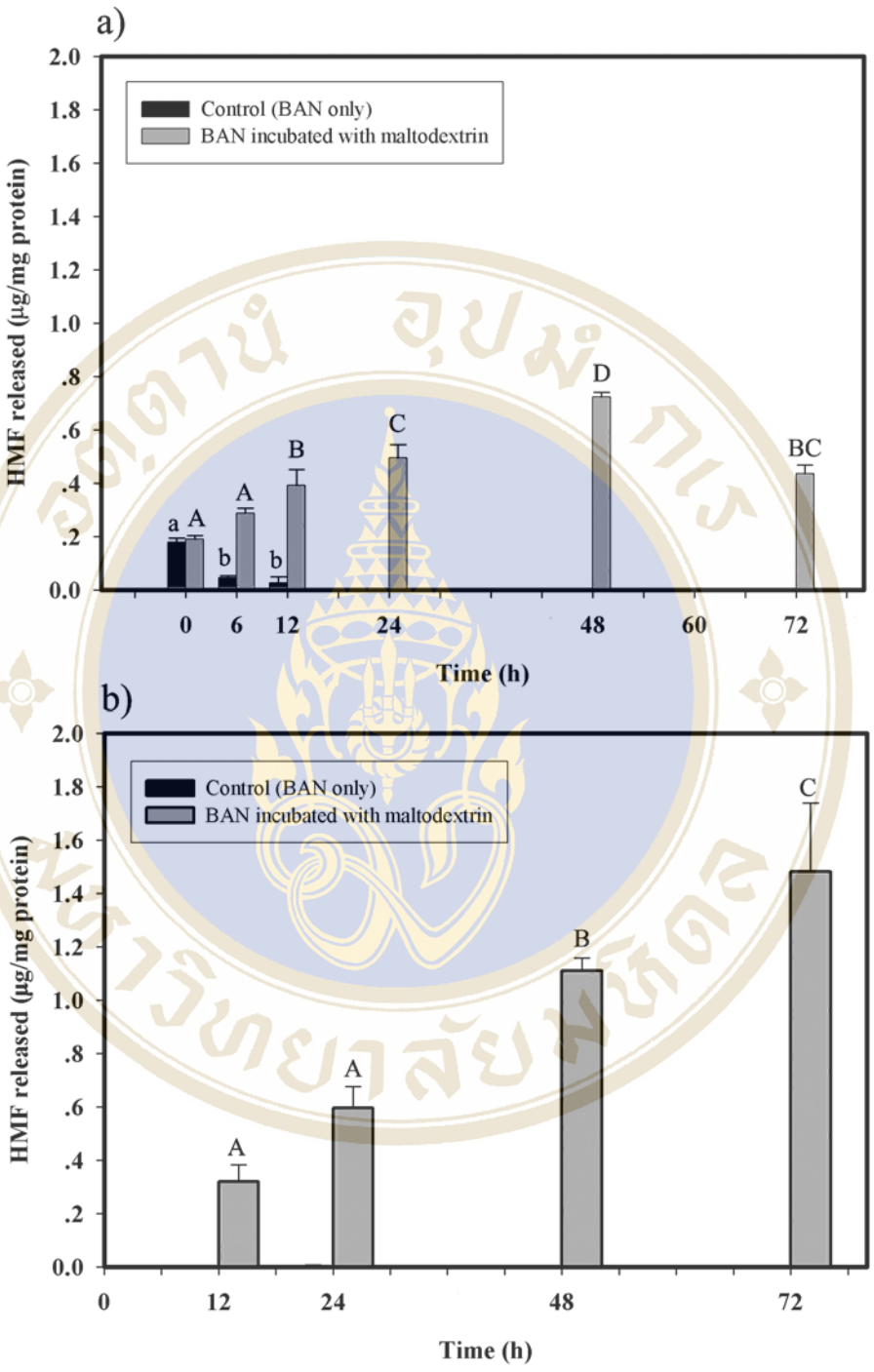


Figure 22 HMF released during incubation BAN (0.6 mg/ml) and maltodextrin (350 mg/ml) at 70^{o}C; a) soluble protein; b) aggregate protein. Different small and capital letters show the significantly difference ($p<0.05$) between time interval of incubation in of each experiment.

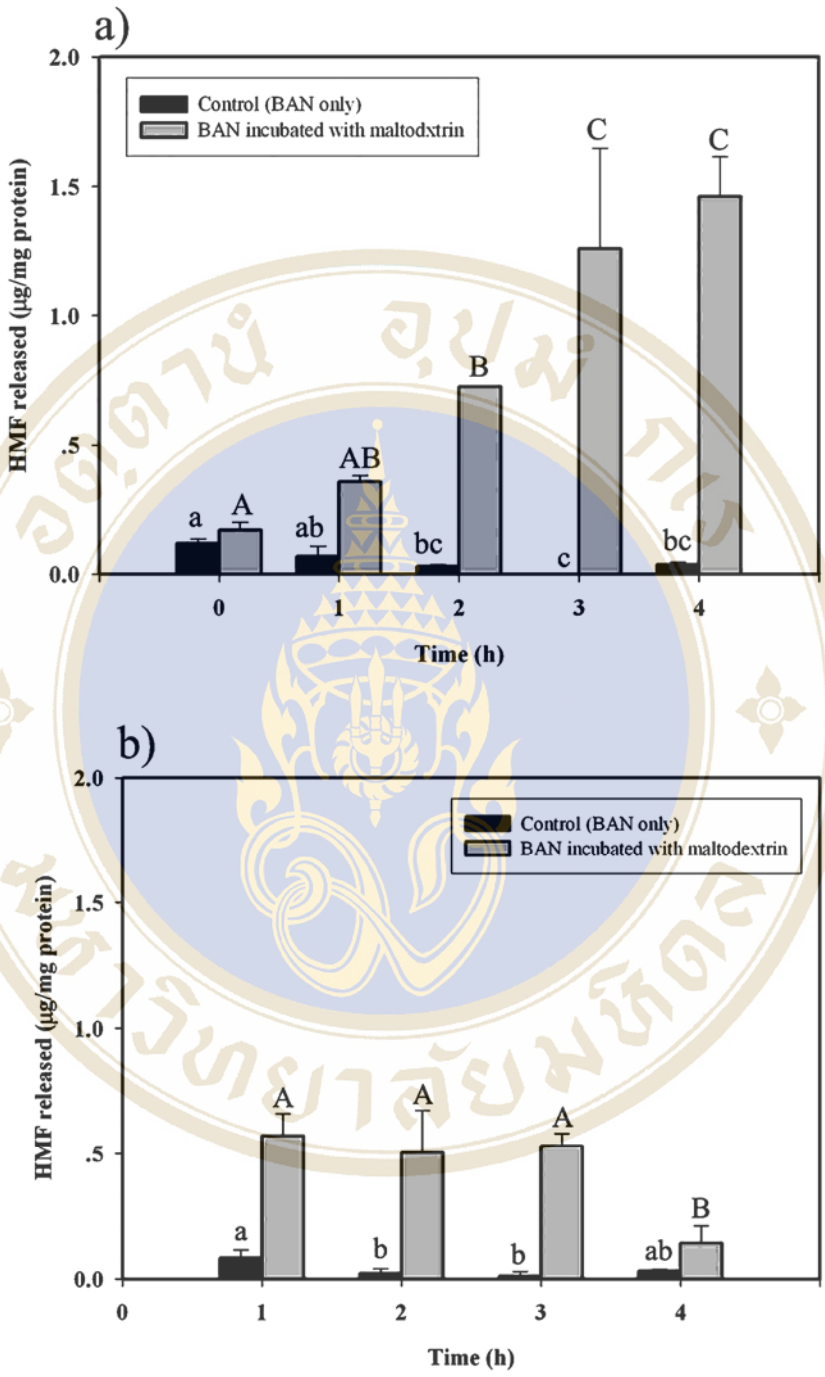


Figure 23 HMF released during incubation BAN (0.6 mg/ml) and maltodextrin (350 mg/ml) at 90^{o}C; a) soluble protein; b) aggregate protein. Different small and capital letters show the significantly difference ($p<0.05$) between time interval of incubation in each experiment.

Table 2 5-(Hydroxymethyl)-2-furfuraldehyde (HMF) released from the soluble protein during incubation of the purified glucoamylase (PG) in the presence of maltodextrin at 60°C

Time (h)	HMF released (μg/mg protein)	
	Control (PG)	PG incubated with maltodextrin
0	0.35±0.01	0.35±0.01[a]
12	no data	0.58±0.05[b]
24	no data	0.69±0.04[c]
48	no data	no data
72	no data	no data

Values are mean±S.D. Different letters (a, b and c) on the same row show the significantly difference ($p<0.05$).

Table 3 5-(Hydroxymethyl)-2-furfuraldehyde (HMF) released from the aggregate protein during incubation of the purified glucoamylase (PG) in the presence of maltodextrin at 60°C

Time (h)	HMF released (μg/mg protein)	
	Control (PG)	PG incubated with maltodextrin
12	0.24±0.02[a]	0.42±0.08[b]
24	0.26±0.02[a]	0.45±0.06[b]
48	0.20±0.00[a]	0.51±0.04[b]
72	0.14±0.01[a]	0.56±0.09[b]

Values are mean±S.D. Different letters (a, b) on the same row show the significantly difference ($p<0.05$).

2.4 Protein migration pattern from SDS-PAGE and Native-PAGE during incubation amylolytic enzyme and reducing sugar

2.4.1 KLE incubated with maltodextrin or glucose

The SDS-PAGE of KLE showed the molecular weight (MW) of 58.1 kDa (Figure 24a). The MW of KLE increased after prolonged incubation with maltodxtrin and was shown to be 59.9 kDa within 2 h of incubation period. However, the intermolecular cross-linking was not observed on the glycated KLE. The different patterns of KLE incubated with maltodextrin were observed on Native-PAGE (Figure 24b). The soluble protein taken from reaction mixture between KLE and maltodextrin migrated beneath the soluble protein taken from the control experiment.

In case of incubation with glucose, the MW of KLE increased to 59.4 kDa within 2 h incubation period (Figure 25). In control experiment (KLE only), the protein band could not be observed on the SDS-PAGE in all incubation periods. The protein bands (KLE+glucose) on Native-PAGE also migrated beneath the protein band from the control experiment. Moreover, the bands were different pattern when compared between incubation with maltodextrin or glucose especially after incubation for 3 h.

2.4.2 BAN incubated with maltodextrin

After incubation of BAN with maltodextrin at 70 and 90^{o}C for 48 and 2 h, the MW increased from 58.4 kDa to 61.2 and 60.7 kDa, respectively. In control experiment (BAN only), the protein band could not be observed on the SDS-PAGE in all incubation periods. The protein bands (BAN+maltodextrin) on Native-PAGE showed different migration patterns in both of different incubation periods and temperatures (Figure 26).

2.4.3 PG incubated with maltodextrin

Incubation of PG and maltodextrin at 60^{o}C for 24 h did not show any effect on both MW on SDS-PAGE and the migration pattern on Native PAGE (Figure 27). The protein was also stained with toluidine blue. However, there were no difference between the glycated PG and intact form.

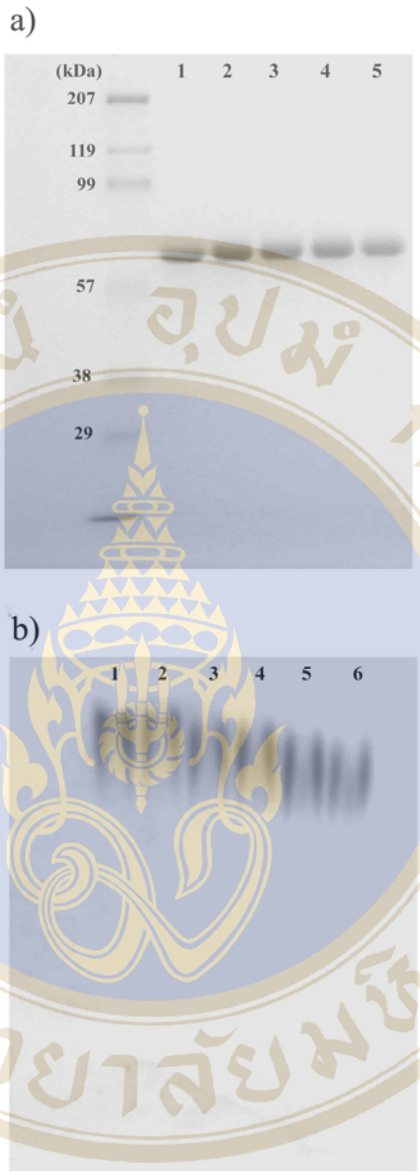


Figure 24 a) SDS-PAGE (10% gel) of KLE with maltodextrin addition during various incubation periods at 95°C. Lane 1 to 5, KLE-maltodextrin incubated at 0, 30, 60, 120 and 180 min, respectively (3 µg).
b) Native-PAGE (10% gel) of KLE with maltodextrin addition under the same conditions. Lane 1, native form; Lane 2 to 6, KLE-maltodextrin incubated at 0, 30, 60, 120 and 180 min, respectively (3 µg).

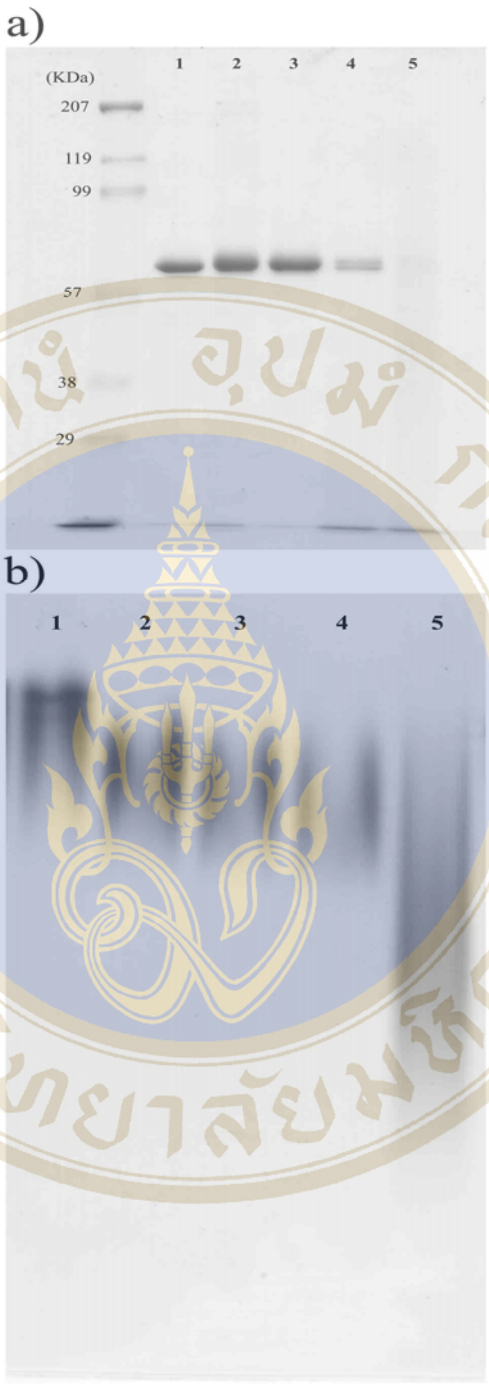


Figure 25 a) SDS-PAGE (10% gel) of KLE with glucose addition during various incubation periods at 95°C. Lane 1 to 5, KLE-glucose incubated at 0, 30, 60, 120 and 180 min, respectively (3 µg).

b) Native-PAGE (10% gel) of KLE with glucose addition under the same conditions. Lane 1 to 5, KLE-glucose incubated at 0, 30, 60, 120 and 180 min, respectively (3 µg).

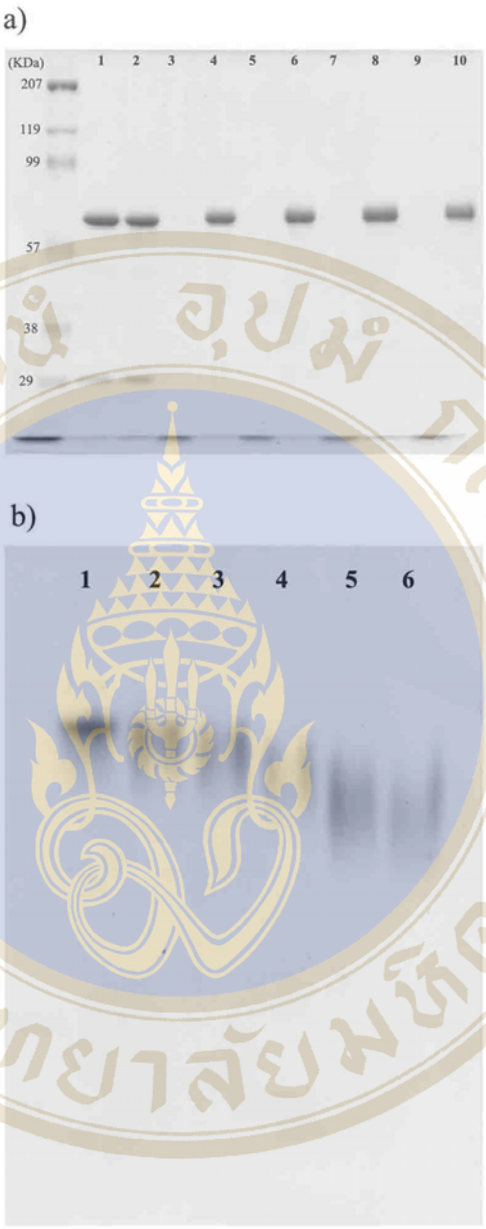


Figure 26 a) SDS-PAGE (10% gel) of BAN with maltodextrin addition during various incubation periods at 70°C. Lane 1, 3, 5, 7 and 9, BAN incubated at 0, 6, 12, 24 and 48 h (3 µg). Lane 2, 4, 6, 8 and 10, BAN-maltodextrin incubated at 0, 6, 12, 24 and 48 h (3 µg).

b) Native-PAGE (10% gel) of BAN with maltodextrin addition during various incubation periods at 70°C (3 µg). Lane 1 to 6, BAN-maltodxtrin incubated at 0, 6, 12, 24, 48 and 72 h, respectively (3 µg).

Figure 26 c) SDS-PAGE (10% gel) of BAN with maltodextrin addition during various incubation periods at 90°C. Lane 1, 3, 5, 7 and 9, KLE incubated at 0, 30, 60, 120 and 180 min (3 µg). Lane 2, 4, 6, 8 and 10, BAN-maltodextrin incubated at 0, 30, 60, 120 and 180 min (3 µg).

d) Native-PAGE (10% gel) of BAN with maltodextrin addition during various incubation periods at 90°C (3 µg). Lane 1 to 5, BAN-maltodextrin incubated at 0, 30, 60, 120 and 180 min, respectively (3 µg).

Figure 27 a) SDS-PAGE (10% gel) of PG. Lane 1, PG, Lane 2, PG incubated with maltodextrin at 60°C for 24 h (3 µg). b) Native-PAGE (10% gel) of glucoamylase. Lane 1, crude enzyme, lane 2, crude enzyme after passed through ultrafiltration (MW cutoff = 10 kDa) (6 µg); lane 3, PG; lane 4, PG incubated with maltodextrin at 60°C for 24 h (3 µg).

2.5 Determination of pI value during incubation of amylolytic enzyme with reducing sugar

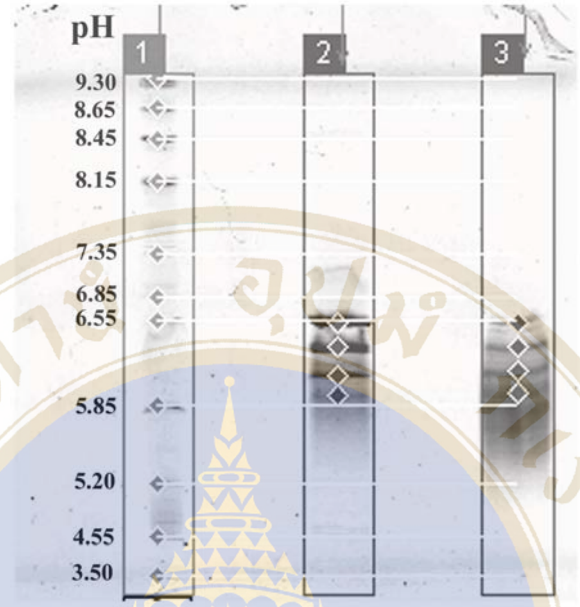
2.5.1 KLE incubated with maltodextrin

Figure 28 shows the pI value of KLE and gKLE. The results showed that the pI value of KLE was altered from the range of 6.54-5.63 to 6.54-5.20 due to the glycation. Not only the lower pI value range was found in gKLE, but also the profile of pI value was different (Figure 28b and 28c).

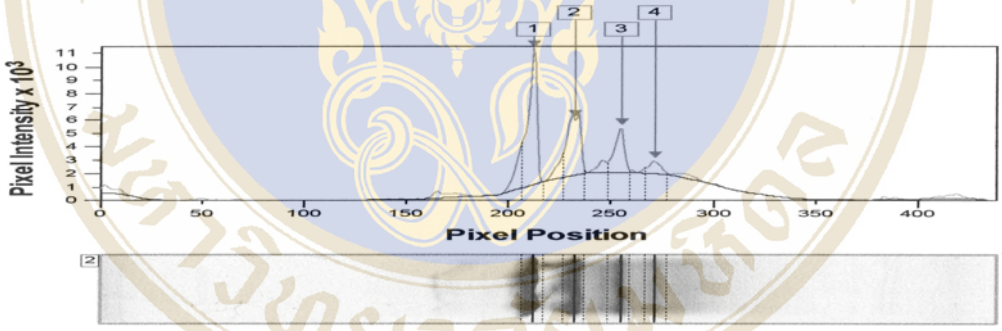
2.5.2 PG incubated with maltodextrin

The pI values of gPG and PG were 3.45 and 3.47, respectively (Figure 29). It was found that the glycation did not strongly affect on the pI values of the glucoamylase.

a)



b)



c)

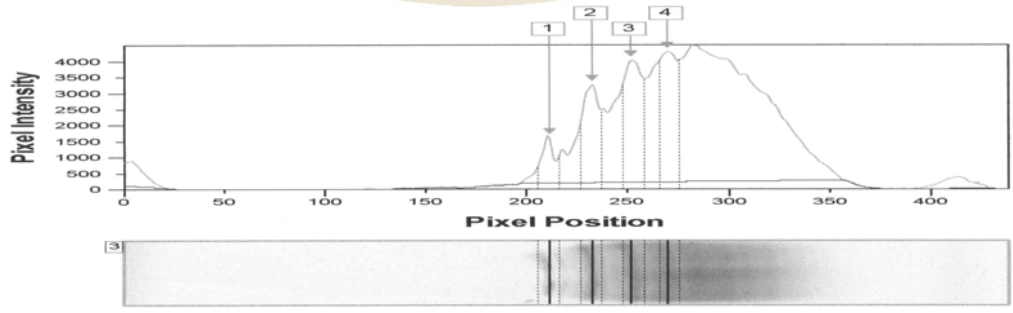


Figure 28 a) Isoelectric focusing profile at pH 3 to 9 of KLE. Lane 1, marker; lane 2, the intact KLE; lane 3, KLE incubated with maltodextrin at 95°C for 120 min; b) pI pattern of KLE; c) pI pattern of KLE-maltodextrin.

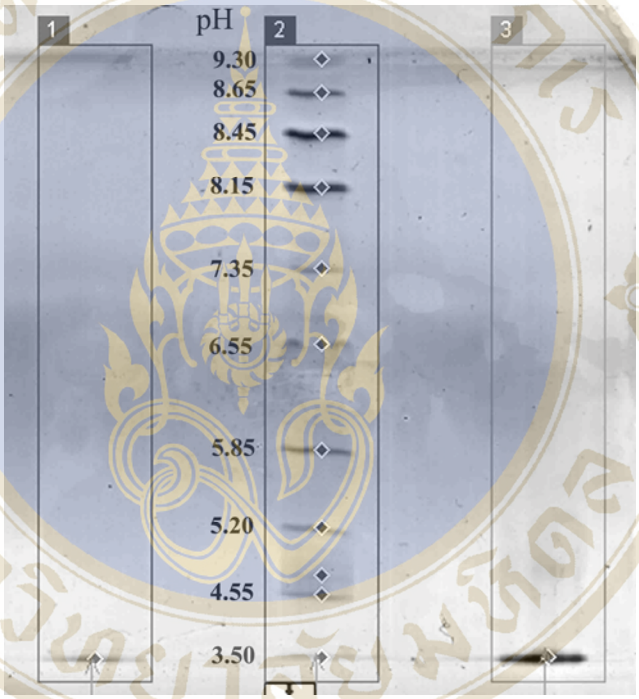


Figure 29 Isoelectric focusing profile at pH 3 to 9 of PG. Lane 1, PG incubated with maltodextrin at 60^{o}C for 24 h; lane 2, marker; lane 3, PG.

3. Thermostability of the glycated amylolytic enzyme

3.1 Effect of heat on the stability of glycated KLE

It was found that the soluble protein was rapidly aggregated in both glycated KLE with maltodextrin (gKLE) and control experiment, and slightly increased after 5 min of incubation (Figure 30). However, soluble protein content taken from the reaction mixture of gKLE was higher than the control experiment (KLE only), $p<0.05$. The results corresponded to the enzyme activity. The specific activity of both experiments quickly decreased after prolonged incubation period. After 5 min incubation, gKLE could maintain more than 50% of its activity whereas the intact KLE lost 60% of its activity. The results also showed that the activity of glycated enzyme was significantly higher than that of the intact enzyme, $p<0.05$.

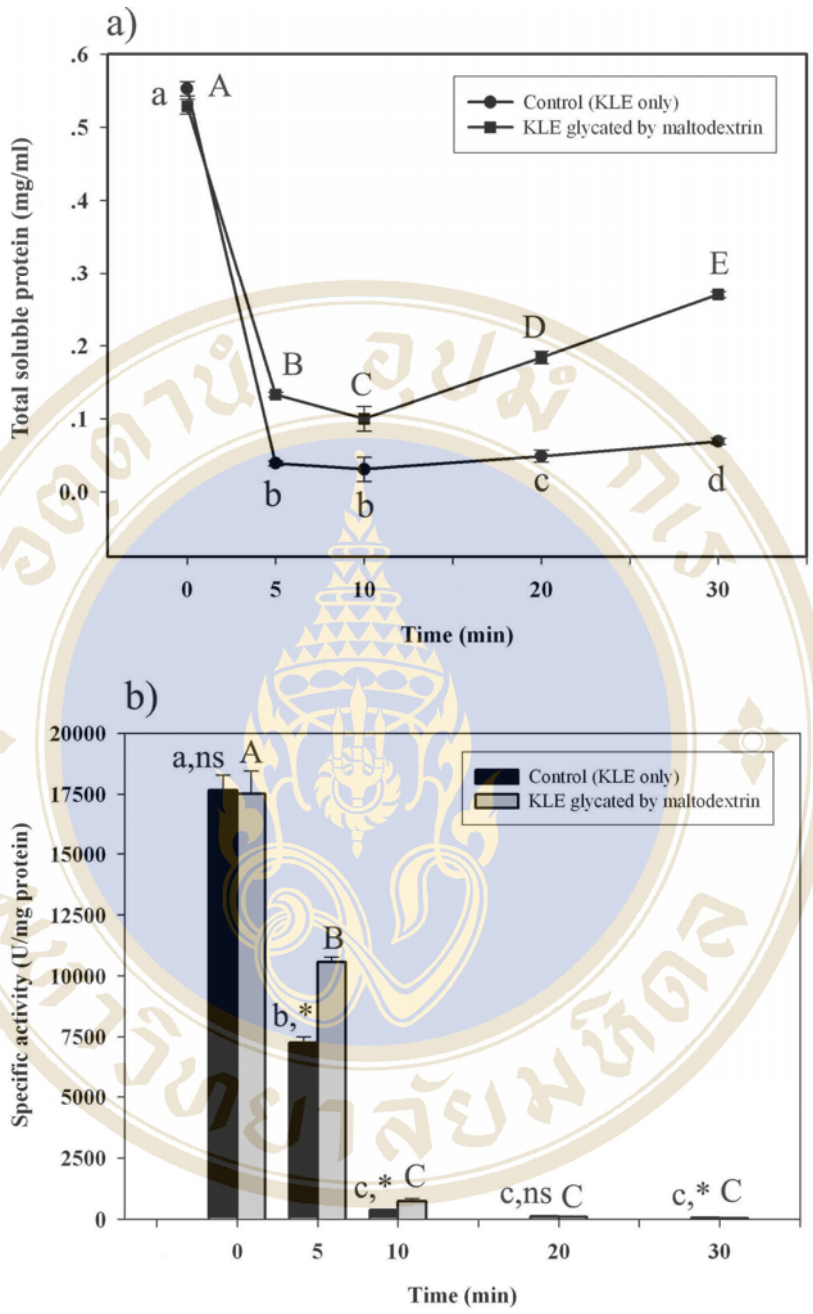


Figure 30 a) Effect of heat on total soluble protein; and b) Specific activity of KLE and KLE glycated by maltodextrin (gKLE). Bars with the different small letters show significant difference ($p<0.05$) in total soluble protein content or specific activity of the control experiment (KLE). Bars with the different capital letters show significant difference ($p<0.05$) in total protein content and specific activity of the glycated KLE. The * stands for the significant different ($p<0.05$) in the specific activity between the intact and glycated form. The "ns" stands for "not significant difference" ($p>0.05$).

3.2 Effect of heat on the stability of glycated BAN

The time course obtained for heat stability of the glycated enzymes comparing to the intact form is shown in Figure 31. In case of incubation at 70^{o}C, the soluble protein of the glycated BAN slightly decreased from 0.50 to 0.43 mg/ml within 5 min incubation period and seemed to be constant after prolonged incubation. The contrary effect was observed in the control experiment. The soluble protein in control experiment gradually decreased from 0.56 to 0.40 mg/ml within 30 min incubation period. The results were contrary to the specific activity of both intact and glycated forms. The specific activity of the gBAN lost rapidly within 5 min of incubation period (less than 2% of relative activity still remained). On the contrary, in the intact BAN, the specific activity could maintain higher than 80% of its activity after prolonged incubation for 10 min. Above 50% of enzyme activity still remained within 30 min of incubation period. After prolonged incubation, the specific activity of control experiment was significantly higher than that of glycated BAN in all incubation period.

In case of incubation at 90^{o}C, the soluble protein of the control experiment rapidly decreased from 0.60 to 0.08 mg/ml within 5 min (Figure 32). On the other hand, the soluble protein was slightly decreased after 5 min incubation time and becoming constant after prolonged incubation. Only 0.06 mg/ml of soluble protein was lost after 30 min incubation period. The enzyme rapidly lost its activity in both of intact and glycated BAN within 5 min. However, the intact enzyme could maintain higher activity after 5 min incubation in comparison with the glycated form.

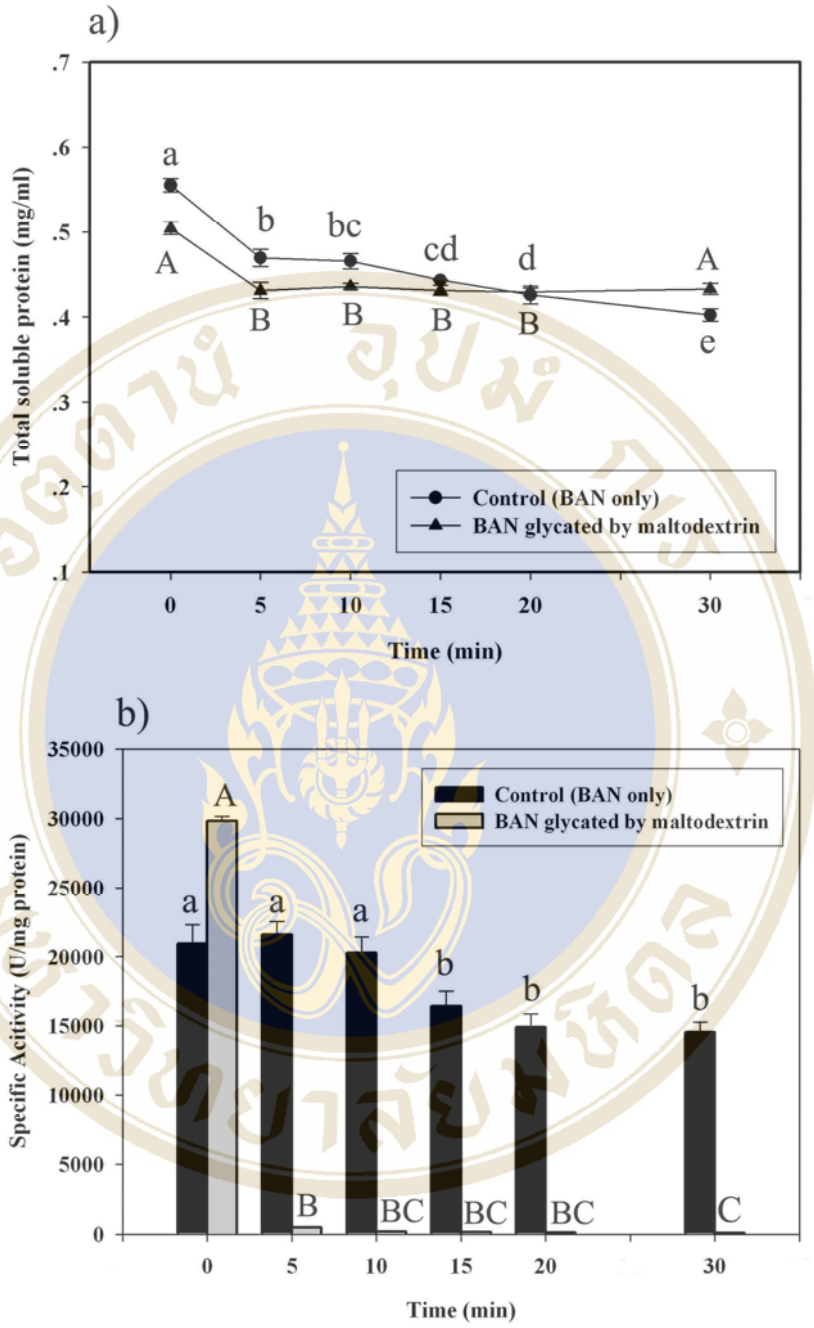


Figure 31 a) Effect of heat on total soluble protein; and b) Specific activity of BAN and BAN glycated by maltodextrin (gBAN) at 70^{o}C for 48 h. The enzyme solution (0.6 mg/ml) was incubated at 70^{o}C and withdrawn at specific time intervals. Bars with the different small letters show significant difference ($p<0.05$) in total soluble protein content or specific activity of the control experiment (BAN). Bars with the different capital letters show significant difference ($p<0.05$) in total soluble protein content and specific activity of the glycated BAN.

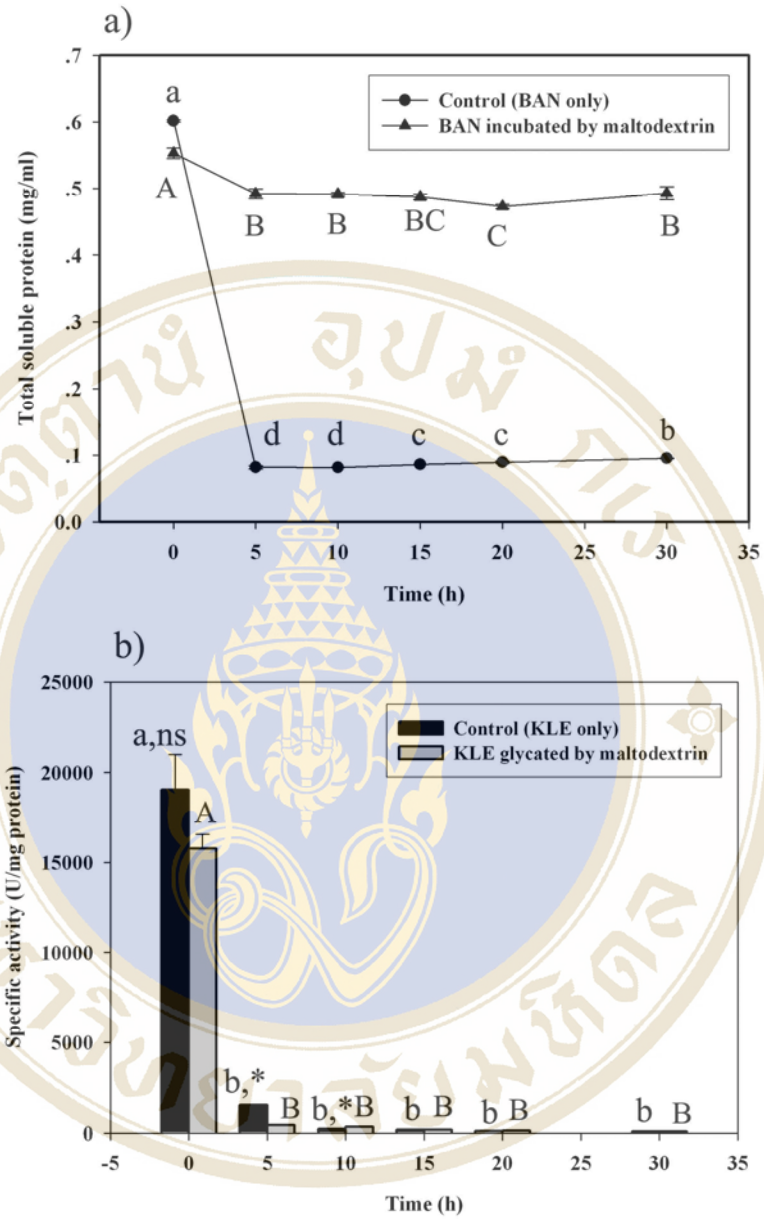


Figure 32 a) Effect of heat on total soluble protein; and b) Specific activity of BAN and BAN glycated by maltodextrin (gBAN) at 90°C for 2 h. The enzyme solution (0.6 mg/ml) was incubated at 90°C and withdrawn at specific time intervals. Bars with the different small letters show significant difference ($p<0.05$) in total soluble protein content or specific activity of the control experiment (BAN). Bars with the different capital letters show significant difference ($p<0.05$) in total protein content and specific activity of the glycated BAN. The * stands for the significant difference ($p<0.05$) in the specific between the intact and glycated form. The "ns" stands for "not significant difference" ($p>0.05$).

3.3 Effect of heat on the stability of glycated PG

Figure 33 shows that the soluble protein rapidly decreased in all experiments after incubating for 2 h. The activity of glucoamylase glycated by maltodextrin (gPG) and glycated by glucose were significantly higher than PG, $p<0.05$ (Figure 33b). The enzyme activity of all experiments decreased during prolonged incubation. After 6-h incubation, the soluble protein in the mixture was not significantly different ($p<0.05$) among the three experiments (Figure 33a). PG and PG glycated by glucose completely lost its activity. However, only the PG glycated by maltodextrin could maintain the enzyme activity.

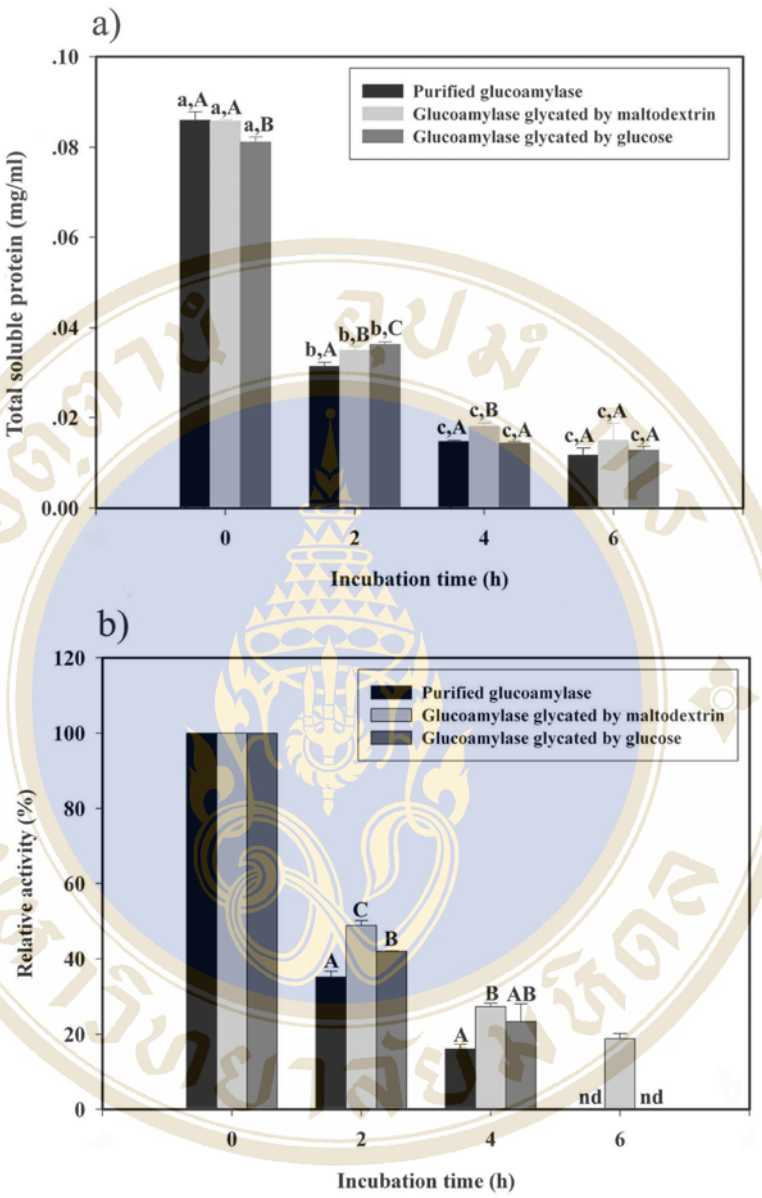


Figure 33 The thermostability of purified glucoamylase compared with the glycated forms. a) Total soluble protein content; b) Relative activity. Bars with the different small letters (a, b, c) show significant difference ($p<0.05$) in total soluble protein content during incubation time along the experiments (purified glucoamylase, glucoamylase glycated by maltodextrin and glucoamylase glycated by glucose). Bars with the different capital letters (A, B, C) show significant difference ($p<0.05$) in both total soluble protein content and relative activity of each incubation time. The "nd" stands for "not detectable".

4. pH stability of the glycated amylolytic enzyme

4.1 Effect of pH on stability of the glycated KLE

The study on pH stability was performed by incubating the glycated enzyme at different pH for 5 min. The effects of pH on soluble protein and specific enzyme activity are shown in Figure 34. The soluble protein started aggregating at pH 4.5 to 6.5. The glycated enzyme was more stable under pH tested (5.5, 6.5 and 8.5) than non-glycated one, whereas there was no significant difference ($p \geq 0.05$) at pH 7.5. Interestingly, the glycated KLE could maintain the higher activity than the intact enzyme at all pH ($p < 0.05$).

4.2 Effect of pH on stability of the glycated BAN

The effect of pH on stability of the glycated BAN was compared with the intact enzyme (Figure 35). Incubation at 70^{o}C, the intact enzyme was more stable in the pH range of 5.5 to 7.5 comparing to the glycated BAN. The amount of soluble protein (the intact enzyme) at pH 4.5 and 8.5 was higher than that of the glycated form. However, the specific activity of the intact form was higher than the glycated in all the pH range. At 90^{o}C, the rapid aggregation was found in both experiments (the intact and glycated forms) (Figure 36). The higher activity was observed in glycated BAN at pH 6.5 and 8.5, $p<0.05$. Although the soluble protein of the intact enzyme was higher than the glycated BAN at pH 4.5 and 7.5, but the specific activity was not different ($p<0.05$).

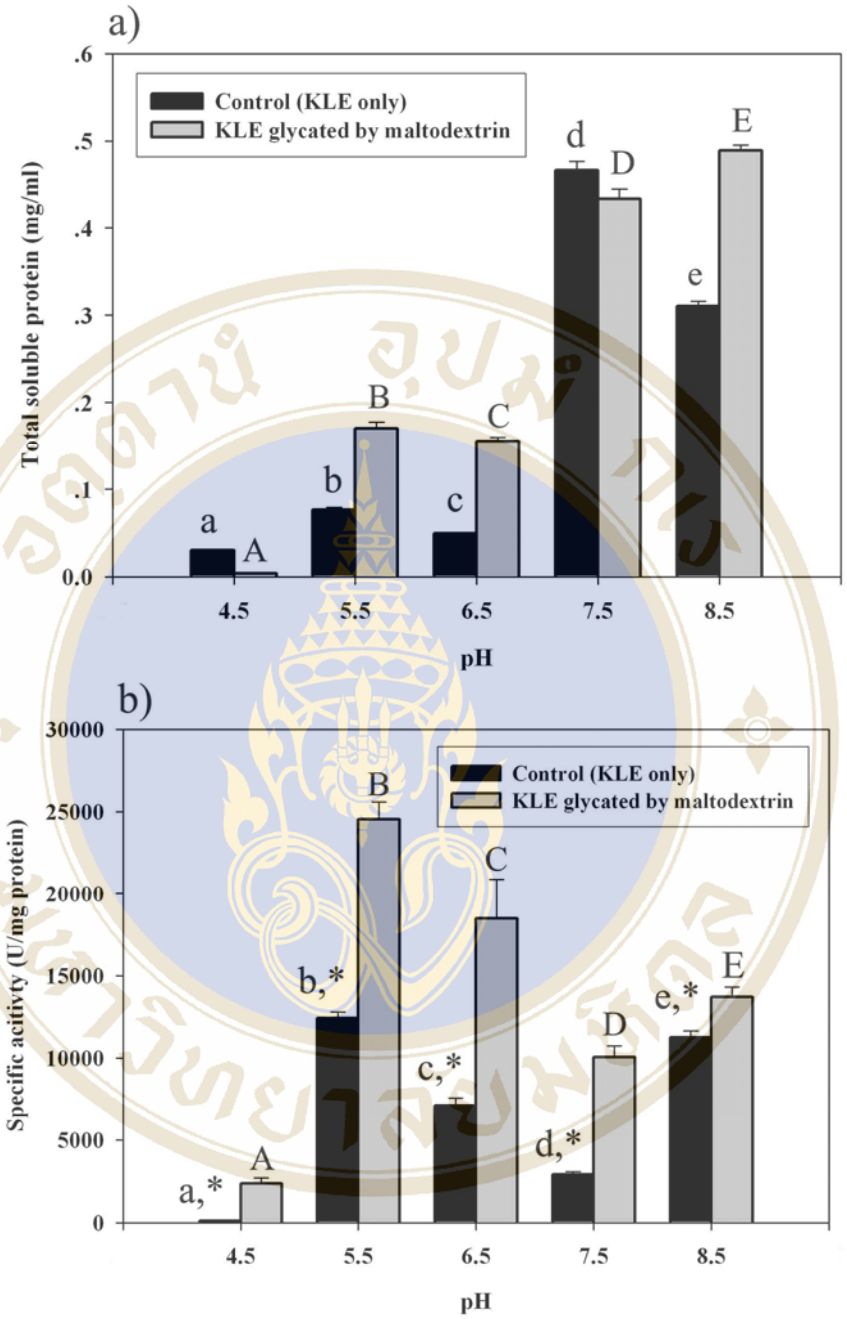


Figure 34 The pH stability of KLE compared with the glycated forms, incubated at 95°C for 5 min. a) Total soluble protein content; b) Specific activity. Bars with the different small letters show significant difference ($p<0.05$) in total soluble protein content or specific activity of the control experiment (KLE). Bars with the different capital letters show significant difference ($p<0.05$) in total soluble protein content and specific activity of the glycated KLE. The * stands for the significant difference ($p<0.05$) in the specific activity between the intact and glycated form.

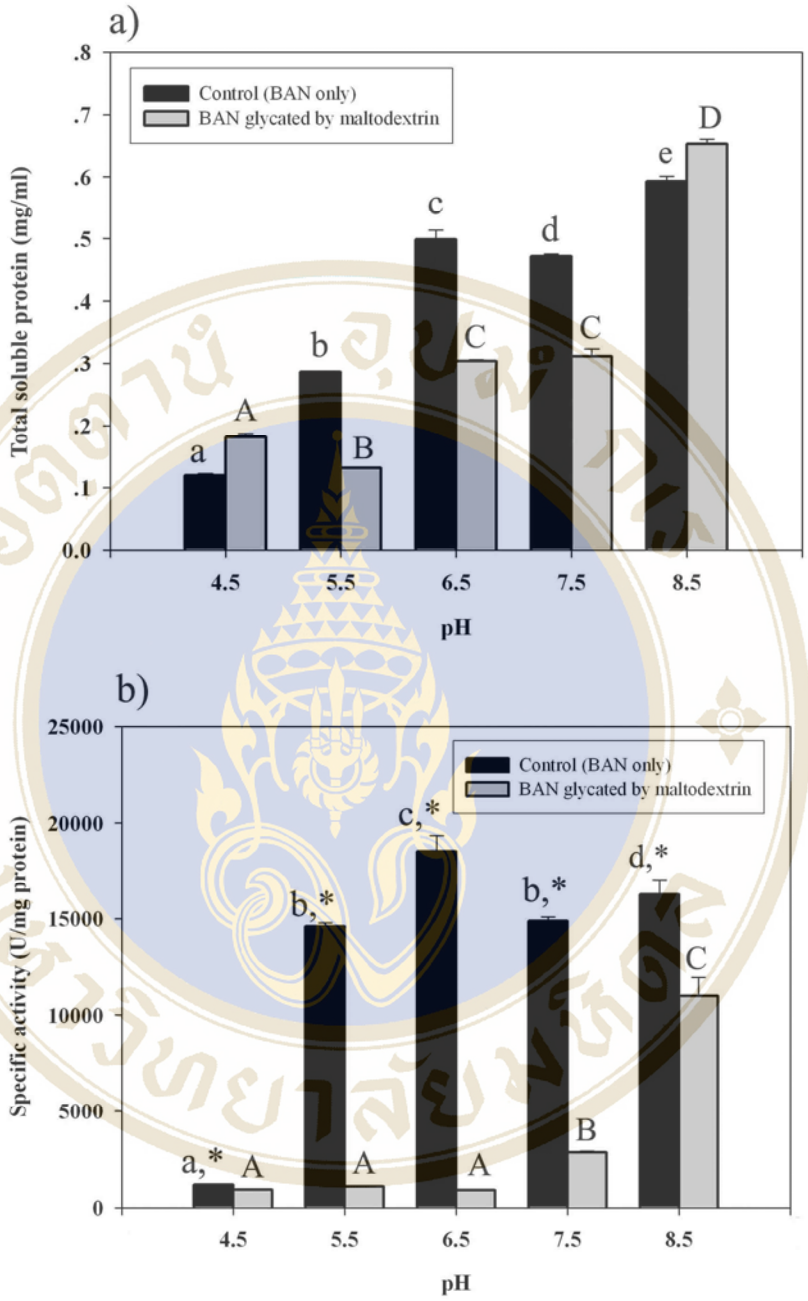


Figure 35 The pH stability of BAN compared with the glycated forms (70^{o}C, 5 min). a) Total soluble protein content; b) Specific activity. Bars with the different small letters show significant difference ($p<0.05$) in total soluble protein content or specific activity of the control experiment (BAN). Bars with the different capital letters show significant difference ($p<0.05$) in total soluble protein content and specific activity of the glycated BAN. The * stands for the significant difference ($p<0.05$) in the specific activity between the intact and glycated form.

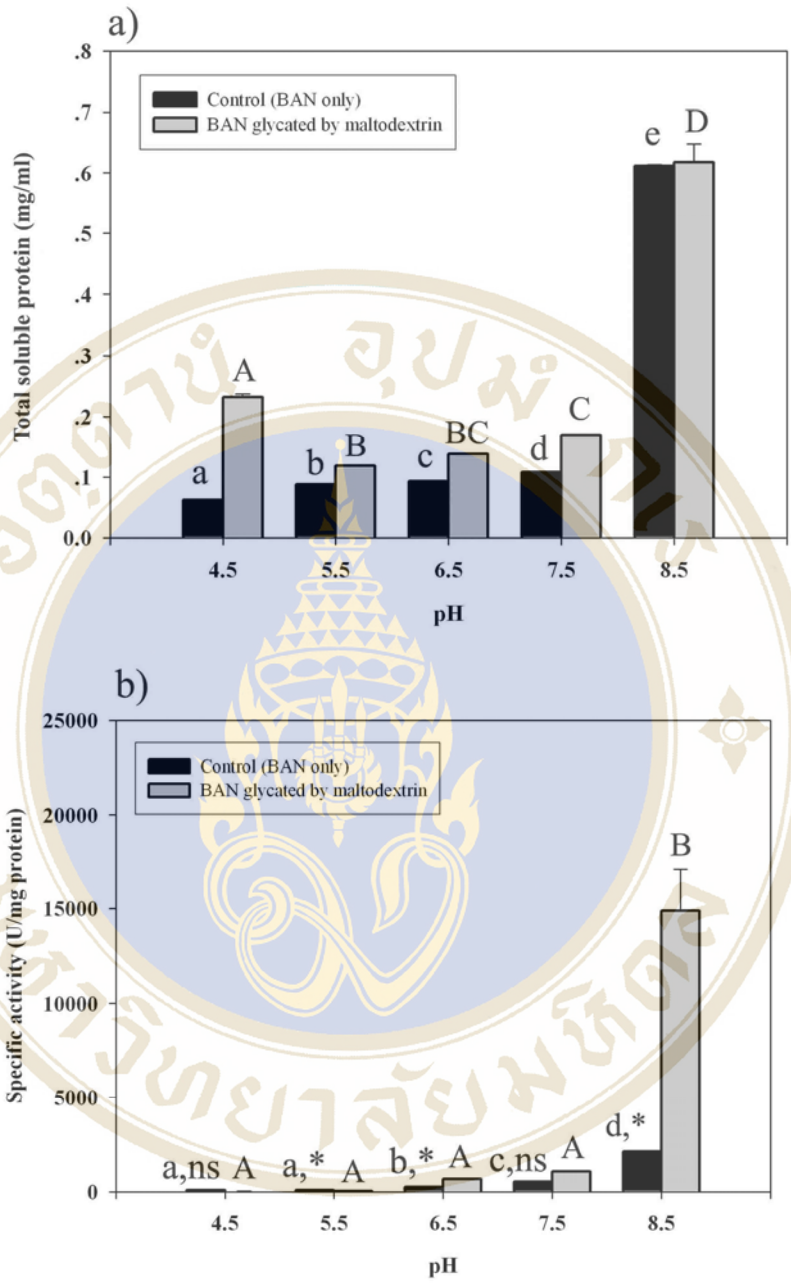


Figure 36 The pH stability of BAN compared with the glycated forms (90^{o}C, 5 min). a) Total soluble protein content; b) Specific activity. Bars with the different small letters show significant difference ($p<0.05$) in total soluble protein content or specific activity of the control experiment (BAN). Bars with the different capital letters show significant difference ($p<0.05$) in total soluble protein content and specific activity of the glycated BAN. The * stands for the significant difference ($p<0.05$) in the specific activity between the intact and glycated form. The "ns" stands for "not significant difference" ($p>0.05$).

4.3 Effect of pH on stability of the glycated PG

Figure 37 shows that the soluble protein started aggregating at pH 3.5 and the soluble protein was higher with increases in pH range (5.5 and 6.5). The enzyme rapidly lost activity at pH 5.5 and completely inactivated at pH 6.5. However, the glycated glucoamylase could maintain the higher activity than the intact enzyme at pH 5.5 ($p<0.05$).

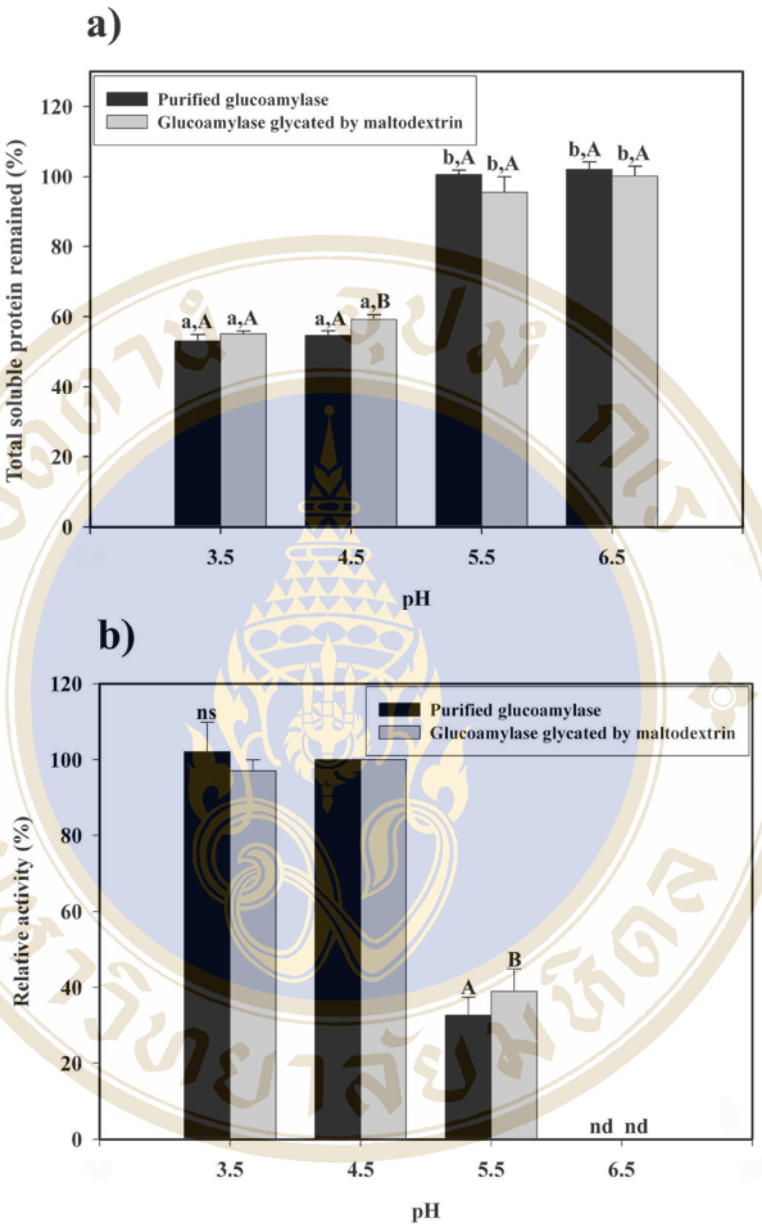


Figure 37 The pH stability of purified glucoamylase compared with the glycated forms (60^{o}C, 1 h). a) Total soluble protein remained, b) Relative activity. Bars with the different small letters (a, b) show significant difference ($p<0.05$) in total soluble protein remained with various pH during experiments (purified glucoamylase and glucoamylase glycated by maltodextrin). Bars with the different capital letters (A, B) show significant difference ($p<0.05$) in both total soluble protein remained and relative activity of each pH. The "ns" and "nd" stand for "not significant difference" ($p>0.05$) and "not detectable", respectively.

5. Effect of glycation on enzyme kinetic parameters

5.1 Glycated KLE by maltodextrin (gKLE)

Table 3 shows the changes of enzyme kinetic parameters due to the glycation of KLE. The results showed that the glycation did not strongly affect the kinetic parameters. The binding affinity (K_m), catalytic rate (V_{max}) and turnover number (K_{cat}) of gKLE were not significant difference in comparison with the intact form, ($p>0.05$).

5.2 Glycated BAN by maltodextrin (gBAN)

In case of BAN, the glycation did not strongly affect the binding affinity and catalytic rate in both glycated at 70 and 90^{o}C (Table 4). However, the turnover number was significantly decreased due to the glycation, ($p>0.05$). The gBAN glycated at both temperatures showed the same capacity to convert gelatinized cornstarch into maltodextrin, ($p>0.05$).

5.3 Glycated PG by maltodextrin (gPG)

In the presence of maltose as substrate, the binding affinity (K_m), catalytic rate (V_{max}) and turnover number (K_{cat}) of gPG were not significantly different to the intact form (Table 5), ($p>0.05$). In case of substrate maltodextrin, The K_m of gPG was higher than of the intact form indicating the lower binding affinity of the enzyme to substrate due to the glycation. The V_{max} of the glycated glucoamylase was higher than the intact enzyme, ($p>0.05$). These indicated that the glycation elevated the rate of reaction. Moreover, the turnover number (K_{cat}) of the enzyme glucoamylase increased due to the glycation in both substrates maltodextrin ($p>0.05$).

Table 4 Kinetic parameters of KLE and KLE glycated (gKLE) at 95°C

Enzyme	K_m (mg/ml)	V_{max} (mM/min)	K_{cat} ($x10^6$ min^{-1})
KLE	13.00^{ns}	3.30^{ns}	3.62^{ns}
gKLE	14.22	3.81	3.53

*The "ns" stands for "not significant difference" ($p>0.05$).

Table 5 Kinetic parameters of BAN and BAN glycated (gBAN) at 70 and 90°C

Enzyme	K_m (mg/ml)	V_{max} (mM/min)	K_{cat} ($x10^2$ min^{-1})
BAN	33.49^{ns}	8.87^{ns}	4.90^{a}
BAN glycated at 70°C	31.17	6.57	2.10^{b}
BAN glycated at 90°C	30.19	8.31	3.36^{b}

*Different letters (a, b) show the significantly difference ($p<0.05$) between intact and glycated enzyme. The "ns" stands for "not significant difference" ($p>0.05$).

Table 6 Kinetic parameters of purified glucoamylase (PG) and glycated glucoamylase (gPG)

Substrate	Enzyme	K_m	V_{max} (mM/min)	K_{cat} $x10^3$ (min^{-1})
Maltose	PG	12.49 mM^{ns}	0.85^{ns}	8.25^{ns}
	gPG	9.19 mM	0.84	9.44
Maltodextrin	PG	0.049 mg/ml^{a}	2.74^{a}	26.60^{a}
	gPG	0.069 mg/ml^{b}	3.64^{b}	31.41^{b}

*Different letters (a, b) show the significantly difference ($p<0.05$) between the intact and glycated enzyme. The ns stands for "not significant difference" ($p>0.05$).

6. Hydrolysis of cornstarch with KLE in 4-L scale

Four liters of 35% cornstarch were hydrolyzed with KLE (0.09 mg protein/ml). The reducing power of mixture was rapidly increased within 60 min of incubation (Figure 38). Prolonged incubation caused slightly increase in reducing power that reached to the maximum after 120 min. KLE quickly lost its activity after hydrolysis form 0 to 60 min. After that, it could maintain 50% enzyme activity until 180 min. HMF released from glycated protein increased with increases in hydrolysis time.

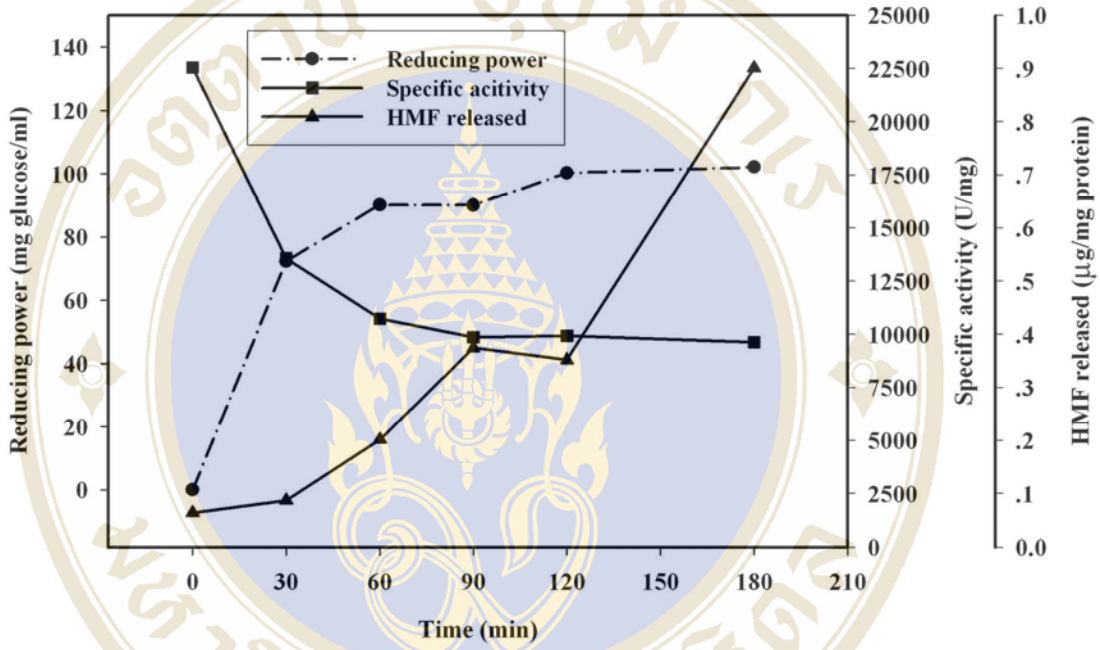


Figure 38 Hydrolysis of cornstarch by KLE at 95°C

CHAPTER VI
DISCUSSION

Thermostable α-amylase (KLE) and glucoamylase, the commercial amylolytic enzymes used in starch hydrolysis process, were studied for the effect of glycation on their properties and kinetic parameters. In case of α-amylase, KLE and mesophilic counterpart (BAN) were compared. The KLE and BAN enzyme mixtures showed a single band on SDS-PAGE with the MW of 58.1 and 58.4 kDa, respectively. This was similar to those previously reported (Mamo and Gessesse, 1999; Fitter *et al.*, 2001; Tsao *et al.*, 2003). On the other hand, the crude glucoamylase showed the three protein bands on SDS-PAGE. After the crude glucoamylase was submitted to the Q-sepharose Fast Flow column, the chromatogram showed two peaks of glucoamylase activity (Figure 14). Saure and coworkers (2001) reported that there are two forms of glucoamylase. The form with lacking of starch binding domain is less able to hydrolyze insoluble starch in comparison with the complete form. The first peak showed the single protein band on SDS-PAGE. The second peak showed two bands with MW above the first peak (Figure 15). Hence, the first peak eluted was glucoamylase with lacking of starch binding domain. Because of the substrate in the saccharification process is soluble maltodextrin, the form with lacking of starch binding domain was used for further purification. After the partially purified glucoamylase was applied onto the hydrophobic interaction column, the purity was assessed by SDS-PAGE and shown to be homogeneously pure. SDS-PAGE indicated a MW of 77.6 kDa. This was correspondent to the previous reports (Ohnishi *et al.*, 1991; Mase *et al.*, 1996).

Since most of research has been performed relevantly to glycated enzymes occurring in biological systems, under physiological conditions. There was no report on the inactivation of amylolytic enzymes due to the glycation, even the amylolytic enzymes are employed under high temperature and high reducing sugar conditions. The amylolytic enzymes can easily undergo glycation and might lead to the alteration of enzyme properties and functions under such conditions. The following discussion was described based on the glycation of amylolytic enzymes with saccharides as concluded in Table 7.

Table 7 Summary of glycation conditions of amylolytic enzymes.

Enzyme	Saccharide	Saccharide concentration (mg/ml)	Incubation temperature (°C)	pH of reaction mixture
KLE	maltodextrin	350	95	6.5[a]
(0.6 mg/ml)	glucose	350	95	6.5[a]
BAN	maltodextrin	350	90	6.5[a]
(0.6 mg/ml)	maltodextrin	350	70	6.5[a]
PG	maltodextrin	350	60	4.5[b]
(0.1 mg/ml)	xylose	350	60	4.5[b]

[a] Reaction conducted in 20 mM phosphate buffer solution.

[b] Reaction conducted in 20 mM acetate buffer solution.

Maltodextrin or glucose was incubated with KLE at 95°C for 180 min. The soluble protein gradually aggregated during incubation periods. The aggregation was due to the thermal treatment of protein resulted in unfolded state of three-dimensional conformation and protein denaturation. In general, the high temperature tends to increase the thermal motions of the atoms in the enzyme molecule. This leads to increasing rate of enzyme denaturation (Sadana, 1991). The rapid aggregation was observed in both of glucose addition and control experiment (KLE only). However, the rate of aggregation in the presence of glucose was slower than the control experiment by approximately 2 folds. This indicated that the sugar could prevent the thermal aggregation of KLE at some degree. Grossman (1976) reported that purified rat liver glucokinase exhibits remarkable thermal stability when the enzyme was incubated with 0.5 M glucose at 48.5°C for 30 min. The thermal stability of glucokinase with glucose addition was due to a substrate protecting influence and a polyhydric alcohol stabilizing effects. The stability of lysozyme with sugar addition against thermal treatment was studied by Back (1979). The temperature (T_m) of the maximum rate of denaturation was measured by the adiabatic scanning calorimeter. The T_m of lysozyme increased with 50% w/w glucose or sucrose addition, indicating the stability of enzyme against thermal treatment. They also reported that various forces and interactions, *i.e.* hydrogen bonding, electrostatic interaction and hydrophobic interaction, between enzyme and sugar affected the thermal stability of lysozyme. The thermal stability of purified pectin methylesterase was studied by Guiavarc'h *et al*. (2003). They found that the addition of sucrose to enzyme resulted in an increase in the thermal stability about six-fold. The protection of α-amylase against thermal aggregation was also observed in this study by incubation BAN with maltodextrin at 70 and 90°C. The results were similar to the KLE. The presence of maltodextrin also prevented the PG aggregation during maltodextrin hydrolysis. However, with the presence of xylose, the PG completely aggregated within 24 h at 60°C. This was due to the high reactivity to the glycation of pentose promoting the thermal aggregation of protein.

It is well known that the substrate can prevent the thermal denaturation of enzyme. This is due to the formation of weak interaction between enzyme and substrate in form of enzyme-substrate complex. Such weak interaction protects enzyme against thermal denaturation. Moreover, glucose which is the final product from maltodextrin hydrolysis can function as an enzyme stabilizer (Bryjak *et al*., 2004). The effect of sugars on the thermal stability of protein was discussed by Back *et al.* (1979) as follows:

1. *Hydrogen bonding* The thermal treatment results in the breaking of the hydrogen-bonded structure in which water successfully competes as both donor and acceptor with backbone and side-chain groups in the protein. The individual hydroxyl groups of sugars may also compete as indicated by the stabilizing effect in case of sugar addition.

2. *Electrostatic interactions* Glucose has lower dielectric constants than pure water. Thus, electrostatic interaction should be stronger in glucose solution than in water. However, this contribution to the stabilizing effect must be relatively small.

3. *Hydrophobic interaction* Hydrophobic interactions are generally considered to be the major factors in stabilizing the three dimensional structure of protein. The effect of sugar on protein stability depends on the stronger hydrophobic interaction between pairs of hydrophobic groups in sugar solution, comparing to pure water.

During incubation of both KLE and BAN with maltodextrin, the reducing power increased due to the action of α-amylase. This enzyme randomly hydrolyzes the α-1,4-glucosidic bond at internal position, releasing maltodextrin and oligosaccharides. In general, maltodextrin is the product from α-amylase hydrolysis and the maximum D.E. obtainable using bacterial α-amylase is around 40. In this case, the D.E. of commercial maltodextrin chain was in the range of 17-19. It was long enough to be further hydrolyzed by KLE and BAN, resulting in the increase in reducing power. In case of BAN incubated at 90^{o}C, the reducing power slightly increased from 158 to reach the maximum at 192 mg glucose/ml within 3 h, comparing to those incubated at 70^{o}C which increased from 138 to 260 mg glucose/ml within 48 h. This was due to the rapid aggregation of BAN at higher temperature. The protein aggregation due to the thermal treatment led to low level of active enzyme in the solution at higher temperature. Moreover, the thermal treatment also led to the alteration of protein

structure resulting in the inactivation of soluble enzyme. In case of PG, it hydrolyzes maltodextrin chain from non-reducing end yielding glucose units. Hence, the reducing power increased to reach the maxima at 340 mg glucose/ml.

Since the amylolytic enzymes can easily undergo glycation especially under high temperature and high reducing sugar conditions, these might alter the stability and kinetic parameters of enzymes. In this thesis, the glycation of amylolytic enzymes under industrial conditions was firstly reported and the alteration of enzyme properties and function due to the glycation was investigated.

When protein and reducing sugar present together, the reaction "so-called the glycation" between free amino group and carbonyl group naturally occurs. The extension of the glycation can be assayed based on the HMF released from mild acid hydrolysis of the glycated protein. As the aggregation of amylolytic enzymes was observed during incubation with reducing sugar, *i.e.* maltodextrin and glucose, we assumed that the glycation occurred. Under such circumstance, HMF released from both soluble and aggregate protein was investigated in order to monitor the progress of the glycation. The HMF released from both soluble and aggregate KLE (maltodextrin or glucose addition) increased with prolonged incubation time, indicating the progress of the glycation. The α-amylase from *B. subtilis* consists of 34 lysine, 21 arginine and 17 histidine residues from 659 amino acid residues locating in the 3 domains (Jayakumararaj *et al.*, 2005). These amino acid residues could react with reducing sugar to form the Amadori products. The glycation of KLE by maltodextrin was limited at some degree comparing to glycated by glucose. This might be due to the low reactivity to glycation of maltodextrin comparing to glucose. Another possibility might be that maltodextrin chain attached to enzyme molecule obstructed the covalently bound of the other maltodextrin chain. The progress of glycation was also observed in the incubation of BAN with maltodextrin in both temperatures (70 and 90^{o}C). BAN contains 30 lysine, 20 arginine and 14 histidine residues from 483 amino acid residues. The amount of HMF released from soluble glycated protein seemed to be constant after prolonged incubation period. This indicated that above some degree of glycation promoted the protein aggregation occurred. The HMF released from both soluble and aggregate enzymes indicated the higher degree of glycation of BAN incubated at 90^{o}C with maltodextrin for 2 to 3 h in comparison with KLE at the same

incubation period. Fujimoto *et al*. (1998) reported that domain A and domain C are conserved for almost of α-amylase while domain B is the most variable region. The amount of lysine residues of KLE locating on domain B was lower than BAN. This might result in the lower degree of glycation on KLE by approximately 1.2-1.6 folds in comparison with BAN. Unfortunately, amino acid sequences of domain C of BAN are not complete to predict the glycation of this domain. In case of PG, the extension of the glycation was also observed in both of soluble and aggregate enzymes. Glucoamylase from *A. niger* consists of 11 lysine, 18 arginine and 4 histidine residues from 534 amino acid residues (Svensson *et al.*, 1986). These amino acid residues could undergo glycation with the reducing sugar. The low level of glycation observed in all control experiments might occur during the enzyme production process.

From the increases in the amount of HMF released, it can be concluded that the glycation progressed during incubation of amylolytic enzyme with reducing sugar. However, the activity of amylolytic enzymes still remained even the enzyme underwent glycation. The results were in agreement with those of Seidler and Seibel (2000). They studied the glycation of aspartate aminotransferase by glyceraldehyde and ribose 5-phosphate. The results showed that briefly incubation of aspartate aminotransferase with glyceraldehydes or ribose 5-phosphate at 37^{o}C for 3.5 h resulted in an increase in enzyme activity. This was due to the glycation promoted conformational change of protein that increases domain flexibility, which translated into greater catalytic rates. However, prolonged incubation at 37^{o}C for 64 h showed that the conformation of glycated aspartate aminotransferase changed to a more rigid structure resulting in the lower catalytic rates. These are contrary to those reported in Cu-Zn-superoxide dismutase and glutathione peroxidase glycated under physiological conditions by Arai *et al.* (1987) and Baldwin, *et al*. (1995), respectively. Arai and coworkers reported that when lysine residues located in an active site liganding loop of Cu-Zn-superoxide dismutase molecule were glycated by glucose, the enzyme loosed its activity. Baldwin, *et al*. (1995) found that enzyme activity decreased when glutathione peroxides was glycated by glucose. The inactivation of enzyme due to the glycation depended on two major factors, *i.e.* the amino acid residue position that glycation occurred and the progress of the glycation. When the amino acid residue involved in catalytic mechanism is glycated, the enzyme lost its activity. On the other

hand, if the glycated amino acid residues did not involve in catalytic mechanism, the enzyme could maintain its activity. This was due to the glycated enzyme existed in a more open or flexible state resulting in the greater catalytic rates. On the other hand, the higher degree of glycation resulted in more rigid of enzyme structure leading to the contrary effect.

The MW of the glycated protein in the form of the Amadori products would increase due to the attachment of sugar moieties to the protein molecule. The MW of KLE incubation with maltodextrin and glucose increased after 2-h incubation about 1.8 and 1.4 kDa, respectively. These confirmed that the sugar moieties covalently bound to the enzyme molecule. The result was correspondent to Luthra and Balasubramanian (1993). They detected the increase in MW of both γ- and α-crystallins by approximately two-fold during incubation with fructose under physiological conditions. This was due to the formation of dimer by covalent non-disulfide intermolecular cross-links. Nevertheless, the intermolecular cross-link did not observe in case of glycated KLE. The protein band on SDS-PAGE of KLE incubation with glucose became broad band covering MW in the range of 29 to 99 kDa at 95^{o}C for 3 h incubation period. This was due to the fragmentation of protein resulted from exposure to glucose. The Amadori products of proteins with glucose subsequence yield reactive oxygen species with trace level of heavy metal as catalyst. These free radicals can induce protein degradation (Hunt, *et al*., 1993). In the presence of glucose, KLE might degrade resulted in the soluble peptide and showed the broad band on SDS-PAGE. This phenomenon would not occur in KLE incubated with maltodextrin. The increase in MW was also observed on BAN molecule incubated with maltodextrin at both 70 and 90^{o}C. The MW increased from 58.4 kDa to 61.2 and 60.7 kDa when BAN was incubated at 70 and 90^{o}C for 48 and 2 h, respectively. The higher MW of BAN glycated at 70^{o}C was due to the higher amount of maltodextrin attached to the enzyme molecule. The protein band could not be observed in control experiment (KLE or BAN only). This was due to the protein aggregation. In case of PG, the increase in MW due to the glycation could not be detected on SDS-PAGE. This might be due to the lower degree of glycation on glucoamylase molecule. Moreover, glucoamylase is a glycoprotein containing mono-, di- or trisaccharides by approximately 1-20% (w/w) saccharides. The different sugar

content on the enzyme molecule resulted in the varying MW, indicated by the broad band on SDS-PAGE. This was difficult to observe the increase in MW due to the sugar moieties attachment. Glucoamylase stained with toluidine blue on SDS-PAGE showed the similar purple bands in both PG incubated with maltodextrin and control experiment (PG only). This might be due to the low degree of glycation of saccharide glucosylated on PG molecule and resulted in the inability to observe the difference between glycated and intact form.

Theoretically, the HMF released from glycated amylolytic enzymes relates to number of sites of the enzyme molecule attached to saccharide. The relation between HMF released from glycated bovine serum albumin (BSA) and total sugar content in the glycated BSA was investigated. From the equation derived from 2.2, amount of HMF released could be converted to number of saccharide molecule attached to protein molecule. We assumed that number of saccharide molecules glycated on amylolytic enzymes indicated the site number of saccharide molecules attached to the enzyme molecule. The estimation is shown in Table 8, 9 and 10 for KLE, BAN, and PG, respectively. The glycated sites were increased with increases in incubation period. After incubation KLE with dextrin for 2 h, 3 sites of enzyme molecule were glycated. The result was similar to glycated BAN at 70 and 90^{o}C for 48 and 2 h, respectively. The glycation site decreased when the glycated KLE and BAN aggregated. This was due to the degradation of the Amadori products. The results indicated that at some degree of glycation, it promoted the aggregation and the Amadori product further degraded. The number of glycated site and the increase in MW were calculated for the amount of glucose unit per 1 glycated site. It was estimated that 4-glucose-unit saccharide attached to 1 glycated site. The results were correspondent to D.E. and average MW of maltodextrin reported by Carvajal *et al*. (1999). They reported that the D.E. of 18 and 25 showed the average MW of 1000 and 720 Da, and referred to the maltodextrin at such D.E. consisting of 6 and 4 glucose units, respectively. In this study, D.E. of maltodextrin was 17-19. Maltodextrin was further hydrolyzed resulting in shorter saccharide covalently bound with the enzyme molecule. In case of PG, only 1 site was glycated. This might be due to the lower amount of lysine residues and lower incubation temperature, comparing to glycated KLE and BAN. In case of glycated KLE and BAN, there was some

limitation of saccharide to covalently bind with enzyme molecule. This is suggested by the high free amino groups on enzyme molecule (as shown before) but only 1-6 sites were glycated. This was due to the position of free amino acid residue which side chain located outer or inner of enzyme molecule. Another possibility was the adjacent amino acid residues promoted or obstructed the sugar binding.

Table 8 Average glycation site that saccharide attached to glycated KLE molecule

Time (min)	Average site/molecule KLE	
	Soluble glycated KLE	Glycated KLE aggregate
0	1	-
30	2	2
60	2	2
120	3	2
180	4	3

Table 9 Average glycation site that saccharide attached to glylated BAN molecule

Time (h)	Average site/molecule BAN BAN glycated at 70°C		Time (h)	Average site/molecule BAN BAN glycated at 90°C	
	Soluble gBAN	gBAN aggregate		Soluble gBAN	gBAN aggregate
0	1	-	0	2	-
6	2	-	1	2	3
12	2	2	2	4	3
24	3	3	3	5	3
48	3	5	4	6	2
72	3	6			

Table 10 Average glycation site that saccharide attached to glycated PG molecule

Time (h)	Average site/molecule PG	
	Soluble glycated PG	Glycated PG aggregate
0	3	-
12	4	3
24	4	4
48	no data	4
72	no data	4

The different migration pattern of protein band was observed on Native-PAGE of both KLE and BAN. In case of KLE, the different pattern of migration was observed between KLE incubation with maltodextrin or glucose, especially at 3-h incubation time. This suggested that the greater progress of the glycation when enzyme was glycated by glucose was greater and led to the protein denaturation. The migration of protein in Native-PAGE depends on native charge, shape and size of the protein. This indicated the difference in migration pattern of KLE due to the glycation. This reaction causes the neutralization of positive charges and results in more negative charge on the protein molecule (Luthra and Balasubramanian, 1993), leading to the faster migration on the Native-PAGE of glycated enzyme. The different migration patterns were observed during incubation BAN at different temperatures. The protein band of BAN incubated at 70^{o}C for 48 h was migrated below the protein band of BAN incubated at 90^{o}C for 2 h indicating the lower positive charges on BAN molecule. These results were in agreement with the increase in MW of BAN from SDS-PAGE. However, the migration pattern of glycated PG on Native-PAGE was similar to that of the intact form.

Since the pK_a of lysine and arginine is above the pH of reaction mixture. In such condition, the side chains of those amino acid residues were protonated and resulting in the positive charges side chains on the protein molecule. The glycation causes the neutralization of positive charges side chain and might be resulted in the change of pI value. Hence, the pI value of KLE became more acidic due to the glycation. Luthra and Balasubramanian (1993) reported that the pI values of both γ and α-crystalline chains, when underwent glycation with fructose under physiological conditions, are shifted from the usual range of 7-8.5 to 5-6, and 4.4-4.85 to 3.1-4.6, respectively. We assumed that the protein became more acidic upon the glycation since the glycation leads to the neutralization of positive charges on the protein molecule. However, glycation did not strongly affect on the pI values of the PG. The lowest pI value of standard protein is 3.5. Since the pI value of PG might be lower than that standard protein resulted in limitation to detect the pI value at pH below 3.5. Another possibility, it might be due to the low degree of glycation in this experiment. Generally, glucoamylase contains 11 lysine residues which were fewer amounts in

comparison with KLE. Moreover, the incubation temperature was lower than that of KLE. These resulted in the low degree of glycation on glucoamylase molecule.

Table 11 Summary of stability and kinetic parameters of glycated enzymes comparing to the intact forms.

Enzyme	Glycation temperature (°C)	Thermal stability		pH stability		K_m	V_{max}	K_{cat}
		solubility	activity	solubility	activity			
gKLE	95	+	+	+	+	nd	nd	nd
gBAN	90	+	-	+	+	nd	nd	-
gBAN	70	+	-	-	-	nd	nd	-
gPG	60	nd	+	nd	+			
-maltose						nd	nd	nd
-maltodextrin						+	+	+

+ = Greater stability or increased in kinetic parameter values

- = Less stability or decreased in kinetic parameter values

nd = not different

-Gelatinized cornstarch was used as the substrate for the glycated KLE and BAN for kinetic parameters determination.

-Maltose and maltodextrin were used as substrate for kinetic parameters determination of gPG.

Thermal stability of glycated enzyme was investigated comparing to the intact form. In case of KLE, the glycated enzyme was more resistant to thermal treatment resulted in greater stability in protein solubility and increased relative enzyme activity in comparison with the intact form. The greater thermal stability of gKLE was due to the glycation of maltodextrin on the protein molecule. We proposed that maltodextrin covalently bound with the KLE molecule resulted in the formation of hydrogen bond between oxygen atom on maltodextrin and acidic amino acid side chain when the glycated site was near those side chains. The hydrophobic interaction could form in case of the position of maltodextrin attached site was close to the aromatic ring of amino acid side chain. Those interactions led to the more rigid of protein structure resulting in the resistance to the thermal treatment. Another possibility may be that the saccharide attachment limited the conformational space available to the unfolded state. The unfolded state leads to the protein denaturation. The results were correspondent with the work of Seidler and Seibel (2000). They found that prolonged incubation of aspartate aminotransferase with glyceraldehyde at 37^{o}C for 14.5 h resulted in a higher temperature required to aggregate the glycated enzyme. The thermal stability increased with increasing concentration of glyceraldehyde. They suggested that the glycated form might exist in a more compact and rigid conformation resisted the denaturing effects of temperature. In general, hyperthermophilic and mesophilic homoloques have a common basic stability afforded by the hydrophobic interaction and core residues involved in secondary structure and better conserved than surface area feature. The interaction involving in the stability of the hyperthermophilic protein is often found in the less conserved area of the protein (Vieille and Zeikus, 2001). Moreover, the crystal structure of extremophilic enzymes also indicates increases in the number and strength of the weak interactions involving in protein stability form mesophilic to thermophilic proteins (D'Amico *et al*., 2003). Enough experimental data (i.e., sequence, mutagenesis, three-dimensional structure of protein and thermodynamics) has been accumulated to conclude that hydrogen bond, ion pairs, Van der Waals and hydrophobic interactions are responsible for the stability of hyperthermophilic protein (Vieille and Zeikus, 2001). In case of glycation, the presence of saccharide might form the interactions between saccharide and protein molecule to promote those interactions resisting to the

thermal denaturation. Fernandez and coworker (2003) reported that thermal stability of trypsin chemically modified by amino cyclodextrin derivatives increased by about 2.4 to 14.5^{o}C after modification. The amino cyclodextrin derivatives were specifically attached to the carboxylate group locating at Asp153 and Glu186. They suggested that this was due to intramolecular cross-link between the cyclodextrin moieties and the aromatic amino acid residues located near the covalent modification points. It is expected that the hydrophobic nature of these cross-link must confer resistance to the enzyme at higher temperature.

The increase in thermal stability of KLE due to the glycation was contrary to BAN glycated by maltodextrin. The results showed that the glycation led to the inactivation of the BAN both incubated at 70 and 90^{o}C while the protein was still solubilized in the reaction mixture. This suggested that the protein glycation of BAN caused conformational changes, leading to resistance of aggregation due to thermal treatment. However, this conformational change lowered rate of catalytic activity. The results were contrast to glycated KLE. This was due to the conformational change of BAN due to the progress of the glycation. BAN underwent glycation easier than KLE. Because BAN contains higher amount of lysine residues in domain B and C. Fitter *et al*. (2001) reported that one of the mainly features contribute to thermostability of α-amylase is some additional salt bridges in the enzyme molecule. Those interactions result in the higher structural rigidity of thermostable enzyme, leading to the slower rate of the glycation reaction to alter the three-dimensional of protein structure in comparison with the mesophilic enzyme. The slower rate of the glycation resulted in the greater thermal stability of glycated KLE. On the other hand, it might be due to the position of amino acid which sugar moiety covalently bound and the amount of free amino group containing in the enzyme molecule. Fujimoto *et al*. (1998) reported that domain A and domain C are conserved for almost of α-amylase while domain B is the most variable region (Figure 39 and 40). Figure 7 shows the ribbon model of α-amylase from *B. subtilis*. Hence, domain C locates on the C-terminal side of the central β-barrel of domain A and is overlaid by domain B, the free amino groups composed in both domain B and C might be easy for being attached by the sugar moieties due to the glycation. The amount of lysine residues of KLE locates on domain B and C was lower than BAN, resulted in the lower degree of glycation of

KLE in comparison with BAN. The degree of glycation affected the enzyme activity was also observed in glutathione reductase glycated by fructose. As a result, the increase in mole of fructose bound to the enzyme molecule in form of the Amadori products results in the progress of enzyme inactivation (Blakytny and Harding, 1997).

Since the glycation involved in thermal stability of thermostable α-amylase, the amount of amino acid residues containing free amino group, *i.e*. lysine, arginine and histidine, might relate to the ability of the enzyme to resist to the heat. Table 6 shows the amount of amino acid residues containing free amino group of α-amylase from different sources (GENBANK). The amount of lysine residues locating almost in domain A and B tends to increase form psychrophilic to thermostable α-amylase in a chain length of 448 amino acids. This suggested that the amino acid compositions of α-amylase are easy to undergo glycation to stabilize the enzyme from thermal denaturation. Especially in case of KLE, 17 lysine residues locating on the C-terminal side (domain C) of the central β-barrel of domain A, were easy to undergo glycation. Because the position of domain C locates freely from domain A and B (Figure 7).

In case of thermal treatment of glycated PG, the enzyme activity of all experiments decreased during prolonged incubation. These were similar to the results reported in thermostable glucoamylase at 70^{o}C with different pH (Munch and Tritsch, 1990). However, the glycated form could maintain higher activity than the intact form. Hence, the glycation led to the more thermal resistance. Not only the thermal stability resulted form the weak interaction formation between sugar and protein molecule, but also the glycation on the protein molecule promoted conformational change and increases in domain flexibility. This might lead to the greater catalytic rate. The presence of sugar with *O*-glycosylated linkage on glucoamylase molecule affected the enzyme stability. This linkage is essential for maximum stability of both catalytic domain and starch binding domain on glucoamylase enzyme because of the limitation of conformation space to unfold the structure which lead to the greater stability on enzyme structure (Williamson *et al.*, 1992). The glycation leading to the sugar covalently bound to amylolytic enzymes with *N*-glycated linkage might cause the limitation of space to unfold structure resulting in the resistance to the thermal treatment of glycated enzyme.

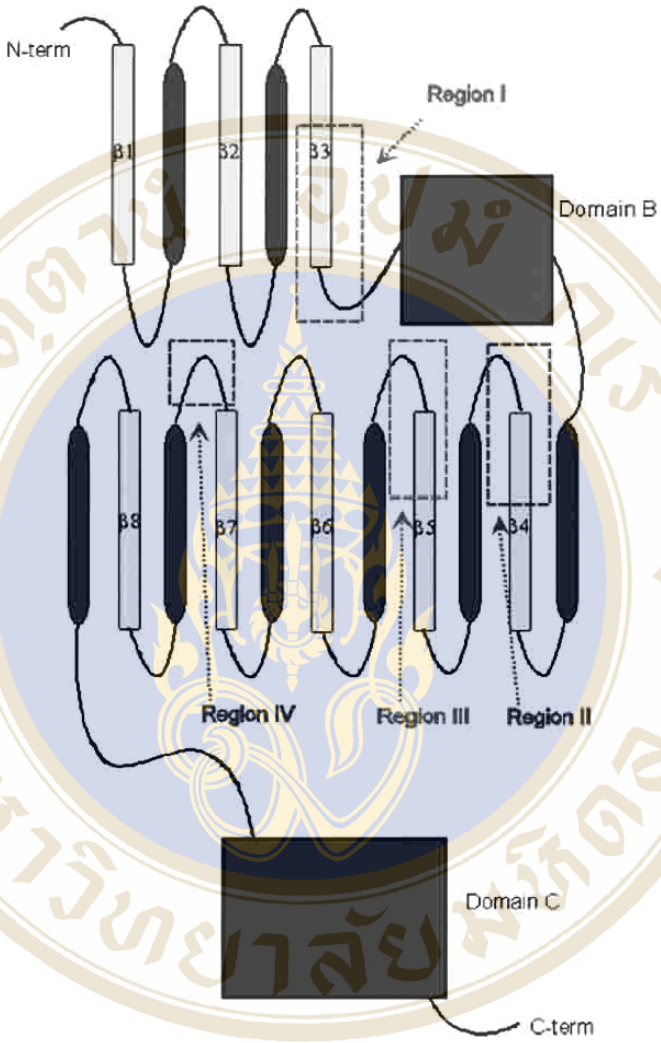


Figure 39 Topology diagram of α-amylases. The positions of the four conserved sequenced patterns are indicated with dashed boxes (Nielsen and Borchert, 2000).

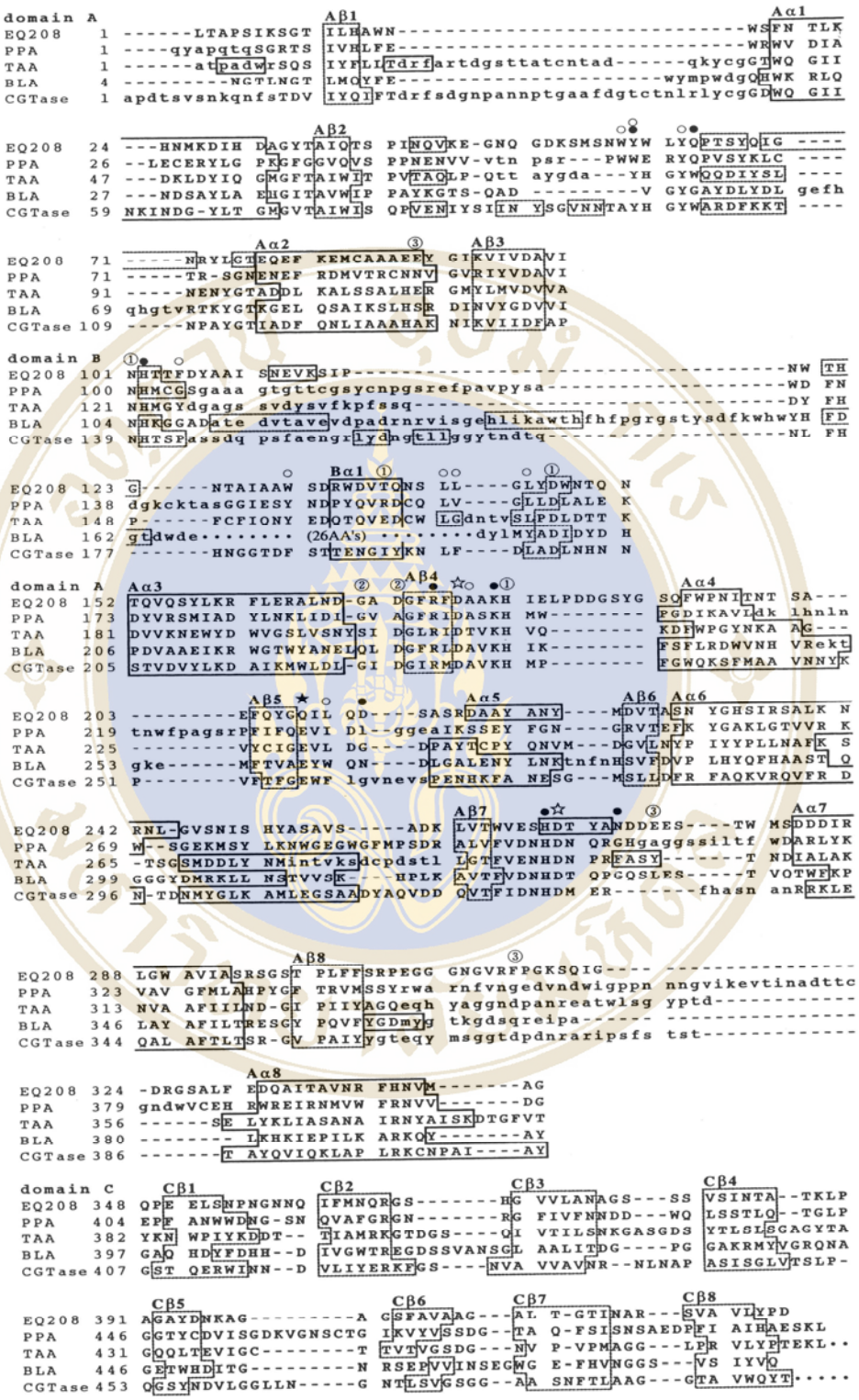


Figure 40 Topological alignment of α-amylase from *Bacillus subtilis* (EQ208), pig pancreatic (PPA), *B. licheniformis* (BLA) and CGTase from *B. circulans*. Small letters indicate unpaired residues in this alignment. Residues that belong to α-helices and β-strands are surrounded by ▭ and ▭, respectively. Residues involved in each calcium binding are indicated with ① ② and ③. The catalytic site residues are indicated with ☆ or ★. Residues involved in the hydrophilic and hydrophobic interactions with substrate are indicated with ● and ○, respectively (Fujimoto *et al.*, 1998).

The results from pH stability study suggested that the glycated α-amylase was more tolerant to wider range of pH than the intact form. The results are similar to those of Darias and Villanga (2001). They studied the chemical modification of cellulase by chitosan and reported that the pH stability was increased in modified enzyme for the range of pH between 1.0 and 3.2. The neutralization of positive charges in the α-amylase molecule is due to the glycation led to more acidic in protein. Both KLE and BAN would appear to be more stable at neutral pH in the range of 5.63-6.54 than at its pI. The neutralization of the free amino groups might be expected to increase the conformational stability of the protein. In case of PG and gPG, the enzyme rapidly lost its activity at pH 5.5 and completely inactivated at pH 6.5 while the protein still solubilized. The hydrolytic action of glucoamylase is acid-base catalysis. This involves transferring of proton to the glycosidic oxygen from a general acid catalyst (Glu179), following with formation of an oxocabonium ion, and a nucleophilic attack of water assisted by a general base catalyst (Glu400). In general, glucoamylase is active when acid catalyst is un-ionized (Sauer, *et al.*, 2000). As the results, we speculated that the inactivation at pH above optimum was due to ionization of Glu179 above pKa (pH 4.4).

It can be speculated that the glycation led to the greater thermal stability and wider range of pH tolerance of amylolytic enzymes in term of solubility and activity. This was possibly due to the formation of new interactions *i.e.* hydrogen bonding, electrostatic interactions and hydrophobic interaction, between covalently bound saccharides and enzyme molecule. Moreover, the glycation led to the conformational flexibility which translated to the greater catalytic activity. However, the higher degree of glycation resulted in the contrary effects. The high degree of glycation led to the more rigidity of protein structure and promoted the thermal aggregation of protein.

The glycation did not strongly affect on kinetic parameters of KLE. The binding affinity (K_m), catalytic rate (V_{max}) and turnover number (K_{cat}) of gKLE were not significant different in comparison with the intact form, ($p>0.05$). These results were contrary to Baldwin *et al.* (1995). They found that the glycation of glutathione peroxidase under physiological conditions increased the K_m by approximately 3 folds. This is due to the glycation of Lys110, locating near the active site. The glycation of

this lysine residue results in changes in the hydrogen bond lengths of other residues in its active site helix and also influences the interatomic distances between other pairs of active site residues leading to the lower binding affinity to the substrate. However, V_{max} of glutathione peroxidase was not affected by the glycation. The glycation did not affect that kinetic parameters of KLE since the glycation possibly did not occur on amino acid residues involving in catalytic mechanism or substrate binding mechanism. The glycation did not affect the K_m and V_{max} of BAN glycated by maltodextrin at both incubation temperatures (70 and 90°C) as well. However, the K_{cat} of glycated BAN was lower than the intact form. This indicated that the glycation lowered the capacity of BAN to convert gelatinized cornstarch into maltodextrin. The lower turnover number of gBAN might be due to the conformational changes of protein resulting from the glycation, becoming more open or flexible state which might translate into greater conversion capacity. The kinetic parameters were the same in case of BAN glycated by maltodextrin at 70 and 90°C. This was due to the similar level of degree of glycation between two glycated forms, indicating by the amount of HMF released from glycated protein.

In case of PG, the effect of glycation on kinetic parameters was different depending on types of substrate. In case of substrate maltose, the glycation did not affect the kinetic parameters of PG. Meanwhile, the glycation lowered the binding affinity to substrate maltodextrin. It was possible that the sugar moieties attached on PG molecule obstructed the formation of enzyme-substrate complex. The glycation elevated the rate of reaction. Moreover, the glycation resulted in a higher efficiency of glucoamylase to convert substrate into glucose. This might be due to the glycation causing the more open and flexible state of the structure in comparison with the intact enzyme.

The progress of glycation was investigated in 4-L scale of cornstarch hydrolysis. The reducing power reached the maxima with in 1 h-incubation due to the action of thermostable α-amylase and became constants after prolonged incubation. The results were in agreement with specific activity of the enzyme. The specific activity decreased with increases incubation period. The amount of HMF increased when incubation time increased. This indicated that the glycation also occurred in the large-scale experiment and progressed after prolonged incubation period.

Since the progress of the glycation was observed in amylolytic enzymes and showed the greater thermal stability due to the glycation under industrial starch hydrolysis conditions, especially in KLE and PG. The compromising conditions among temperature, hydrolysis duration, pH, concentration of substrate and enzyme properties should be realized in order to give a maximum thermal stability of the enzymes. In liquefaction process, the operating temperature might be decreased to stabilize the glycated α-amylase and increased the hydrolysis period to give the maximum yeild. Because calcium ion plays an important role on α-amylase stability (Takase *et al.*, 1988). The synergistic effect of glycation and calcium ion on the stability should be study in order to reduce the concentration of calcium ion.

For the further studies on the effect of glycation on properties and kinetic parameters of amylolytic enzymes under high reducing sugar conditions are recommended to conduct such as;

1. Identification of the glycation site on glycated amylolytic enzymes by tryptic digestion, following by isolation of glycated peptides. Using peptide mapping technique to identify the glycation site which lead to the clear discussion on the effect of glycation on enzyme properties and kinetic parameters.

2. In this study, we used the same degree of glycation to study the effect of glycation on kinetic parameters. The different degree of glycation might affect the kinetic parameters. Hence, the correlation between degree of glycation and kinetic parameters should be investigated.

3. The MW changes of enzyme due to the attachment of saccharides must be confirmed by various techniques such as, LC-MS or MALDI-TOF. By these techniques, the amount of saccharides attached to the enzyme molecule should be calculated directly from the MW changes. This is also to confirm the implication of HMF released as evidence of glycation.

4. In order to detect the glycation on PAGE, many visualization techniques should be used such as, glycoprotein staining.

5. The effect of glycation on amylolytic enzymes should be studied in the other enzymes such as, glucose isomerase, invertase, and xylanase in order to describe the properties and functions of those enzymes due to the glycation.

Table 12 The amount of amino acid residues containing free amino group in α-amylase from different sources. The data were obtained from GENBANK.

Enzyme	Type	The amount of amino acid residues			Total	Total amino acid contained	Sources
		Lysine	Arginine	Histidine			
α-Amylase	Thermostable	42	33	11	86	690	*Pyrococcus furiosus*
α-Amylase	Thermostable	21	14	12	47	460	*Pyrococcus woesei*
α-Amylase	Thermostable	22	13	12	47	457	*Thermococcus hydrothermalis*
KLE	Thermostable	34	21	17	72	659	*B. subtilis*
α-Amylase	Thermostable	31	24	25	80	512	*B. licheniformis*
α-Amylase	Thermostable	32	21	12	65	549	*B. sterothermophilus*
α-Amylase	Thermostable	16	30	20	66	650	*Thermomonospora curvaya*
BAN	Mesophilic	30	20	14	64	483	*B. amyloliquefacians*
Taka	Mesophilic	20	10	7	37	499	*A. oryzae*
α-Amylase	Mesophilic	23	28	12	63	496	*Human saliva*
α-Amylase	Mesophilic	23	28	11	62	511	*Human pancreatic*
αAmylase	Mesophilic	20	28	9	57	496	*Pig pancreatic*
α-Amylase	Psychrophilic	12	13	12	37	448	*Pseudoalteromonas haloplanktis*

CHAPTER VII
CONCLUSIONS

1. The glycation occurred and progressed during incubation of amylolytic enzymes with reducing sugar under high temperature conditions. This was indicated by the increases in amount of HMF released from enzyme glycated. The extension of glycation was observed in both aggregate and soluble enzymes. The degree of glycation was depended on the type of enzyme, type of reducing sugar, temperature and pH. Both SDS-PAGE and Native-PAGE, and pI value changes supported the evidences for the attachment of sugar moieties on the enzyme molecule due to the glycation. However, the glycation on glucoamylase molecule did not show strongly effect on the MW change on SDS-PAGE and protein migration pattern on Native-PAGE and pI value changes.

2. The glycation led to the greater thermal stability and more tolerance to wider range of pH of amylolytic enzymes. KLE and PG were more resistant to thermal treatment when enzymes were glycated. Meanwhile, BAN showed the contrary effects. This might be due to the position of amino acid residues that the sugar moieties were covalently bounded. The glycated amylolytic enzymes were more tolerant to pH than the intact form.

3. The glycation did not show strong effect on kinetic parameters of KLE and BAN. However, the glycation led to the lower capacity to convert gelatinized cornstarch of BAN. The lower affinity to bind substrate maltodextrin was observed when PG was glycated. The glycation also led to the elevation of catalytic activity and greater capacity to convert maltodextrin into glucose.

4. The progress of glycation of thermostable α-amylase was also observed in the 4-L scale cornstarch hydrolysis, indicating by the increases in amount of HMF released.

BIBLIOGRAPHY

Aoki, T., Hiidome, Y., Kitahata, K., Sugimoto, Y., Ibrahim, H.R. and Kato, Y. (1999). Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the Maillard reaction. *Food Res Inter*, 32, 129-133.

Apar, D.K. and Ozbez, B. (2004). α-Amylase inactivation during corn starch hydrolysis. *Process Biochem*, 39, 1877-1892.

Arai, K., Maguchi, S., Fujii, S., Ishibashi, H., Oikawa, K., and Taniguchi, N. (1987). Glycation and inactivation of human Cu-Zn-superoxide dismutase. *J Biol Chem*, 262, 16969-16972.

Ashoor, A.H. and Zent, J.B. (1984). Maillard browning of common amino acids and sugars. *J Food Sci*, 49, 1206-1207.

Back, J.F., Oakenfull, D. and Smith, M.B. (1979). Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry*, 18, 5191-5196.

Baldwin, J.S., Lee, L., Leung, T.K., Muruganadam, A., and Mutus, B. (1995). Identification of the site of non-enzymatic glycation of glutathaione peroxidase: rationalization of the glycation-related catalytic alterations on the basis of the three-dimensional protein structure. *Biochim Biophys Acta*, 1247, 60-64.

Belitz, H.D. and Grosch, W. (1999). *Food Chemistry*. (2nd ed.). Germany: Springer-Verlag.

Benfeld, P. (1955). Amylase. In S.P. Colowick and N.O. Kaplan, *Methods in enzymology*, (pp. 149-158). New York: Academic Press.

Blankytny, R. and Harding, J.J. (1997). Bovine and human α-crystallins as molecular chaperones: prevention of the inactivation of glutathione reductase by fructation. *Exp Eye Res*, 64, 1051-1058.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-254.

Brady, R.L., Brzozowski, A.M., Derewanda, Z.S., Dodson, E.J. and Dodson, G.G. (1991). Solution of the structure of *Aspergillus niger* acid α-amylase by combined molecular replacement and multiple isomorphous replacement methods. *Acta Crystallog sect B*, 47, 527-535.

Bryjak, J., Ciesielski, K., and Zbicinski, I. (2004). Modeling of glucoamylase thermal inactivation in the presence of starch by artificial neural network. *J Biotechnol*, 114, 177-185.

Carney, S.L. 1994. Proteoglycan. In M.F. Chaplin and J.F. Kennedy, *Carbohydrate analysis: A practical approach*, (pp. 125-180). New York: Oxford University Press.

Carvajal, P.A., MacDonald, G.A. and Lanier, T.C. (1999). Cryostabilization mechanism of fish muscle proteins by maltodextrin. *Cryobiology*, 38, 16-26.

Chiou, Y., Tomer, K.B. and Smith, P.C. (1999). Effect of nonenzymatic glycation of albumin and superoxide dismutase by glucuronic acid and suprofen acyl glucuronide on their functions in vitro. *Chemico-Biol Interact*, 121, 141-159.

Crabb, W.D. and Mitchinson, C. (1997). Enzymes involved in the processing of starch to sugars. *Trends Biotechnol*, 15, 349-352.

D' Amico, C., Gerday, C. and Feller, G. (2003). Temperature adaptation of proteins: engineering mesophilic-like activity and stability in a cold-adapted α-amylase. *J Mol Biol*, 332, 981-988.

Damodaran, S. (1996). Amino acid, peptides, and proteins. In O.R. Fennema, *Food Chemistry*. (3rd ed.), (pp. 321-429). New York: Marcel Dekker, Inc.

Darias, R. and Villanga, R. (2001). Functional stabilization of cellulase by covalent modification with chitosan. *J Chem Technol Biotechol*, 76, 489-493.

Dubois, M., Gilles, K.A., Hamilton, J.K, Rebers, P.A and Smith, F. (1956). Calorimetric method for determination of sugars and related substances. *Anal Chem*, 28, 350-356.

Fennema, O.R. (1996). *Food Chemistry*. (3rd ed.), New York: Marcel Dekker, Inc.

Fernandez, M., Fragoso, A., Cao, R. and Villalonga, R. (2003). Improved functional properties of trypsin modified by monosubstituted amino-β-cyclodextrins. *J Mol Catalysis B: Enzymatic*, 21, 133-141.

Fitter, J., Herrmann, R., Dencher, N.A., Blume, A., and Hauss, T. (2001). Activity and stability of thermostable α-amylase compared to it mesophilic homologue: mechanisms of thermal adaptation. *Biochemistry*, 40, 10723-10731.

Fujimoto, Z., Takase, K., Doui, N., Momma, M., Matsumoto, T. and Mizuno, H. (1998). Crystal structure of a catalytic-site mutant α-amylase from *Bacillus subtilis* complexed with maltopentose. *J Mol Biol*, 277, 393-407.

Gerhartz, W. (1990). *Enzymes in Industry*. New York: VHC Publishers.

Glomb, M.A. and Monnier, V.M. (1995). Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J Biol Chem*, 270, 10017-10026.

Grossman, S.H. (1976). A note on the dual role of glucose in the protection of glucokinase against inactivation. *Biochim Biophys Acta*, 452, 392-397.

Guiavarc'h, Y., Sila, D., Duvetter, T., Loey, A.V. and Hendrickx, M. (2003). Influence of sugars and polyols on the thermal stability of purified tomato and cucumber pectinmethylesterase: a basis for TTI development. *Emz Microb Technol*, 33, 544-555.

Humany, A., Kislinger, T., Becker, C. and Pischetsrieder, M. (2002). Qualitative determination of specific protein glycation products by matrix-assisted laser desorption/ionization mass spectrometry peptide mapping. *J Agric Food Chem*, 50, 2153-2160.

Hunt, J.V., Bottoms, M.A. and Mitchinson, M.J. (1993). Oxidative alterations in the experimental model of diabetes mellitus are due to protein-glucose adduct oxidation. *Biochem J*, 291, 529-535.

Jayakumararaj, R., Senthikumar, V. and Gunasekaran, P. (2005). Cloning and sequencing of alpha-amylase gene from *Bacillus subtilis*-233. *Genetic*, India.

Knorr, D. (1987). *Food Biotechnology*. New York: Marcel Dekker.

Kondo, A and Urabe, T. (1995). Temperature dependence of activity and conformation changes in α-amylases with different themostability upon adsorption on ultrafine silica particles. *J Colloid Interface Sci*, 174, 191-198.

Kraulis, P.J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallog*, 24, 946-950.

Kuriki, T. and Imanaka, T. (1999). The concept of the α-amylase family: Structure similarity and common catalytic mechanism. *J Biosci Bioeng*, 87, 557-565.

Lapolla, A., Traldi, P. and Fedele, D. (2005). Importance of measuring products of non-enzymatic glycation of proteins. *Clinical Biochem*, 38, 103-115.

Leammli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Lertsiri, S., Maungma, R., Assavanig, A. and Bhumiratana, A. (2001). Roles of the Maillard reaction in browning during Moromi process of Thai soy sauce. *J Food Process Preserv*, 25, 149-162.

Lumdubwong, N. and Seib, P.A. (2001). Low- and medium-DE maltodextrins from waxy wheat starch: preparation and properties. *Starch/Starke*, 53, 605-615.

Luthra, M. and Balasubramanian, D. (1993). Nonenzymatic glycation alters protein structure and stability. *J Biol Chem*, 268, 18119-18127.

MacGregor, E.A., Janecek, S. and Svensson, B. (2001). Relationship of sequence and structure to specificity in the α-amylase family of enzymes. *Biochim Biophys Acta*, 1546, 1-20.

Machius, M., Weigand, G. and Huber, R. (1995). Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å resolution. *J Mol Biol*, 246, 545-559.

Mamo, G. and Gessesse, A. (1999). Purification and characterization of two raw-starch-digesting thermostable α-amylase from a thermophilic *Bacillus*. *Enz Microb Technol*, 25, 433-438.

Martins, S.I.F.S., Jongen, W.M.F. and Boekel, M.A.J.S. (2001). A review of Maillard reaction in food and implications to kinetic modeling. *Trends FooD Sci Technol*, 11, 364-373.

Mase, T., Matsumiya, Y., Mori, S., and Matsuura, A. (1996). Purification and characterization of a novel glucoamylase from *Acrenonium* sp. YT-78. *J Ferment Bioeng*, 81, 347-359.

Miller, A.G., Meade, S.J. and Gerrard, J.A. (2003). New insights into protein crosslinking via the Maillard reaction: structural requirement, the effect of enzyme function, and predicted efficacy of crossling inhibitors as anti-ageing therapeutics. *Biorganic Med Chem*, 11, 843-852.

Molares, F.J. and Jimenez-Perez, S. (1998). Study of hydroxymethylfurfural formation from acid degradation of the Amadori product in milk-resembling systems. *J Agric Food Chem*, 46, 3885-3890.

Munch, O. and Tritsch, D. (1990). Irreversible thermoinactivation of glucoamylase from Aspergillus niger and thermostabilization by chemical modification of carboxyl groups. *Biochim Biophys Acta*, 1041, 111-116.

Namiki, M. (1988). Chemistry of Maillard reaction: recent studies on the browning reaction mechanism and the development of antioxidants and mutagens. *Adv Food Res*, 32, 115-184.

Nielsen, J.E. and Borchert, T.V. (2000). Protein engineering of bacterial α-amylases. *Biochim Biophys Acta*, 1543, 253-274.

O'Brien, J. and Massey, P.A. (1989). Nutritional and toxicological aspects of the Maillard browning reaction in foods. *Crit Rev Food Sci Nutr*, 28, 211-248.

Odibo, F.J.C. and Ulbrich-Hofmann, R. (2001). Thermostable α-amylase and glucoamylase from *Thermomyces lanuginosus* F1. *Acta Biotechnol*, 21, 141-153.

Ohnishi, H., Sakai, and H. Ohta, T. (1991). Purification and some properties of glucoamylase from *Clostridium* sp. G500. *Agric Biol Chem*, 55, 1901-1902.

Ozbek, B. and Yuceer, S. (2001). α-Amylase inactivation during wheat starch hydrolysis process. *Process Biochem*, 37, 87-95.

Pomeranz, Y. (1991). *Functional Properties of Food Components*. (2nd ed.). California: Academic Press.

Ray, R.R., Jana, S.C. and Nanda, G. (1994). Biochemical approaches of increasing thermostability of β-amylase from *Bacillus megaterium* B_6. FEBS Lett, 356, 30-32.

Sadana, A. (1991). *Biocatalysis*: fundamentals of enzyme deactivation kinetics. New Jersey: Prentice-Hall, Inc.

Sauer, J., Sigurskjold, B.W., Christensen, U., Frandsen, T.P., Mirgorodskaya, E., and Harrison, M. (2000). Glucoamylase: structure/function relationships, and protein engineering. *Biochim Biophys Acta*, 1543, 275-293.

Schmitt, A., Gasic-Milenkovic, J. and Schmitt, J. (2005). Characterization of advanced glycation end products: mass changes in correlation to side chain midification. *In press*.

Seidler, N.W. and Seibel, I. (2000). Glycation of aspartate aminotransferase and conformational flexibility. *Biochem Biophys Res Commun*, 277, 47-50.

Shaw, C.J., Huang, A., and Zhang, X. (2003). Quantitation and test of enantiomeric purity of the L-ketohexose by liquid chromatography with dual refractive index and laser-based chiroptical detection. *J Chromatogr A*, 987, 439-443.

Suzuki, Y., Ito, N., Yuuki, T., Yamagata, H. and Udaka, S. (1989). Amino acid residues stabilizing a *Bacillus* α-amylase against irreversible thermoinactivation. *J Biol Chem*, 264, 18933-18938.

Svensson, B., Larsen, K. and Gunnarsson, K. (1986). Characterization of a glucoamylase G2 from *Aspergillus niger*. *Eur J Biolchem*, 154, 497-502.

Swamy, M.V. and Seenayya, G. (1996). Thermostable pullulanase and α-amylase activity form *Clostridium thermosulfurogenes* SV9-optimization of culture conditions for enzyme production. Process Biochem, 31, 157-162.

Swift, H. J., Brady, L., Derewanda, Z.S., Dodson, E.J., Dodson, G.G., Turkenburg, J.P. and Wilkinson, A.J. (1991). Structure and molecular model refinement of *Aspergillus oryzae* (TAKA) α-amylase: an application of the simulated-annealing method. *Acta Crystallog sect D*, 47, 535-544.

Takahashi, K., Lou, X.F., Ishii, Y. and Hattoro, M. (2000). Lysozyme-glucose stearic acid monoester conjugate formed through the Maillard reaction as an antibacterial emulsifier. *J Agric Food Chem*, 48, 2044-2049.

Takase, K., Mizuno, H. and Yamane, K. (1988). NH2-terminal processing of *Bacillus subtilis* α-amylase. J Biol Chem, 263, 11548-11553.

Thornally, P.J., Yurek-George, A. and Argirov, O.K. (2000). Kinetics and mechanism of the reaction of aminoquanidine with the α-oxoaldehydes

glyoxal, methylglyoxal and 3-deoxyglucosone under physiological conditions. Biochem Pharmacol, 60, 55-65.

Tsao, C., Pan, Y. and Jiang, S. (2003). Purification and characterization of amylases from small abalone (*Sulculus diversicolor aquatilis*). *J Agric Food Chem*, 51, 1064-1070.

Vieille, C. and Zeikus, G.J. (2001). Hyperthermophilic enzyme: sources, uses, and molecular mechanisms for thermostability. *Microbiol Mol Biol*, 65, 1-43.

Wells-Knecht, K.J., Zyzak, D.V., Litchfield, J.E., Thorp, S.R. and Baynes, J.W. (1995). Identification of Glyoxal and Arabinose as Intermediates in the autoxidative Modification of Proteins by Glucose. *Biochemistry*, 34, 3702-3709.

Williamson, G., Belshaw, N.J., Noel, T.R., Ring, S.G. and Williamson, M.P. (1992). O-Glycosylation and stability: unfolding of glucoamylase induced by heat and quanidine hydrochloride. *Eur J Biochem*, 207, 661-670.

Wong D.W.S. (1995). *Food Enzymes*: structure and mechanism. New York: Chapman&Hall.

Wong, D.W.S. (1986) Mechanism and Theory in Food Chemistry. USA: Van Nostrand Reinhold.

Woolf, S.P. and Dean, R.T. (1987). Glucose autoxidation and protein modification: the potantial role of autoxidative glycosylation in diabetes mellitus. *Biochem J*, 245, 243-250.

Yeboah, F.K., Alli, I., and Yaylayan, V.A. (1999). Reactivities of D-glucose and D-fructose during glycation of bovine serum albumin. *J Agric Food Chem*, 47, 3164-3172.

Yeboah, F.K., Alli, I., Yaylayan, V.A., Konishi, Y. and Stefanowicz, P. (2000). Monitoring of lysozyme by Electrospray Ionization Mass Spectrometry. *J Agric Food Chem*, 48, 2766-2774.

APPENDIX

APPENDIX A

Solution for Gel Electrophoresis

SDS-PAGE

1. Acrylamide/bis (30% T, 2.67% C, Solution A)

87.6 g acrylamide, 2.4 g N'N'-bis-methylene-acrylamide, made up volume to 300 ml with deionized water and stored at 4°C in the dark.

2. 1.5 M Tris-HCl, pH 8.8 (Solution B)

27.23 g Tris-base, 80 ml DW, adjusted to pH 8.8 with 5 N HCl. Made to 150 with deionized water and stored at 4°C.

3. 0.5 M Tris-HCl, pH 6.8 (Solution C)

6 g Tris-base, 60 ml deionized water, adjusted to pH 6.8 with 5 N HCl. Made to 150 ml with deionized water and stored at 4°C.

4. 10% SDS

10 g SDS in 90 ml deionized water with gentle stirred and adjusted to 100 ml with deionized water.

5. Sample buffer (SDS reducing buffer)

200 µl appropriated dilute sample, 100 µl 0.5 M tris-HCl pH 6.8, 20 µl 10% SDS and 2 µl 2-mercaptoethanol were mixed together and heated in boiling water-bath for 2 min. 20µl of heated sample, 5 µ glycerol and 5 µl marker stain were mixed together before loaded into the well (20 µl).

6. Running buffer, pH 8.3

3 g Tris-base, 14.4 g glycine, 10 ml of 10% SDS and adjusted to 1 L with deionized water. Stored at 4°C.

7. Catalyst (Solution D)

10% ammonium persulfate, freshly prepared.

Preparation of separating gel (10% gel)

1. Assemble the ATTO vertical slab gel unit in the gel casting stand.

2. Mix 3.75 ml of deionized water, 3 ml solution A and 2.25 ml solution B.

3. Add 50 µl solution D and 12.5 µl tetramethylethyldiamine (TEMED), and gently swirl to mix.

4. Pipette the solution down the gel casting.

5. Fill the gel casting with deionized water and polymerized by exposed to the light. The gel should be fully polymerized within 1 h.

Preparation of staking gel (4.5% gel)

1. Mix 1.3 ml of deionized water, 0.45 µl solution A and 0.75 µl solution C.

2. Add 25 µl solution D and 6.25 µl (TEMED), and gently swirl to mix.

3. Pour off deionized water overlay from the gel. Remove all liquid before proceeding.

4. Fill the gel casting with stacking gel solution and insert a comb, taking care not to trap any bubbles below the teeth of the comb.

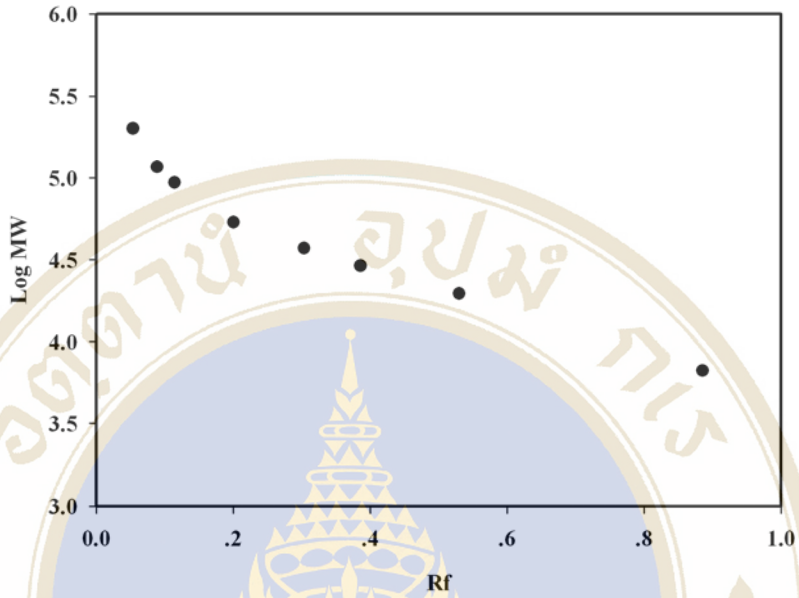
5. Allow the gel to sit for at least 30 min.

6. Slowly remove the comb from the gel and rinse each well with running buffer.

Native-PAGE

The solution for Native-PAGE was the same as SDS-PAGE, with the absence of sodium dodesyl sulfate and no 2-mercaptoethanol reduction. The separating and stacking gels were prepared as described above.

Calibration curves of protein standards taken from the gel



Estimation of an unknown protein MW

To estimate the unknown protein MW, the curve was fitted with power equation. The MW of unknown protein was calculated by placing the unknown mobility in to the equation and calculating log MW. The equation can be described as:

$$y = 3.9459x^{-0.1066}$$

where x and y stand for Rf and log MW, respectively.

APPENDIX B

Properties of commercial amylolytic enzymes from product specification

1. Properties of commercial thermostable α-amylase (KLEISTASE T10S)

Microoganism	: *Bacillus subtilis*
Optimum pH	: pH 6.0-7.0
pH-Stability	: 6.0-10.0
Optimum temperature	: Effective at 90-105^{o}C

2. Properties of commercial α-amylase (BAN 480L)

Microoganism	: *Bacillus amyloliquefciencs*
Optimum temperature	: 70-90^{o}C

3. Properties of commercial glucoamylase (DAIZYME GL4.2)

Microoganism	: *Aspergillus niger*
Optimum pH	: Optimum pH 4.5
Optimum temperature	: 60^{o}C

BIOGRAPHY

NAME	Mr. Pornpong Sutthirak
DATE OF BIRTH	11 March 1972
PLACE OF BIRTH	Suratthani, Thailand
INSTITUTIONS ATTANDED	Prince of Songkla University, 1991-1994 Bachelor of Science (Agro-Industry) Prince of Songkla University, 1994-1997 Master of Science (Food Technology) Mahidol University, 2000-2005 Doctor of Philosophy (Biotechnology)
OFFICE ADDRESS	Department of Bioindustry, Faculty of Technology and Management, Prince of Songkla University, Suratthani Tel. 077-355040 Email: supornpo@hotmail.com
HOME ADDRESS	154 Moo 1, Kadae, Kanchanaditha, Suratthani, Thailand 84160 Tel. 06-5387994