การเปลี่ยนแปลงความจำเพาะต่อซับสเตรตของแอลฟาอะไมเลส โดยวิธีไบโออิมพรินทิงด้วยบีตาไซโคลเดกซ์ทริน

นายพิศิษ ศิริสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ALTERING SUBSTRATE SPECIFICITY OF ALPHA-AMYLASE BY BIOIMPRINTING WITH BETA-CYCLODEXTRIN

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พิศิษ ศิริสุวรรณ : การเปลี่ยนแปลงความจำเพาะต่อซับสเตรตของแอลฟาอะไมเลสโดยวิธีไบโออิมพรินทิงด้วยบีตาไซโคลเดกซ์ทริน. (ALTERING SUBSTRATE SPECIFICITY OF ALPHA-AMYLASE BY BIOIMPRINTING WITH BETA-CYCLODEXTRIN) **อ.ที่ปรึกษา : ผศ.ดร.มัญชุมาส เพราะสุนทร, อ.ที่ปรึกษาร่วม : รศ.ดร. เปี่ยมสุข พงษ์สวัสดิ์**, 78 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อ ทำการศึกษาการเปลี่ยนแปลงความจำเพาะต่อซับสเตรตของแอลฟาอะไมเลส (alpha-amylase) โดยใช้วิธีไบโออิมพรินทิง (bioimprinting) ด้วยบีตาไซโคลเดกซ์ทริน (beta-cyclodextrin, β-CD) ร่วมกับวิธีการตรึงเอนไซม์ เมื่อทำการเติมหมู่ไวนิลบนโมเลกุลของเอนไซม์ (derivatization) เพื่อให้มีตำแหน่งเชื่อมต่อที่มากพอในขั้นตอนของการตรึงเอนไซม์ พบว่าอัตราส่วนที่เหมาะสมระหว่างเอนไซม์กับอิทาโคนิคแอนไฮไดรด์เท่ากับ 1:5 ซึ่งทำให้เกิดการเติมหมู่ไวนิล (derivatization degree) เท่ากับ 60.7% และมีแอกทิวิตีคงเหลือ 93% เมื่อทำการไบโออิมพริน (bioimprint) แอลฟาอะไมเลสด้วย β-CD และตรึงด้วยเอทิลีนไกลคอลไดเมทาคริเลท (ethylene glycol dimethacrylate, EGDMA) และ 2,2'-เอโซบิส 2-เมทิลโพรพิโอไนตรายล์ (2,2'-azobis (2-methylpropionitrile), AIBN) พบว่าภาวะที่เหมาะสมในการทำไบโออิมพรินทิงคือ บ่มแอลฟาอะไมเลส 30 มิลลิกรัมต่อมิลลิลิตร กับ β-CD เข้มข้น 25 มิลลิกรัมต่อมิลลิลิตร ในโซเดียมอะซิเตทบัฟเฟอร์เข้มข้น 50 มิลลิโมลาร์ pH 6.0 เป็นเวลา 30 นาที และภาวะที่เหมาะสมในการตรึงเอนไซม์คือ ใช้แอลฟาอะไมเลส 10 มิลลิกรัมต่อมิลลิลิตร ในไซโคลเฮกเซน แล้วเติม EGDMA ให้มีความเข้มข้นสุดท้ายเป็น 0.82 โมลาร์ และ AIBN 4 มิลลิกรัม บ่มภายใต้แสงยูวีความยาวคลื่น 240 นาโนเมตร เป็นเวลา 3 ชั่วโมง จากนั้นทำการศึกษาปฏิกิริยาการย่อย β-CD (CDase activity), การสร้าง β-CD (cyclization activity) และการย่อยแป้ง (dextrinizing activity) ของแอลฟาอะไมเลส ที่ผ่านการทำไบโออิมพรินทิงและการตรึง (crosslinked imprinted protein, CLIP-α-amylase) ข้อมูลที่ได้บ่งชี้ว่าการทำไบโออิมพรินทิงแอลฟาอะไมเลสด้วย β-CD ไม่สามารถทำให้เอนไซม์ย่อย β-CD และสร้าง β-CD ได้ อย่างไรก็ตามพบว่าปฏิกิริยาการย่อยแป้งของ CLIP แอลฟาอะไมเลสมีการเปลี่ยนแปลง โดยเกิดปฏิกิริยาเพียง 42.4% เมื่อเปรียบเทียบกับแอลฟาอะไมเลสรูปแบบที่ทำการตรึงเพียงอย่างเดียวโดยไม่ได้ทำการอิมพรินด้วยลิแกนด์

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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The aim of this study was to alter the substrate specificity of α-amylase by the combination of bioimprinting with β-cyclodextrin (β-CD) and immobilization method. α-Amylase was first derivatized with itaconic anhydride to have enough attachment points for enzyme crosslinking. It was determined that the optimum ratio (w/w) of protein to itaconic anhydride was 1:5 which resulted in derivatization degree of 60.7% and the remaining activity of the derivatized enzyme was 93%. The derivatized α-amylase was then bioimprinted with β-CD and immobilized using ethylene glycol dimethacrylate (EGDMA) and 2,2'-azobis (2-methylpropionitrile) (AIBN). It was found that the optimal condition for bioimprinting process was to incubate 30 mg/ml of α-amylase with 25 mg/ml of β-CD in 50 mM sodium acetate buffer pH 6.0 for 30 minutes. And the optimal condition for crosslinking procedure was to use 10 mg/ml of α-amylase suspended in cyclohexane with the addition of 4 mg/ml AIBN and EGDMA at the final concentration of 0.82 M under UV irradiation (λ = 240 nm) for 3 hours. The CDase, cyclization and dextrinizing activity of crosslinked imprinted protein (CLIP-α-amylase) were investigated. The results obtained suggested that the CDase and cyclization activity of CLIP-α-amylase could not be generated by imprinting with β-CD under the investigated conditions. Nevertheless, the dextrinizing activity of CLIP-α-amylase could be altered. The dextrinizing activity was found to be only 42.4% in comparison with that of the crosslinked form where the enzyme was crosslinked without the imprinted molecule.

Department:.....BIOCHEMISTRY.....Student's Signature:.....

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LIST OF ABBREVIATIONS

A	Absorbance
AIBN	2,2'-Azobis (2-methylpropionitrile)
CD	Cyclodextrin
CDase	Cyclodextrinase
CLIP	Crosslinked Imprinted Protein
CLEAs	Crosslinked Enzyme Aggregate
CGTase	Cyclodextrin glycosyltransferase
°C	Degree Celsius
DNS	Dinitrosalicylic acid
EGDMA	Ethylene glycol dimethacrylate
et al.	Et. Alii (latin),and others
g	Gram
HPLC	High Performance Liquid Chromatography
μg	Microgram
μl	Microliter
M	Molar
mg	Milligram
Min	Minute
ml	Milliliter
mM	Millimolar
MW	Molecular weight
R_f	Relative mobility
R_t	Retention time
TLC	Thin-layer chromatography
U	Unit(s)
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight

CHAPTER I

INTRODUCTION

1.1 Bioimprinting

In industries where enzymes have been exploited in the production process, the control of enzyme specificity (for example, substrate specificity, enantioselectivity or regioselectivity) is the important goal to improve productivity or achieve new desired products. At present, enzyme engineering is a powerful method that modifiy the enzyme at its genetic level in order to increase the enzyme activity or to improve the enzyme specificity (Perona *et al*., 1995; Shao *et al*., 1996). However, this method requires several equipments and techniques including high cost. Thus, a biochemical method which is called “bioimprinting” has been developed (Mosbach *et al*., 1996) to allow a modification of enzyme properties directly at the protein level. This method is based on the conformational change of enzyme molecule which is induced by a ligand. As a result, the enzyme possesses the bioimprinted memory of that ligand. In this way, the enzymatic activity is tailored because the enzyme can use that ligand as its substrate.

Bioimprinting starts with derivatazation of the enzyme using itaconic anhydride to have enough attachment points for enzyme crosslinking (Figure 1 and 2). Free amino groups of lysines, hydroxyl groups of tyrosines or sulfhydryl groups of cysteines of proteins are able to covalently couple with itaconic anhydride (Fischer *et al*., 1998). The derivatized enzyme is imprinted with ligands such as substrate analogues or inhibitors in aqueous medium (Vaidya *et al*., 2004). By this, the catalytic activity (Rich *et al*., 1997), the substrate selectivity (Johansson *et al*., 1995) or the enantioselectivity (Stahl *et al*., 1991) of enzymes and, the binding properties (Dabulis *et al*., 1992) of proteins were manipulated. Unfortunately, all these examples of tailoring proteins by imprinting techniques are limited by the fact that the new protein properties are available in nearly anhydrous environment only since aqueous environment causes a renaturation of

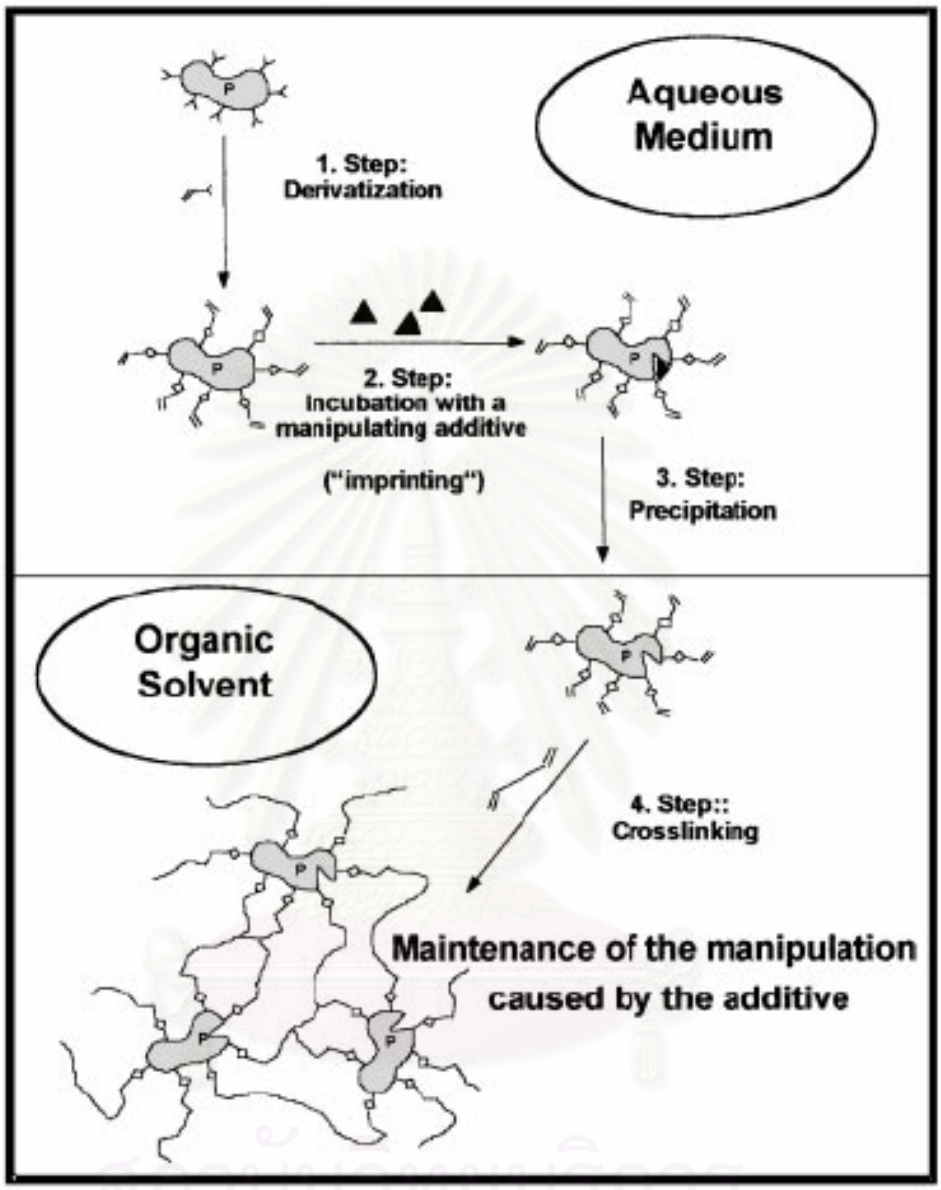


Figure 1. Altering the substrate specificity through the combination of bioimprinting and a covalent immobilization technique (Peiβker *et al.*, 1998)

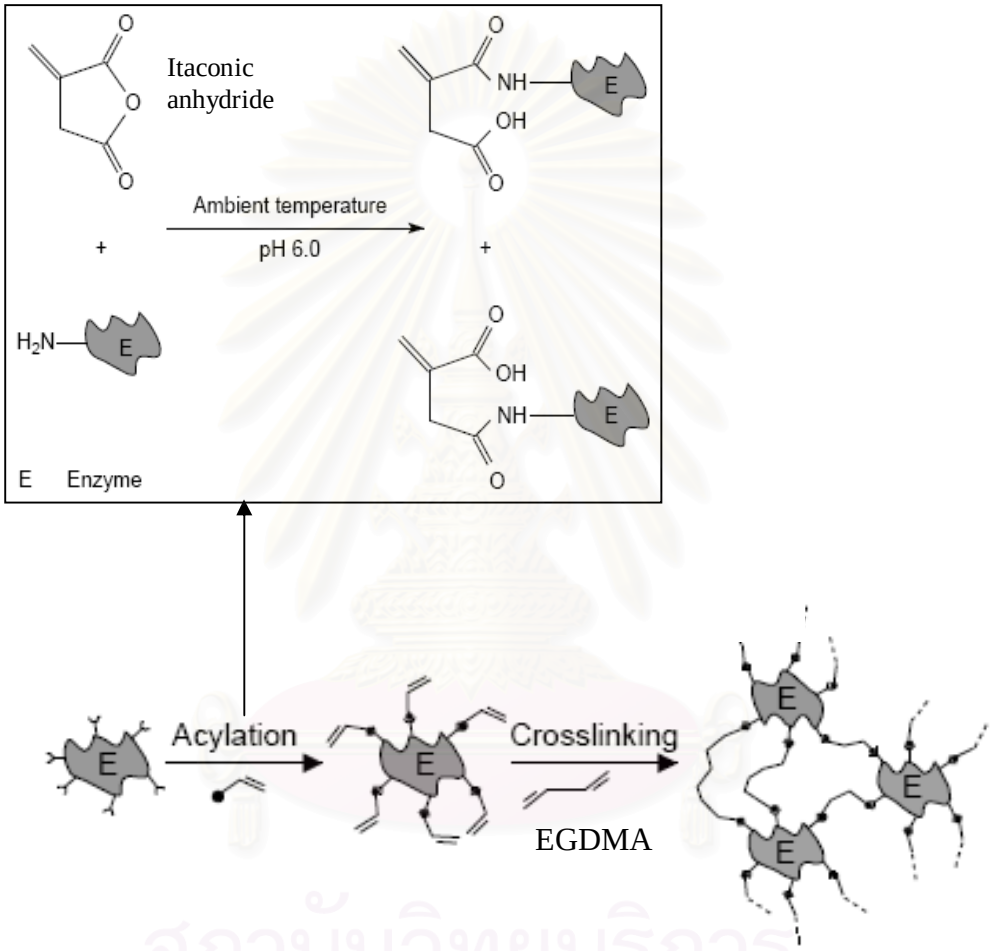


Figure 2. Advanced two-step immobilization technique (Fischer *et al*., 1998)
EGDMA = Ethylene glycol dimethacrylate

the protein and loss of imprinting effect. Thus, maintain a structural memory, the imprinted protein or enzyme is transferred from aqueous into anhydrous environment (Klibanov *et al*., 1995). So, if a native protein is precipitated (Stahl *et al.*, 1991) or lyophilized (Gonzales-Navarro *et al.*, 1998) from aqueous environment in the presence of a defined ligand, the ligand can cause a predictable alteration of the property of the protein in organic solvents. The conformational changed enzyme is maintained by polymerization through crosslinking in water-free organic solvent. This enzyme preparation is called crosslinked imprinted protein (CLIP). Finally, the ligand is removed and the CLIP enzyme permanently maintain its “new” property in aqueous as well as in organic environment (Peißker *et al*., 1998).

Joseph *et al.* (1997) has found that the activity and substrate specificity of subtilisin-catalyzed acylation of nucleosides in organic solvents can be controlled by lyophilizing the enzyme from an aqueous solution containing the substrate. This “molecular imprinting” technique was examined using thymidine as a model nucleoside, and the resulting subtilisin preparation was up to 50-fold more reactive toward thymidine acylation in nearly anhydrous tetrahydrofuran than subtilisin lyophilized from aqueous buffer in the absence of the nucleoside. Although several compounds lyophilized with subtilisin, including thymine and ribose, improved the rate of thymidine acylation, the thymidine-imprinted enzyme was the most efficient catalyst for this reaction. Furthermore, it was possible to alter the substrate selectivity of subtilisin by lyophilizing the enzyme in the presence of a different nucleophilic substrate. For example, imprinting made possible the discrimination between structurally different (i.e., sucrose versus thymidine) as well as structurally similar (i.e., thymidine versus deoxyadenosine) nucleophiles. Molecular modeling studies of the interaction of thymidine or the unrelated sucrose with subtilisin revealed that structural changes upon imprinting in the serine protease’s catalytic triad may be responsible for the observed activation and selectivity changes. Further use of molecular dynamics indicated that structural changes in the catalytic triad occur during imprinting,

and that these changes may be the major factor that contributes to imprinting-induced substrate selectivity. This contrasts with the previously held notion that imprinting influences mainly substrate binding.

Kronenburg *et al*. (2001) have studied the bioimprinting of membrane-associated epoxide hydrolase (EH). Partially purified EH was immobilized in a two-step procedure. In the first step, the proteins were derivatized with itaconic anhydride. In the second step, the derivatized proteins were co-polymerized with ethylene glycol dimethacrylate in water-free cyclohexane to form a bioplastic. Before co-polymerization, the derivatized enzyme had been imprinted by substrates or its analogues as the imprinters in an aqueous phase. After removing the imprinters, an enzyme with rationally modified properties was obtained. This is the first time that the above-mentioned method was successfully performed with a membrane-associated enzyme of the α/β-hydrolase fold family to which EH belongs. The enantioselective conversion of (±)-1,2-epoxyoctane was reversed from a preference for (*R*)-1,2-epoxyoctane to (*S*)-1,2-epoxyoctane when the enzyme had been imprinted with (*S*)-1,2-epoxyoctane prior to co-polymerization. The enzymatic reaction was performed in aqueous media. Other benefits of immobilizing EH into a co-polymer were the ease of recycling of the biocatalyst and the separation of biocatalyst and its products. An unexpected benefit was the enhanced enzyme stability. The half-life of the immobilized and imprinted biocatalyst was enhanced at least 7-fold. Most remarkable was that washing the immobilized EH with HCl, followed by washing it with buffer, resulted in about 50% of the residual activity, while native EH completely lost its activity.

Bio-imprinting of lipases with fatty acids was shown to be a feasible, effective method for obtaining highly active enzymes in organic solvents (Fishman *et al*., 2003). The increase in activity was dependent on the enzyme type, the solvent type and the imprint molecule itself. A correlation between the initial activity of caprylic acid-imprinted *Candida rugosa* lipase (CRL), and solvent hydrophobicity was observed. In addition, the combination of bio-imprinting with adsorption onto an inert support such as celite, proved to be a powerful technique

for obtaining an even more active and stable enzyme preparations. In the case of lipase from *Pseudomonas* sp., the increase in activity resulting from bio-imprinting with caprylic acid and immobilization onto celite, was 20-fold. Porcine pancreatic lipase (PPL), treated in the same manner, retained 70% of its initial activity at the end of 20 consecutive reaction cycles, compared to only 20% residual activity for the non-treated control.

Recently, an alteration of the substrate specificity of glucose oxidase could be achieved by using this CLIP (Vaidya *et al.*, 2004). The resulting CLIP enzyme can then be used to catalyze the reaction either in aqueous medium or organic solvent. Glucose oxidase bioimprinted with D-galactose could accept both D-glucose and D-galactose as substrate to form D-glucono-1,5-lactone and D-galactono-1,4-lactone, respectively whereas the native enzyme only recognized D-glucose as substrate for the synthesis of D-glucono-1,5-lactone. Furthermore, CLIP-glucose oxidase could be reused while losing only 10% of its initial activity. This can be explained by the stabilizing effect of the covalent crosslinking of the CLIP technique.

Kaulpiboon *et al.* (2007) succeeded to manipulate the product specificity of the *Paenibacillus* sp. A11 and *Bacillus macerans* cyclodextrin glycosyltransferases towards the preferential formation of gamma-cyclodextrin (CD8). The efficiency of the crosslinked imprinted enzymes for CD8 synthesis was increased 10-fold, whereas that for cyclodextrin hydrolysis was decreased. The native cyclodextrin glycosyltransferases from *Paenibacillus* sp. A11 and *Bacillus macerans* produced CD6 : CD7 : CD8 : > CD9 ratios of 15 : 65 : 20 : 0 and 43 : 36 : 21 : 0, whereas the CLIP-CGTase from *Paenibacillus* sp. A11 and *Bacillus macerans* produced cyclodextrin in ratios of 15 : 20 : 50 : 15 and 17 : 14 : 49 : 20, respectively. The size of the synthesis products formed by the crosslinked imprinted cyclodextrin glycosyltransferases was shifted towards CD8 and > CD9, and the overall cyclodextrin yield was increased by 12% compared to the native enzymes (Table 1).

Table 1. Effect of the different CGTases preparations on yield and product selectivity of CDs (Kaulpiboon *et al*., 2007)

CGTase preparation	Yield (%)	Product ratio (%)			
		CD_6	CD_7	CD_8	CD_9
A11 CGTase					
native	42	15	65	20	0
immobilized	56	18	39	24	19
CLIP	54	15	20	50	15
BM CGTase					
native	44	43	36	21	0
immobilized	57	28	27	22	23
CLIP	56	17	14	49	20

A11 = *Paenibacillus* sp. A11

BM = *Bacillus macerans*

The application of CLIP enzyme involves its capability to altering the substrate or product specificity (Vaidya *et al.*, 2004; Klibanov *et al*., 1995; Mosbach *et al*., 1996; Peißker *et al.*, 1998) and enantioselectivity (Kronenburg *et al*., 2001; Stahl *et al*., 1991). This beneficial properties are especially useful in the fields of synthetic organic chemistry, pharmaceutical application and other biocatalysis applications.

1.2 a-amylase

1.2.1 Classification of amylases and the related enzymes.

The term “amylase” can be generally defined as the enzyme which hydrolyzes the 0-glycosyl linkage of starch. Starch is the most popular polysaccharide used as food ingredient. It is a mixture of amylose, which is essentially composed only of α-1,4 linked glucose-polymers, and amylopectin, which is composed of α-1,4 linked glucose-polymers branched by α-1,6 linkages. The enzymes which specifically catalyze the hydrolysis or synthesis of glucosidic linkages of starch are represented by four types as follows: (i) hydrolysis of α-1,4 glucosidic linkages; α-amylase (EC 3.2.1.1); (ii) hydrolysis of α-1,6-glucosidic linkages; pullulanase (EC 3.2.1.41) or isoamylase (EC 3.2.1.68); (iii) transglycosylation to form α-1,4 glucosidic linkages; cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19); and (iv) transglycosylation to form α-1,6-glucosidic linkages; 1,4-α-D-glucan : 1,4-α-D-glucan 6-α-(1,4-α-D-glucano)-transferase (branching enzyme, EC 2.4.1.18) (Takashi *et al.*, 1999).

1.2.2 Enzymes belonging to the a-amylase family

Takata *et al.* (1992) demonstrated that α-amylase, pullulanase, isoamylase, CGTase, the branching enzymes, α-glucosidase, oligo-1,6-glucosidase, amylopullulanase, and neopullulanase belong to α-amylase family. Subsequently, Svensson *et al.* (1994) added cyclodextrinase, dextran glucosidase, amylomaltase, and glycogen debranching enzyme to the family. As stated earlier, Janacek *et al.* (1997) demonstrated that amino acid transport-related proteins and 4F2 heavy-chain cell surface antigens also belong to the α-

amylase family. The relationship of enzymes that belong to α-amylase family according to types of reaction, are shown in Figure 3 (Takashi *et al.*, 1999) and Figure 4. (Van der Maarel *et al.*, 2001).

A characteristic feature of the enzymes from the α-amylase family is that they all employ the α-retaining mechanism but they vary widely in their substrate and product specificities. These differences can be attributed to the attachment of different domains to the catalytic core or to extra sugar-binding subsites around the catalytic site (Table 2).

1.2.3 Taka-amylase A (*Aspergillus oryzae* a-amylase)

Taka-amylase A (TAA) is a fungal α-amylase, catalyzing the hydrolysis of α-1,4-glucosidic linkage of α-1,4-glucan. TAA hydrolyzes amylase completely to maltose and glucose. The branched substrates including the branched fractions from corn starch, waxy maize starch, glycogen and β-amylase limit dextrins are hydrolyzed less readily and less extensively than the linear fraction from corn starch (Hanrahan *et al*., 1953). TAA catalyzes the ring opening hydrolysis of cyclodextrins. The linear maltooligosaccharides produced are immediately degraded to smaller oligosaccharides. Suetsugu *et al*. (1974) observed multiple attacks on the hydrolysis of cyclodextrins. Table 3 summarizes the values of the kinetic parameters for the ring opening hydrolysis.

1.2.4 a-amylase of *Bacillus subtilis* and related bacteria

α-Amylases of *Bacillus* and related bacteria (aerobic, spore formimg, mesophile) are divided into two types from their specificity (Kneen *et al*., 1946). One is starch liquefying and the other, saccharifying type (Fukumoto *et al*., 1951). The liquefying α-amylase is one of the industrially important enzymes and utilized in various fields. The enzyme of *B. subtilis* belongs to the liquefying type. The saccharifying α-amylase, however, has not been so applied yet, but this α-amylase is useful for structural elucidation study of branched dextrins and cyclodextrins, as described below:

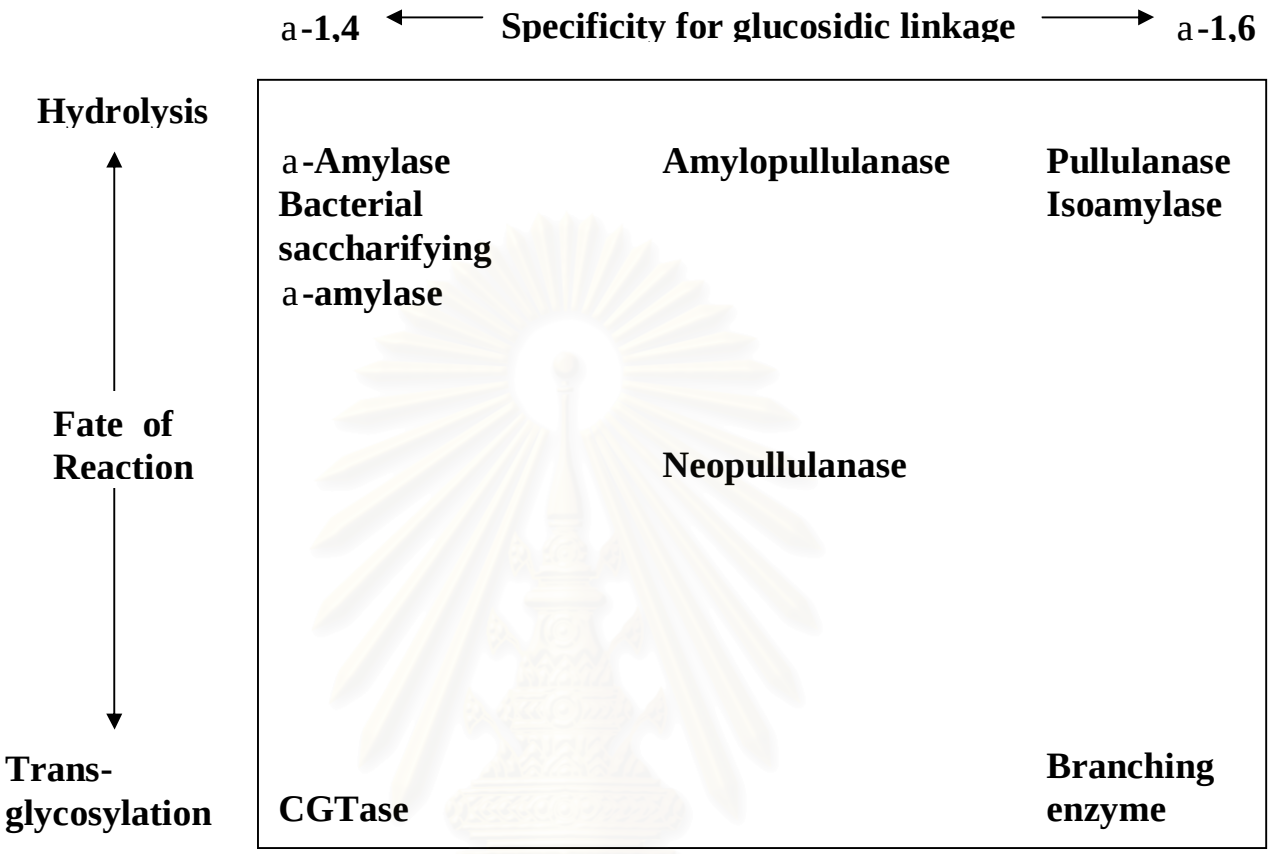


Figure 3. Schematic representation of relationship of enzymes that belong to a-amylase family according to types of reaction (Takashi *et al.*, 1999)

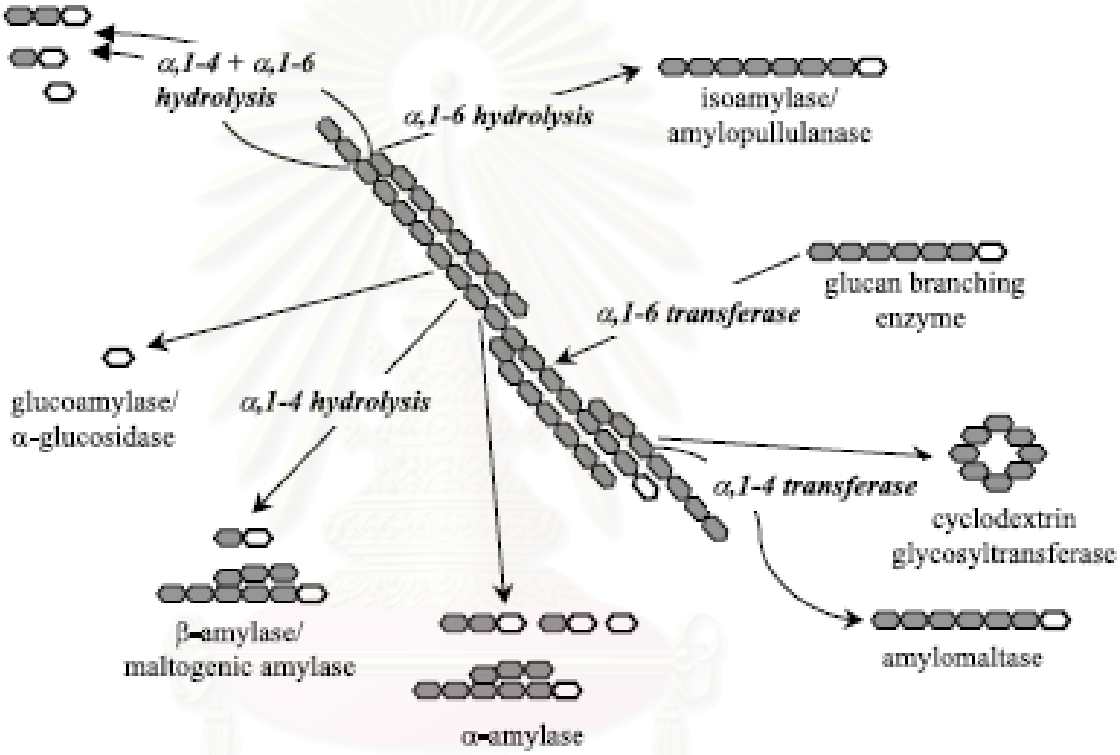


Figure 4. Different enzymes involved in the degradation of starch The open ring structure symbolizes the reducing end of a polyglucose molecule (Van der Maarel *et al.*, 2001).

Table 2. Enzymes of the a-amylase family that act on glucose-containing substrates, their corresponding EC number, the domain organization, and main substrates (Van der Maarel *et al.*, 2001)

Enzyme	EC number	Domains	Main substrate
Amylosucrase	2.4.1.4		Sucrose
Sucrose phosphorylase	2.4.1.7		Sucrose
Glucan branching enzyme	2.4.1.18	A, B, F	Starch, glycogen
Cyclodextrin glycosyltransferase	2.4.1.19	A, B, C, D, E	Starch
Amylomaltase	2.4.1.25	A, B1, B2	Starch, glycogen
Maltopentaose-forming amylase	3.2.1.–	A, B, I	Starch
α-Amylase	3.2.1.1	A, B, C	Starch
Oligo-1,6-glucosidase	3.2.1.10	A, B	Amylopectin
α-Glucosidase	3.2.1.20		Starch
Amylopullulanase	3.2.1.41 or 3.2.1.1	A, B, H, G, 1	Pullulan
Cyclomaltodextrinase	3.2.1.54	A, B	Cyclodextrins
Isopullulanase	3.2.1.57		Pullulan
Isoamylase	3.2.1.68	A, B, F, 7	Amylopectin
Maltotetraose-forming amylase	3.2.1.60	A, B, C, E	Starch
Glucodextranase	3.2.1.70		Starch
Trehalose-6-phosphate hydrolase	3.2.1.93		Trehalose
Maltohexaose-forming amylase	3.2.1.98		Starch
Maltogenic amylase	3.2.1.133	A, B, C, D, E	Starch
Neopullulanase	3.2.1.135	A, B, G	Pullulan
Malto-oligosyl trehalase hydrolase	3.2.1.141		Trehalose
Malto-oligosyl trehalase synthase	5.4.99.15		Maltose

Table 3. Kinetic parameters for the hydrolysis of cyclodextrins by Taka-amylase A at 25^0C and pH 5.3 (Suetsugu *et al.*, 1974)

Substrate	K_m (x10^3M)	K_i (x10^3M)	V/$[E]_0$ (min^{-1})
α-CD	4.7	5.2	3.3
β-CD	10.2	9.3	270
γ-CD	2.4	-	3270

Liquefying α-amylase

$$\text{Starch} + n\,H_2O \longrightarrow \text{G1} + \text{G2} + \text{G3} + \text{G4} + \text{G5} + \text{G6} + \text{various branched oligosaccharides}$$

Saccharifying α-amylase

$$\text{Starch} + n\,H_2O \longrightarrow \text{G1} + \text{G2} + \text{G3} + \text{various branched oligosaccharides}$$

The liquefying α-amylase hydrolyzes amylose to produce glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose. The enzyme splits maltoheptaose and maltooctaose into maltohexaose and glucose, and maltohexaose and maltose, respectively. The glucose to split from maltoheptaose is of the reducing end of the dextrin (Okada *et al*., 1969).The liquefying α-amylase never attacks α-1,4-glucosidic linkages adjacent to α-1,6-glucosidic linkages in starch and glycogen.

The saccharifying α-amylase hydrolyzes amylose to produce glucose, maltose and maltotriose. On a long incubation, the enzyme split maltotriose into glucose and maltose. Under this condition, isomaltose is formed from maltose by this enzyme action. But the amount of isomaltose formed is much less than that of the maltose remaining. Saccharifying α-amylase hydrolyzes α-1,4-linked glucosidic chains existing outside the anomalous linkage of amylopectin, glycogen, etc. and produces glucose, maltose and maltotriose, as described above. The enzyme also hydrolyzes β- and γ-cyclodextrins, but hardly attacks α-cyclodextrin (Fujita, unpublished).

1.3 Cyclodextrin

1.3.1 Properties of cyclodextrin

Cyclodextrins (CD) are cyclic oligosaccharides consisting of six glucose units (α-cyclodextrin : α-CD), seven glucose units (β-cyclodextrin : β-CD), eight glucose units (γ-cyclodextrin : γ-CD) or more glucopyranose units linked by α-(1,4) bonds (Figure 5). They are also known as cycloamyloses, cyclomaltoses and Schardinger dextrins (Martin Del Valle, 2003; Eastburn *et al*., 1994). They are produced as a result of intramolecular transglycosylation reaction from degradation of starch by cyclodextrin glycosyltransferase (CGTase) enzyme (Szetjli, 1998). The main properties of cyclodextrins are given in Table 4.

From the X-ray structures it appears that in cyclodextrins the secondary hydroxyl groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl groups (C6) on the other edge, and that the apolar C3 and C5 hydrogens and ether-like oxygens are at the inside of the torus-like molecules. This result in a molecule with a hydrophilic outside, which can dissolve in water, and an apolar cavity, which provides a hydrophobic matrix, described as a 'micro heterogeneous environment' (Szetjli, 1989). As a result of this cavity, cyclodextrins are able to form inclusion complexes with a wide variety of hydrophobic guest molecules. One or two guest molecules can be entrapped by one, two or three cyclodextrins (Figure 6).

1.3.2 Inclusion complex formation

The most notable feature of cyclodextrins is their ability to form solid inclusion complexes (host–guest complexes) with a very wide range of solid, liquid and gaseous compounds by a molecular complexation (Martin Del Valle, 2003). In these complexes (Figure 6), a guest molecule is held within the cavity of the cyclodextrin host molecule. Complex formation is a dimensional fit between host cavity and guest molecule (Munoz-Botella *et al*., 1995). The lipophilic cavity of cyclodextrin molecules provides a microenvironment into which appropriately

sized non-polar moieties can enter to form inclusion complexes (Loftsson *et al*., 1996). The potential guest list for molecular encapsulation in cyclodextrins is quite varied and includes such compounds as straight or branched chain aliphatics, aldehydes, ketones, alcohols, fatty acids, aromatics, gases, and polar compounds such as halogens, oxyacids and amines (Schmid, 1989).

1.3.3 Applications of cyclodextrins

Since each guest molecule is individually surrounded by a cyclodextrin (derivative), the molecule is micro-encapsulated from a microscopical point of view. This can lead to advantageous changes in the chemical and physical properties of the guest molecules. These characteristics of cyclodextrins or their derivatives make them suitable for applications in food (Fujishima *et al*., 2001), pharmaceuticals (Bhaedwaj *et al*., 2000), cosmetics (Holland *et al*., 1999), environment protection (Lezcano *et al*., 2002), bioconversion (Dufosse *et al*., 1999), packaging and the textile industry (Hedges, 1998).

Table 4. Cyclodextrins properties (Martin Del Valle, 2003)

Properety	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25^0C (%,w/v)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	174	262	427

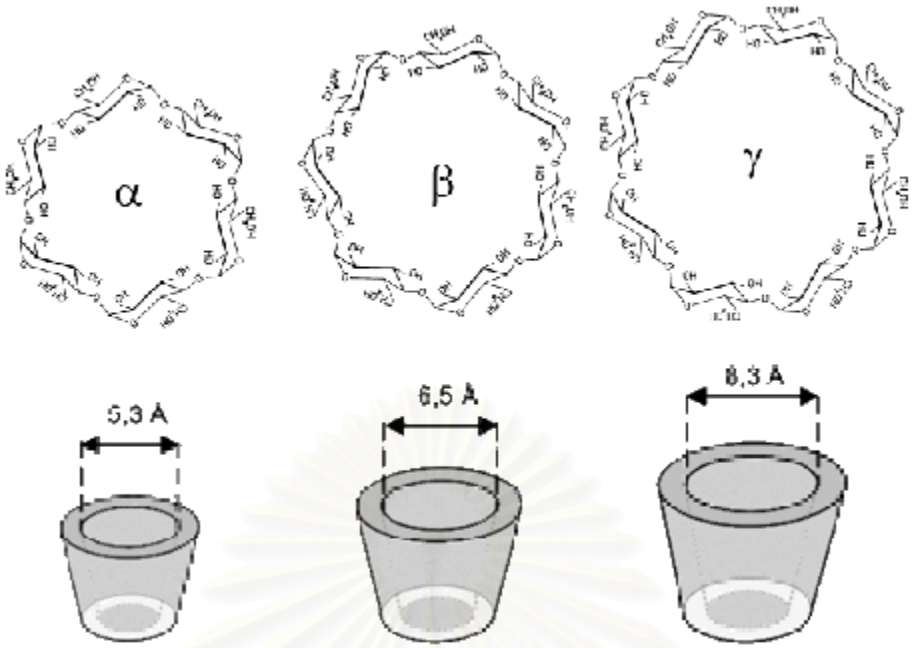


Figure 5. Chemical structure of a, b and g-cyclodextrin

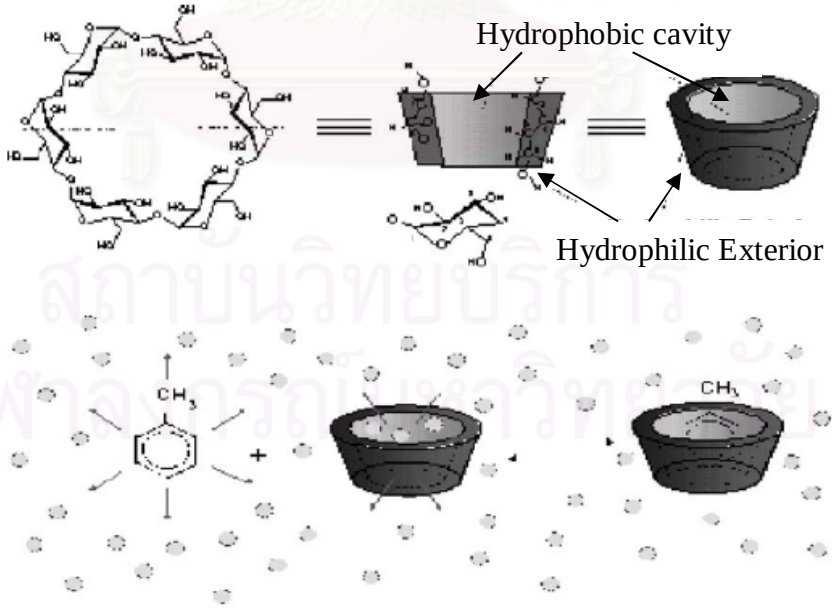


Figure 6. Cyclodextrin structure and inclusion complex formation (Martin Del Valle, 2003)

1.4 Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (CGTase) is a starch degrading enzyme relating to α-amylase family. CGTase belongs to the transferase group of enzymes (EC 2.4.1.19) which is produced by various microorganisms especially *Bacillus* species. However, there are also reports on CGTase production in different kinds of bacteria e.g. *Klebsiella*, *Thermoanaerobacter*. CGTase is an extracellular enzyme which can catalyze transglycosylation reaction in three different types. Most important reaction is cyclization, the conversion of starch to cyclodextrins - CDs are cyclic and non-reducing oligosaccharides which are mainly found in 3 forms: α-, β- and γ-cyclodextrin that are built up from six, seven and eight D-glucopyranosyl units, respectively (Bender, 1986 and Szetjli, 2004). Moreover, this enzyme also catalyzes a coupling reaction (cleavage of α-glycosidic bond of CD rings and transfer glycosyl group of linear maltooligosaccharides to some types of acceptors) and a disproportionation reaction (transfer glycosyl group from linear maltooligosaccharides to some types of acceptors) by ping-pong mechanism. Furthermore, CGTase has a weak hydrolytic action on α-1,4-glucans and CDs. The newly made reducing end is transferred to water. A scheme of CGTase-catalyzed transglycosylation reactions (van der Veen, 2000) is shown in Figure 7. The proposed models of the events taking place in the CGTase-catalyzed are shown in Figure 8.

The efficiency of each mechanism depends on the number of glucopyranosyl residue in reaction mixture. In other words, the cyclization takes place most effectively when substrates have glucopyranosyl units from 16 to 80 molecules. Meanwhile, the coupling and disproportionation reaction have rapidly occurred in reaction mixture which has 5-14 units and more than 100 units of glucopyranose, respectively. Nevertheless, the disproportionation reaction can still poorly occur in reaction mixture with 5-14 units of glucopyranose (Szejtli, 1988). In general, the application of CGTase is CD production by cyclization reaction. However, CGTase can be used for synthesizing derivatives of some compounds so as to improve their disadvantageous properties via disproportionation reaction.

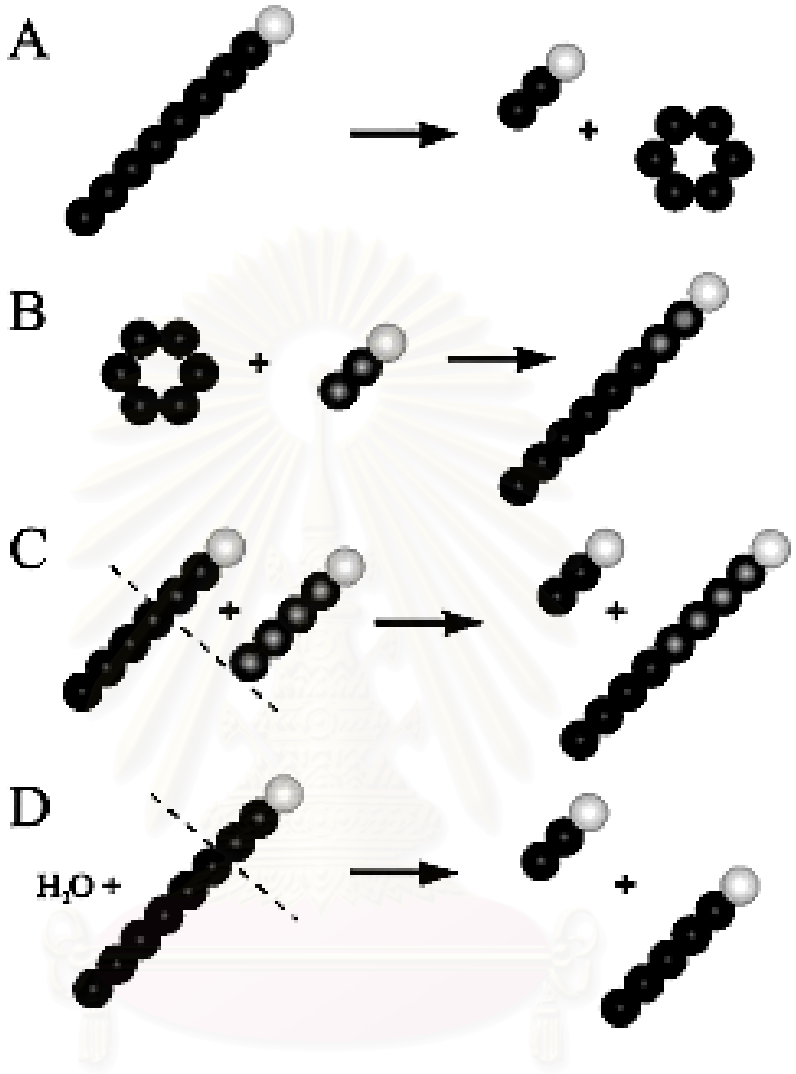


Figure 7. Schematic representations of the CGTase-catalyzed transglycosylation reactions (A) cyclization; (B) coupling; (C) disproportionation and (D) hydrolysis The shaded circles represent glucose residues; the white circles indicate the reducing end glucoses. (van der Veen *et al*., 2000)

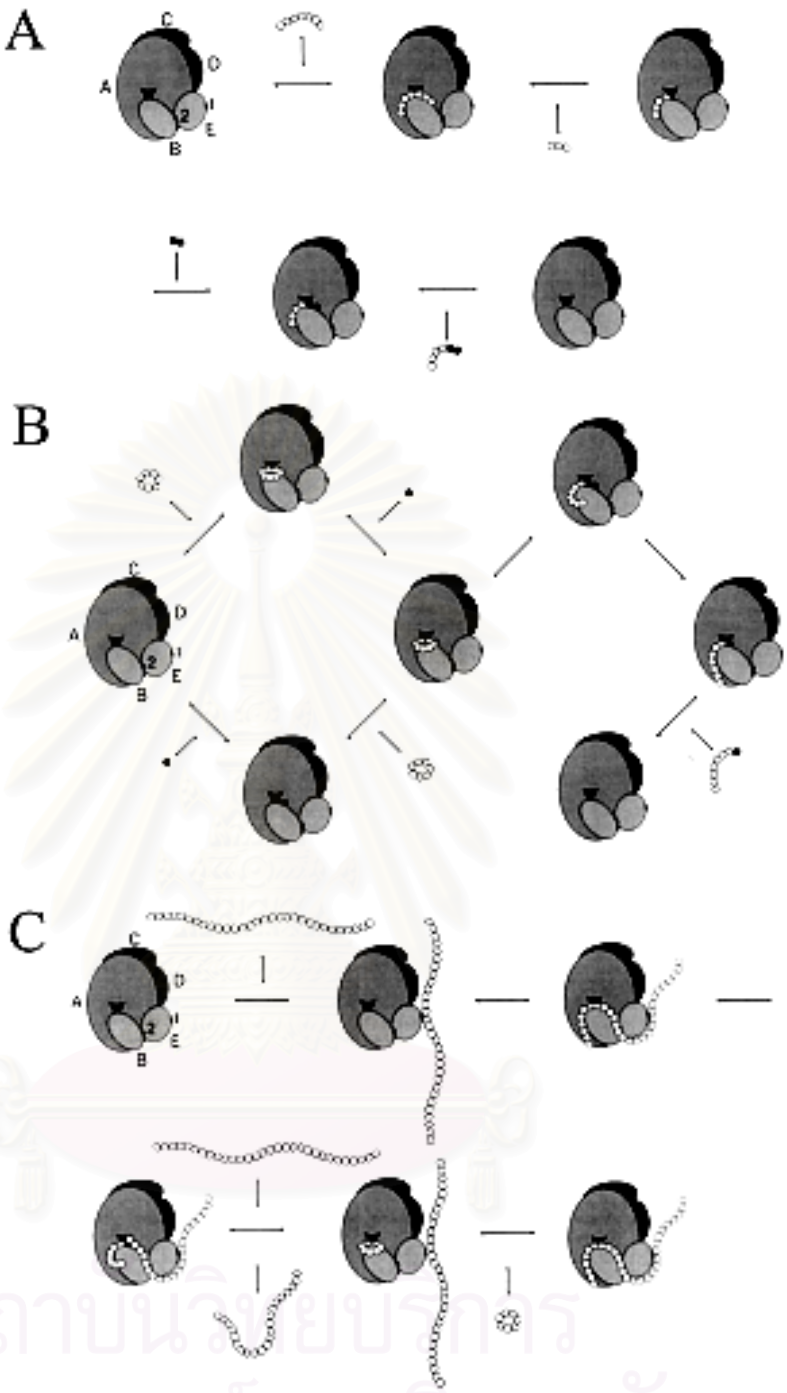


Figure 8. Proposed models of the events taking place in the CGTase-catalyzed reactions (van der Veen *et al*., 2000) (A) disproportionation, (B) coupling and (C) cyclization. The different CGTase domains are indicated (A, B, C, D and E). 1 and 2 indicate the maltose binding sites on the E-domain. The triangle indicates the cleavage site in the active site. Circles represent glucose residue; acceptor residues are represented in black.

1.5 Cyclomaltodextrinase (CDase)

Cyclomaltodextrinase (CDase; EC 3.2.1.54) catalyzes the hydrolysis of cyclodextrins (CDs) to form linear oligosaccharides of α-1,4-linkages (Depinto *et al*., 1968). In general, the hydrolysis rate of cyclodextrins (CDs) by α-amylase is far slower than that of starch (Ohnishi, 1971, Suetsugu *et al*., 1974), and exotype amylases, such as glucoamylase and β-amylase, scarcely hydrolyze CDs (Kobayashi *et al*., 1973, Thoma *et al*., 1960). On the other hand, CDase can rapidly hydrolyze CDs (Depinto *et al*., 1968, Kitahata *et al*., 1983), but has very little activity for polysaccharides such as starch and glycogen.

The objective of this research

The aim of this study is to alter the substrate specificity of α-amylase by the combination of bioimprinting with β-cyclodextrin (β-CD) and immobilization method. It is hoped that the native α-amylase which cannot hydrolyze CD may be able to use it as substrate. The data obtained will be beneficial for the applicable use with other enzymes in order to further improve their specificity. The work focuses on

1. To alter the substrate specificity of α-amylase by bioimprinting with β-cyclodextrin.
2. To determine the optimum condition for bioimprinting of α-amylase.
3. To study the catalytic activities of crosslinked imprinted α-amylase (CLIP-α-amylase) and compared them with the native and immobilized α-amylase.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Analytical column for HPLC: Inertsil®ODS-3 4.6x250 mm I.D. 5 μm, GL Science Inc., Japan

Analytical balance: AB204-S, Mettler-Toledo, Switzerland

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model J-21-C, Beckman Instrument Inc, USA

Centrifuge, microcentrifuge, high speed centrifuge: MIKRO 22R, Hettich Laborapparate, Germany

Freeze-dryer: LYPH-LOCK, LABCONCO, U.S.A.

Heating bath B-490, BUCHI, Switzerland.

High Performance Liquid Chromatography: Hewlett-Packard series 1050, Japan

Incubator: Haraeus, Germany

Incubator shaker: Psyco-Therm, New Brunswick Scientific Co., Ltd., U.S.A.

Laminar flow: Model BVT-124, International Sciencetific Supply Co., U.S.A.

Magnetic stirrer and heater: Model 512p-2, Barnstead/Thermolyne Corporation, U.S.A.

pH Meter: pH900, Precisa, Switzerland

Precision balance: PB303-S, Mettler-Toledo, Switzerland.

UV-VIS Spectrophotometer: DU650 Spectrophotometer, Beckman, USA

VIS Spectrophotometer: 6400 Spectrophotometer Jenway, LABQUIP, England

Viva Flow Concentrator:

Vortex: Model K-550-GE, Scientific Industries, USA

Water bath: Memmert, Germany

2.2 Chemicals

Acetonitrile (HPLC grade): Labscan, Thailand

Amberlite XAD-4: Fluka, Switzerland

Amyloglucosidase (Glucoamylase) from *Aspergillus niger* 70.7 U/mg: Fluka, Switzerland

α-Amylase from *Aspergillus oryzae* 5 U/mg: Sigma, U.S.A

α-Amylase from *Bacillus subtilis* 58.1 U/mg: Sigma, U.S.A

α-Glucosidase (maltase) 5.8 U/mg: Fluka, Switzerland

2,2'-Azobis (2-methylpropionitrile): Sigma-Aldrich Chemie GmbH, Germany

Bacto-peptone: Difco Laboratories, U.S.A.

Bacto-agar: Britania, Argentina

Beef extract: Difco Laboratories, U.S.A.

Bovine serum albumin (BSA): Sigma, U.S.A.

β-Cyclodextrin: Sigma, U.S.A

Calcium chloride: Merck, Germany

Corn starch: Maizena, Thailand

Dialysis tubing: Sigma, U.S.A.

Ethanol absolute: Carlo Erba, France

Ethylene glycol dimethacrylate: Sigma, U.S.A

36.5-38.0% Hydrochloric acid: J.T.Baker, U.S.A.

Iodine: Fluka, Switzerland

Itaconic anhydride: Sigma, U.S.A

Maltose: Conda, Spain

2-Mercaptoethanol: Scharlau, Spain

Methanol: Merck, Germany

Peptone from meat: Merck, Germany

Phenolphthalein: BDH, England

Potassium iodide: Univar, Australia

Slilica gel 60 TLC plate F254, Merck, Germany

Sodium acetate: BDH, England

Sodium hydroxide: BDH, England

Soluble starch, potato: Scharlau, Spain

2,4,6-Trinitrobenzene sulfonic acid: Sigma, USA

Yeast extract: Scharlau, Spain

2.3 Bacteria

Paenibacillus RB01 was isolated from hot spring soil in Ratchaburi province, Thailand and screened for CGTase activity by Tesana, 2001.

2.4 Media preparation

2.4.1 Medium I

Liquid medium I, composed of 0.5% (w/v) beef extract, 1.0% (w/v) peptone from meat, 0.2% (w/v) NaCl, 0.2% (w/v) yeast extract and 1.0% (w/v) soluble starch, was prepared and pH was adjusted to 7.2. In the case of solid medium, 1.5% (w/v) bacto-agar was added. Medium I was sterilized by autoclaving at 121^{O}C for 15 minutes.

2.4.2 Horikoshi medium (HK)

HK was used as enzyme (CGTase) production medium. Medium formula is slightly modified from Horikoshi, 1971, consisted of 1.0% (w/v) peptone from meat, 0.5% (w/v) yeast extract, 1.0% (w/v) soluble starch, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4.7H_2O$ and 0.75% (w/v) Na_2CO_3 with the pH of 10.1-10.2 (Rutchtorn, 1993). Medium was sterilized as described above.

2.5 Cultivation of bacteria

2.5.1 Starter cultivation

A colony of *Paenibacillus* RB01 was grown in 50 mL of starter Medium I in 250 mL conical flask at 37 ^{O}C with 250 rpm incubator shaking until A_{660} measurement by spectrophotometer reached 0.3-0.5 unit.

2.5.2 Enzyme production

Starter *Paenibacillus* RB01 (1.0 % (v/v)) was transferred to HK broth in a 1,000 mL Erlenmeyer flask and was allowed to grow at 40 ^{O}C with 250 rpm incubator shaking for 60 hours. Cells were harvested by refrigerated centrifugation at 3,000 x g for 30 minutes. Crude CGTase was collected as supernatant and kept at 4 ^{O}C for purification.

2.6 Partial purification of CGTase

CGTase was partially purified by starch adsorption method with slight modification from Kato and Horikoshi, 1984 (Laloknam, 1997).

Corn starch was dried at 100 ^{O}C for 2-3 hours, and was cooled to room temperature. It was gradually sprinkled into crude CGTase from the previous step to 5% (w/v) concentration. The mixture was 3-hour-continuous stirred at 4 ^{O}C and then starch cake was recovered by centrifugation at 2,390 x g for 30 minutes at 4 ^{O}C. The starch cake was washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$ pH 8.5 (TB1) and centrifuged at 4,039 x g for 30 minutes at 4 ^{O}C. The CGTase was eluted from the starch cake with TB1 (containing 0.2 M maltose pH 8.5) by three times of stirring for 30 minutes at 4 ^{O}C and centrifugation at 7,745 x g for 30 minutes at 4 ^{O}C. The supernatant was collected, concentrated with Viva flow concentrator and dialyzed twice with dialysis buffer (50 mM acetate buffer containing 5 mM $CaCl_2$ pH 6.0) at 4 ^{O}C.

2.7 Enzyme assay

2.7.1 Dextrinizing activity assay

Dextrinizing activity of α-amylase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (100 μl) was incubated with 0.3 ml starch substrate (0.2 g% soluble potato starch in 50 mM sodium acetate buffer pH 6.0) at 25^{O}C for 3 minutes. The reaction was stopped with 4 ml of 0.2 M HCl. Then 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, HCl was added before the enzyme sample.

One unit of enzyme was defined as the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described conditions.

2.7.2 CDase activity assay

Cyclodextrin degradating (CDase) activity of α-amylase was determined by incubating the enzyme with 1 ml of β-CD (5 mg/ml) in 50 mM sodium acetate

buffer pH 6.0 at 40^{O}C for 60 minutes. The release of reducing sugars was measured by monitoring the amount of cyclodextrin degraded using dinitrosalicylic acid (DNS) method. This method tests for the presence of free carboxyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose and keto functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid (which give the red color at 540 nm) under alkaline conditions.

Reaction mixture sample (0.25 ml) was incubated with 0.25 ml of DNS reagent. The reaction was stopped by boiling for 5 minutes. The mixture was cooled to room temperature and 2 ml of distilled water was added. The absorbance at 600 nm was measured.

2.7.3 Cyclization activity assay

Cyclization activity was determined by the phenolphthalein method of Goel and Nene (1995). Ten milligrams of enzyme was added into 1.5 ml of 6.0% (w/v) soluble potato starch in 50 mM sodium acetate buffer pH 6.0. The reaction mixture was incubated at 40 ^{0}C for 30 minutes. Reaction was stopped by boiling for 5 minutes. To 0.5 ml of sample, 2.0 ml of phenolphthalein solution (1.0 ml of 4 mM phenolphthalein in ethanol, 4 ml of ethanol and 100 ml of 125 mM Na_2CO_3 solution in distilled water) was added and incubated for 10 minutes at 70 ^{0}C. Absorption was measured at 550 nm and β-CD formed was calculated using the calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the corresponding condition.

2.8 Protein determination

Bradford's protein determination (1976) was chosen to determine the amount of protein using bovine serum albumin as standard. Enzyme sample (100 μL) was mixed with 0.1 ml of 1 M NaOH and 5 ml of protein reagent. The absorbance of the mixture was recorded at 595 nm after it was left for 5 minutes.

One liter of protein reagent is composed of 100 mg Coomassie blue G-250, 50 ml of 95% ethanol, 10 ml of 85% H_3PO_4 and distilled water.

2.9 Derivatization of α-amylase by acylation with itaconic anhydride

Thirty milligrams of α-amylase in 5 ml of 50 mM sodium acetate buffer pH 6.0 was acylated by using various amounts of itaconic anhydride. The solution mixtures at different ratios of itaconic anhydride per mg protein were stirred at 4 ^{0}C for 60 minutes. The pH was monitored and maintained at 6.0 using 3 M NaOH. Non-reacted itaconic anhydride and other low-molecular mass compounds were removed by gel filtration (HiTrap Desalting columns, 1.6x2.5 cm, Pharmacia) with distilled water as the eluent. The active α-amylase fractions were combined and finally lyophilized.

2.10 Determination of free amino groups in the α-amylase protein (TNBS assay)

The relative quantitative determination of amino groups of the native and covalently derivatized α-amylase samples (after elution of HiTrap Desalting columns) was performed according to Habeeb (1966) and Hall *et al*. (1973) with 2,4,6-trinitrobenzene sulfonic acid (TNBS). The absorbance obtained with the native protein corresponded to 100% and the extent of derivatization was calculated according to Shetty and Kinsella (1980).

$$\text{Derivatization degree (\%)} = [1 - (A_{der} / A_{nat})] \times 100$$

Where A_{der} and A_{nat} are the absorbance values obtained with derivatized and native protein solution, respectively.

To 0.3 ml of 10 mg/ml of protein solution (native or derivatized), 0.3 ml of 4% $NaHCO_3$ and 0.3 ml of 0.1% TNBS were added. The samples were placed in the thermomixer at 37 ^{0}C. After 60 minutes, 0.47 ml of 1 M HCl was added and the absorption was measured at 335 nm against a blank which was treated as above but 0.3 ml of deionized water was used instead of the protein solution.

2.11 Imprinting of the derivatized a-amylase

A typical procedure for imprinting was as follows. Derivatized enzyme (30 mg/ml) and β-CD (25 mg/ml) was dissolved in 1 ml of 50 mM sodium acetate buffer, pH 6.0. The mixture was incubated at 25 ^{0}C for 30 minutes. The α-amylase-β-CD complex was precipitated by the addition of 4 ml of *n*-propanol (-20 ^{0}C), then it was kept on ice for 10 minutes. The precipitate was then collected by centrifugation at 11,000 rpm for 15 minutes at 4 ^{0}C. The pellet was washed with 1 ml of *n*-propanol (-20 ^{0}C) and kept at 4 ^{0}C until further use. To obtain high imprinting efficiency, several reaction parameters in bioimprinting step including enzyme concentration, β-CD concentration and imprinting time were optimized.

2.12 Crosslinking of imprinted derivatized a-amylase

Imprinted derivatized α-amylase (10 mg/ml) prepared as in section 2.11 was suspended in 1 ml of dry cyclohexane using an ultrasonication bath for 30 minutes. Then 4 mg of 2,2'-Azobis (2-methylpropionitrile) (AIBN) and 0.82 M of ethylene glycol dimethacrylate (EGDMA) were added into this suspension. The radical polymerization was initiated under UV irradiation (λ = 240 nm) at 25 ^{0}C for 3 hours. The resulting polymer was kept in a refrigerator at 4 ^{0}C for 12 hours. Then the polymer was washed with 2 ml of cyclohexane to remove unreacted crosslinker followed by washing with 50 mM sodium acetate buffer, pH 6.0 (3 x 10 ml). The polymer with nonimprinted derivatized α-amylase was also crosslinked (immobilized α-amylase). To obtain high immobilization efficiency, several reaction parameters in crosslinking step including crosslinker concentration and immobilization time were optimized.

2.13 Analysis of the products of CLIP activity

2.13.1 Thin layer chromatography (TLC)

TLC was used to determine the products produced from CLIP. The sample solution was spotted on a TLC silica gel 60 plate and developed with n-propanol : ethyl acetate : water (7:1:2). The glass chamber was equilibrated at 25^0C for 3 hours before used. When the ascending solvent front was near the top margin (about 1 cm), the plate was removed from the chamber and dried for 30 minutes at room temperature. After that spot were detected by spraying with sulfuric acid : ethanol (1:9) (Kennedy and Pagliuca, 1994) followed by heating at 110^0C for 15 minutes to develop color of the sugar spot.

2.13.2 High performance liquid chromatography (HPLC)

The products from CLIP were analyzed by HPLC. The HPLC system was a Shimadzu LC-3A equipped with LUNA-NH_2 column (0.46 x 25 cm) and using Shimadzu RID-3A refractometer as detector. The reaction mixture of α-amylase was filtered through a 13 mm Nylon 0.45 μm disc filter before injection and eluted with acetonitrile : water (70:30, v/v) using flow rate of 1 ml/min. The product peaks were identified by comparing the retention times with those of standard oligosaccharides (10 mg/ml).

CHAPTER III

RESULTS

In this study, we attempted to alter the substrate specificity of α-amylase on the level of mature protein by the combination of bioimprinting with β-cyclodextrin (β-CD) and immobilization method. To maintain the bioimprinted memory of the enzyme not only in organic solvents but also in aqueous environment, the imprinted enyme was first vinylated and then crosslinked with ethylene glycol dimethylacrylate to form a rigid and stable conformation. The technique is called CLIP (crosslinked imprinted protein). In this way, we propose that the ability of the enzyme to hydrolyze β-CD was possible (using a crosslinked α-amylase initially imprinted with β-CD).

3.1 Partial purification of cyclodextrin glycosyltransferase (CGTase)

CGTase from *Paenibacillus* RB01 was first partially purified and was used as a control in this study. The enzyme was obtained after cultivation of this bacterium in Horikoshi's medium at 40^{O}C with 250 rpm continuous shaking for 60 hours. Afterwards, the enzyme in supernatant was separated from cells by centrifugation and collected for partial purification via corn starch adsorption with slight modification from Kato and Horikoshi (1984), as described in Section 2.6. After the enzyme was eluted from corn starch using maltose, concentrated by Viva flow and dialyzed against 50 mM acetate buffer containing 10 mM $CaCl_2$, its activity (dextrinizing activity) and amount of protein were determined according to the method described in Section 2.7 and 2.8 The partial purified enzyme had a specific activity of 3,244.9 U/mg of protein and the % recovery of CGTase was 78% of the total activity in crude enzyme as shown in Table 5.

Table 5 Purification of CGTase from *Paenibacillus* sp. RB01

Purification step	Volume (mL)	Dextrinizing activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1,500	23,012	201.7	114.1	1	100
Starch adsorption	200	21,968	69.4	316.5	2.8	96
Ultrafiltration	30	17,847	5.5	3244.9	28.3	78

3.2 Cyclodextrin degradating (CDase) activity of native a-amylase

In order to study the effect of bioimprinting with β-CD on the substrate specificity of α-amylase, the CDase and dextrinizing activities of each enzyme sources (α-amylase from *Aspergillus oryzae* and *Bacillus subtilis*) were investigated in comparison with cyclodextrin glycosyltransferase (CGTase) and cyclodextrinase (CDase). To measure the CDase activity, the enzyme was incubated with β-CD (5 mg/ml) in 50 mM sodium acetate buffer, pH 6.0.

The released reducing sugar was measured by the dinitrosalicylic acid (DNS) method. The results obtained indicated that the native α-amylase from *Bacillus subtilis* did not degrade β-CD at the certain condition (25^0C, overnight) whereas the α-amylase from *Aspergillus oryzae,* CGTase and CDase had strong CDase activities under the same condition (Table 6). In addition, the incubation time of all enzyme preparations was overnight except α-amylase from *Aspergillus oryzae* which was incubated with β-CD for only 60 minutes. Since it gave very high absorbance at 540 nm under the certain condition. This indicated that α-amylase from *Aspergillus oryzae* had an ability to degrade CD which was in contrast with that of α-amylase from *Bacillus subtilis.* Therefore, in order to investigate the effect of bioimprinting on CDase activity, the α-amylase from *Bacillus subtilis* was chosen for further studies.

3.3 Enzyme derivatization

B. subtilis α-amylase was derivatized according to the described method in Section 2.9.

3.3.1 Enzyme derivatization and purification of derivatized enzyme

In order to have enough attachment points in enzyme crosslinking under UV irradiation of CLIP technique, the α-amylase had to be first derivatized by acylation with itaconic anhydride in aqueous medium. The derivatized enzyme

Table 6 CDase activity of a-amylases, CGTase and CDase

Enzyme	A_{540} [a]	U/mg
H_2O	0	0
A. oryzae α-amylase (10 mg/ml)	1.207	11
B. subtilis α-amylase (10 mg/ml)	0	0
CGTase (0.18 mg/ml)	0.894	454
CDase (6 mg/ml)	1.692	774

[a] The data represent the mean values of three independent experiments

One hundred microliters of each enzyme was incubated with 5 mM β-CD in a total reaction volume of 1.1 ml at 25^0C overnight, except for α-amylase from *A. oryzae* which was incubated for only 1 hour.

preparation was applied onto a gel filtration column (HiTrap® desalting column, Pharmacia). Non-reacted itaconic anhydride and other low-molecular mass compounds were removed with distilled water as the eluent. Fractions of 1.5 ml were collected and protein content was measured by reading the absorbance at 280 nm. The enzyme activity was determined as described in Section 2.7.1. and the purification profile is shown in Figure 9. The active α-amylase fractions (fractions 2, 3 and 4) were combined and finally lyophilized.

3.3.2 Influence of derivatization with itaconic anhydride on a-amylase activity

By varying the protein per itaconic anhydride ratio, different degree of derivatization of α-amylase was obtained. The remaining activity of the enzyme depends on the degree of derivatization as presented in Table 7. The optimum ratio (w/w) of protein to itaconic anhydride was 1:5. The resulting degree of α-amylase derivatization determined by the TNBS assay was 60.7%. The remaining activity of the derivatized enzyme was 93%. For the ratio of 1:2.5, the derivatization degree was somewhat lower than the ratio 1:5. Beyond the ratio of 1:5, higher levels of derivatization were possible by increasing the itaconic anhydride concentration per mg of protein, but resulted in a further decrease in activity. Thus, the protein per itaconic anhydride ratio of 1:5 was chosen for further experiments.

3.4 Optimization of the bioimprinting procedure

After the derivatization step, the derivatized enzyme was then imprinted with imprinter (β-CD). In order to investigate the optimum condition for bioimprinting of α-amylase, the CDase and dextrinizing activity of CLIP-α-amylase should be determined. However, no CDase activity of CLIP-α-amylase could be measured. Thus, only dextrinizing activity of CLIP-α-amylase was investigated. In this study *Bacillus subtilis* α-amylase was incubated with β-CD (imprinter). To obtain high imprinting efficiency, several reaction parameters in

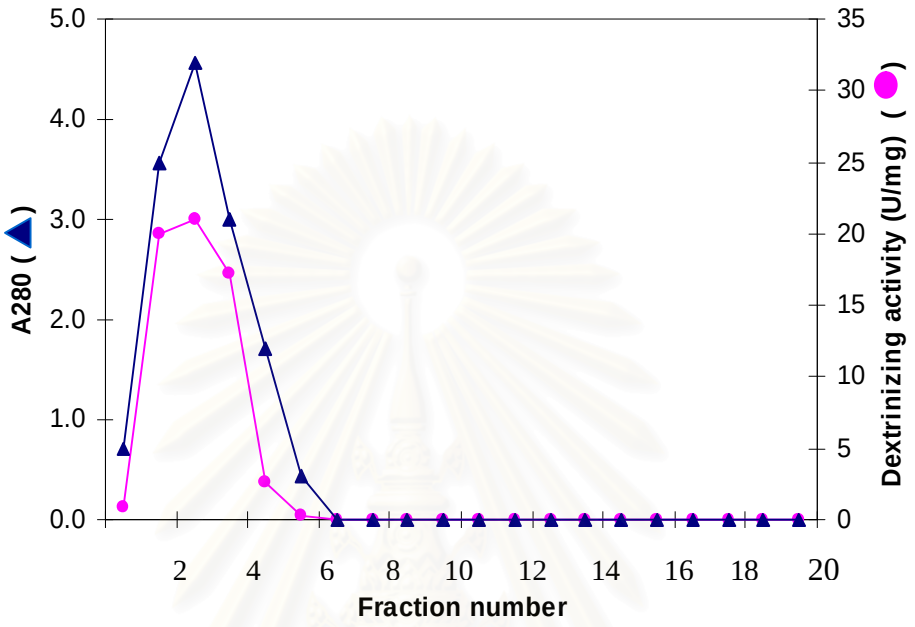


Figure 9 Chromatographic profile of derivatized a-amylase from HiTrap desalting column

The enzyme solution of 1.5 ml was applied onto HiTrap desalting column (1.6x2.5 cm) and washed with distilled water.

Table 7 Effect of itaconic anhydride on derivatization degree and dextrinizing activity of a-amylase

Enzyme:Itaconic anhydride (w/w)	Derivatization Degree[a] (%)	Activity[a] (%)
1 : 0	0	100
1 : 2.5	52.5 ± 2.9	99 ± 0.3
1 : 5.0	60.7 ± 0.9	93 ± 0.3
1 : 7.5	68.7 ± 1.8	72 ± 0.5
1 : 10	78.4 ± 0.2	45 ± 0.6

[a] The data represent the mean values of three independent experiments

bioimprinting step including derivatized enzyme concentration, β-CD concentration and imprinting time were optimized. In this experiment, the immobilized α-amylase which was defined as crosslinked enzyme without imprinter was used as a control and its dextrinizing activity was assigned to the value of 100%.

3.4.1 Effect of derivatized enzyme concentration

To investigate the influence of derivatized enzyme concentration on the CDase and dextrinizing activity of CLIP-α-amylase, the fixed amount of β-CD (25 mg) was incubated with different concentration of derivatized enzyme varying from 10 to 50 mg. Then the imprinted α-amylase was immobilized before the activities were assayed. In the case of successful imprinting, the dextrinizing activity of CLIP-α-amylases should decrease in comparison with the immobilized enzyme (crosslinked without imprinter). It can clearly be seen in Figure 10 that at 20-50 mg of α-amylase used, the resulting CLIP-α-amylases were indifferent with dextrinizing activity of approximately 39-46%. At 10 mg enzyme used, the resulting CLIP-α-amylase could not be collected. However, the amount of 30 mg of α-amylase was considered to be suitable for bioimprinting procedure. Since, it gave enough amount of imprinted enzyme to be used in further steps (immobilization).

3.4.2 Effect of b-CD concentration

Once the optimal concentration of derivatized α-amylase was achieved, another factor that has an effect on the bioimprinting was then investigated. The 30 mg of the derivatized enzyme was added into 1 ml buffer, followed by the addition of different concentration of β-CD, ranging from 1 to 50 mg. The enzyme was then immobilized and the dextrinizing activity was investigated as described in Section 2.12 and 2.7, respectively. The result is shown in Figure 11. When 1 mg/ml of β-CD was used, it gave the dextrizing activity of 77.8% which was higher than other β-CD concentrations used. This could be due to the fact that β-CD at

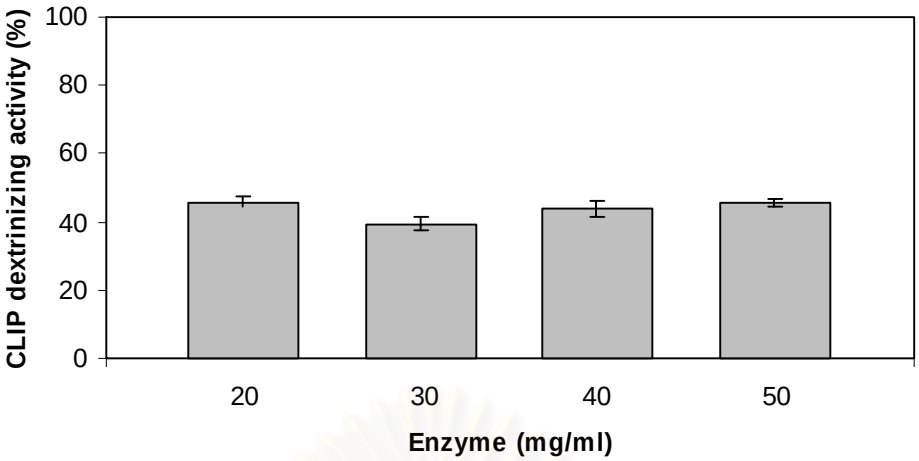


Figure 10. Influence of derivatized a-amylase concentration on dextrinizing activity of CLIP-a-amylase.

Various concentrations of derivatized α-amylase were incubated with 25 mg/ml β-CD in sodium acetate buffer pH 6.0 at 25°C for 30 minutes. The activity of immobilized α-amylase (without imprinter) was defined as 100%. Results shown were average values of triplicate experiments.

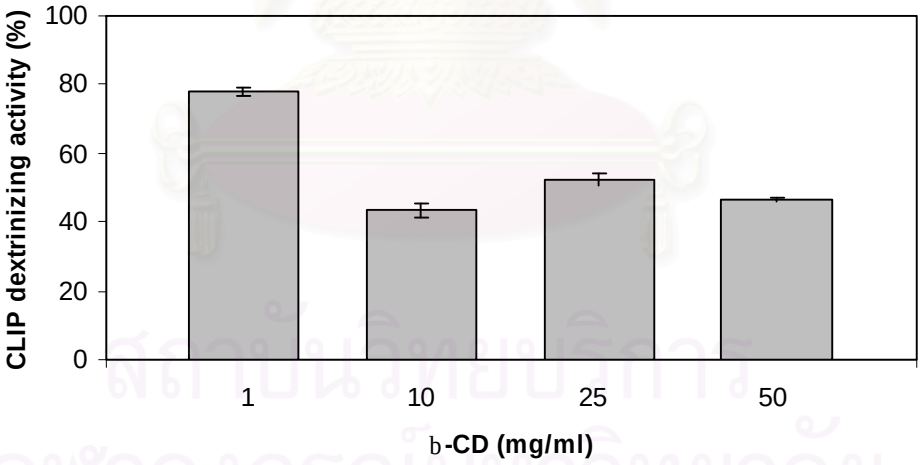


Figure 11. Effect of the b-CD concentration on CLIP-a-amylase activities

Derivatized α-Amylase of 30 mg/ml was incubated with various concentrations of β-CD in sodium acetate buffer pH 6.0 at 25°C for 30 minutes. The activity of immobilized α-amylase (without imprinter) was defined as 100%. Results shown were average values of triplicate experiments.

the concentration of 1 mg/ml did not reach the maximum solubility of β-CD in water (18 mg/ml). And hence, low imprinter molecules. Although at other concentrations of β-CD used, there were not significantly different in CLIP dextrinizing activity, the amount of 25 mg/ml of β-CD was considered to be suitable for bioimprinting procedure since it exceeded the maximum solubility of β-CD in water.

3.4.3 Effect of the imprinting time

Further optimization of bioimprinting procedure for a higher imprinting yield was performed by varying the imprinting time. The imprinting time was varied from 5 to 60 minutes. As shown in Figure 12, there were no effects of imprinting time on the dextrinizing activity of CLIP-α-amylase. The dextrinizing activities obtained from various imprinting times ranging from 42.8% to 49.7%. However, the imprinting time of 30 minutes was chosen since it has previously been shown from other studies to be adequate to modify the enzyme (Peiβker *et al*., 1998; Vaidya *et al*., 2004).

3.5 Optimization of the immobilization procedure

The bioimprinted enzyme was produced using the optimized condition as performed in Section 3.4. The next step was to immobilize the bioimprinted enzyme to obtain high immobilization efficiency, reaction parameters in crosslinking step including crosslinker concentration and immobilization time were optimized.

3.5.1 Effect of crosslinker concentration

To investigate the influence of crosslinker concentration on the CDase and dextrinizing activity of CLIP-α-amylase, various amounts of ethylene glycol dimethacrylate (EGDMA) were used in the immobilization step. The results are shown in Figure 13. It was concluded that the resulting polymer (CLIP-α-amylase) could not be collected when a crosslinker at a final concentration of 0.05 and

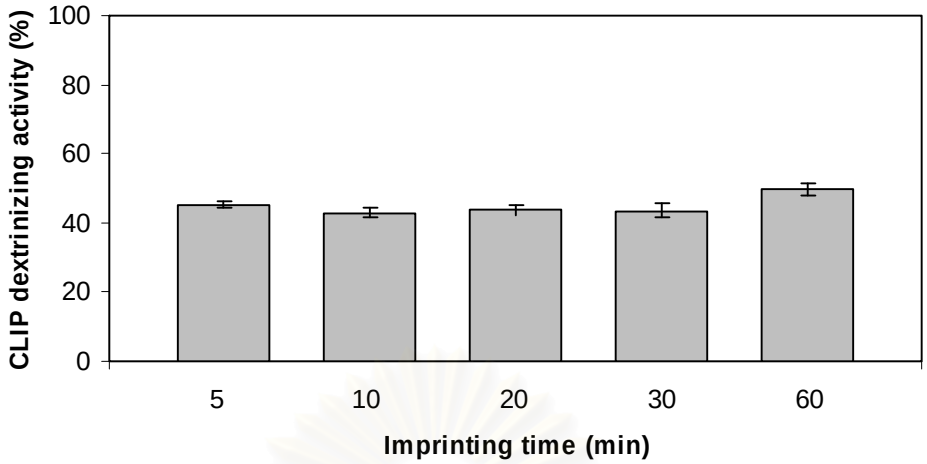


Figure 12. Effect of the imprinting time on CLIP-α-amylase activities
α-Amylase of 30 mg/ml was incubated with 25 mg/ml of β-CD in sodium acetate buffer pH 6.0 at 25°C for various times. The activity of immobilized α-amylase (without imprinter) was defined as 100%. Results shown were average values of triplicate experiments.

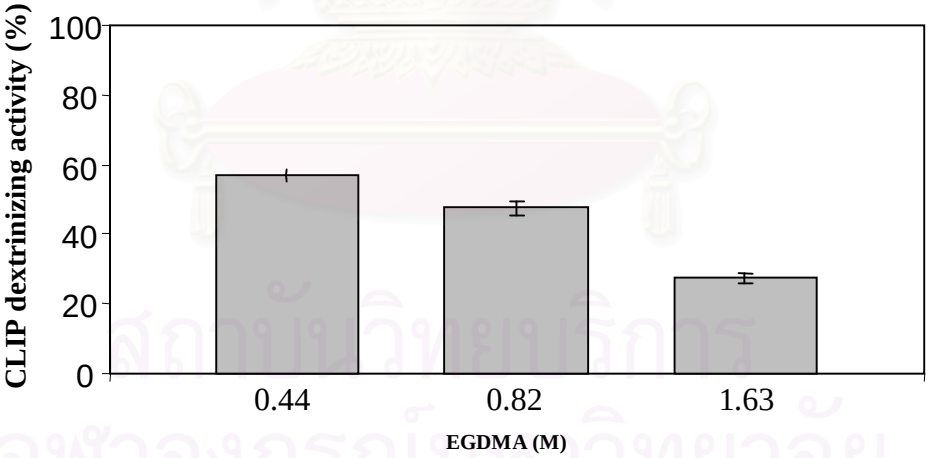


Figure 13. Effect of the EGDMA concentration on CLIP-α-amylase activities
Imprinted α-amylase of 10 mg/ml was suspended in cyclohexane with various amounts of EGDMA at 25°C for 3 hours. The activity of immobilized α-amylase (without imprinter) was defined as 100%. Results shown were average values of triplicate experiments.

0.23 M were used. The optimal EGDMA concentration was found to be 0.44 and 0.82 M, which resulted in dextrinizing activities of 57.0% and 47.6%, respectively. However, the EGDMA concentration of 0.82 M was considered to be suitable for crosslinking procedure. Since it ensured to maintain an imprinted property of the enzymes and did not result in too rigid conformation of the enzyme (which can markedly decrease the enzyme activity). At other concentrations of EGDMA used (1.63 M), the CLIP-α-amylase dextrinizing activity was markedly decreased (27.3%) and thus, was not suitable to be used in immobilizing the imprinted enzyme.

3.5.2 Effect of crosslinking time

The effect of immobilization time on dextrinizing activity of CLIP-α-amylase was investigated by varying the UV irradiation time (λ=240 nm) from 0.5 to 4 hours. The results are shown in Figure 14. The immobilization time of 0.5 hour was not enough to initiate the adequate UV irradiation, and no CLIP-α-amylase was collected. At longer crosslinking times (1-4 hrs), there were slightly different in dextrinizing activities obtained ranging from 46.7% to 52.7%. And at crosslinking time of 5 hrs, the dextrinizing activity obtained was markedly decreased (33.5%). It was concluded that the enzyme activity was significantly destroyed by UV irradiation of 5 hours or more. Therefore, the suitable crosslinking time was 2 to 4 hours. However, the UV irradiation time of 3 hours was considered to be used for crosslinking procedure.

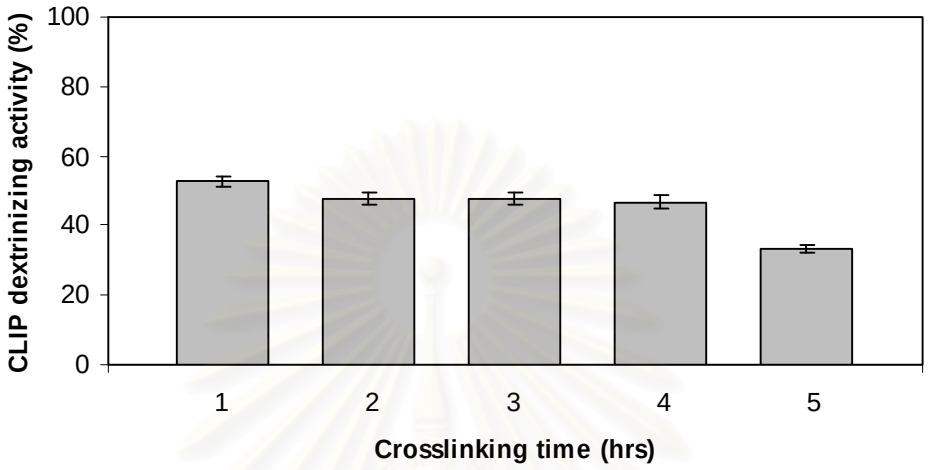


Figure 14. Effect of the crosslinking time on CLIP-α-amylase activities
Imprinted α-amylase of 10 mg/ml was suspended in cyclohexane with a final concentration of 0.82 M EGDMA at 25°C for various crosslinking times. The activity of immobilized α-amylase (without imprinter) was defined as 100%. Results shown were average values of triplicate experiments.

3.6 Effect of bioimprinting on CDase activity of a-amylase

To investigate the influence of bioimprinting process on CDase activity of α-amylase, the enzyme (10-30 mg/ml) and β-CD (25 mg/ml) were dissolved in 1 ml of buffer. The mixture was incubated at 25 ^{0}C for 30 minutes. The resulting α-amylase-β-CD complex was precipitated and washed. The CDase activity of CLIP-α-amylase (immobilized imprinted α-amylase) was determined as described in Section 2.7.2 and compare to that of the native, derivatized (coupling with itaconic anhydride only) and crosslinked (immobilization without imprinting) α-amylase. The results are shown in Table 8. For all α-amylase preparations, there were no CDase activities obtained under the investigated condition (40^{0}C for 60 min and 40^{0}C overnight). Assay of CGTase activity confirmed positive reaction on hydrolysis of CDs. It was concluded from the result obtained that the CDase activity of α-amylase could not be generated by imprinting with β-CD under the investigation conditions.

3.7 Effect of bioimprinting on cyclization activity of a-amylase

The cyclization activity of CLIP-α-amylase (immobilized imprinted α-amylase) was determined as described in Section 2.7.3 and compare to that of the native, derivatized (coupling with itaconic anhydride only) and crosslinked (immobilization without imprinting) α-amylase. The results are shown in Table 9. For all α-amylase preparations, there were no cyclization activities obtained under the investigated condition (40^{0}C for 30 min and 40^{0}C overnight). Assay of CGTase activity confirmed positive reaction on formation of CDs. It was concluded from the result obtained that the cyclization activity of α-amylase could not be generated by imprinting with β-CD under the investigation conditions.

Table 8 Effect of bioimprinting on CDase activity of crosslinked and CLIP-α-amylases

Enzyme Samples	Specific activity[a] (U/mg)
CGTase (0.18 mg/ml)	6.99
Native α-amylase	0
Crosslinked α-amylase	0
CLIP-α-amylase	0

[a] The data represent the mean values of three independent experiments

One hundred microliters of CGTase and 10 mg of each α-amylase preparations were incubated with 5 mM β-CD in a total reaction volume of 1.1 ml at 25^0C overnight.

Table 9 Effect of bioimprinting on cyclization activity of immobilized and CLIP-α-amylase

Enzyme Samples	Activity[a] (U/mg)
CGTase (0.18 mg/ml)	162
Native α-amylase	0
Crosslinked α-amylase	0
CLIP-α-amylase	0

[a] The data represent the mean values of three independent experiments

One hundred microliters of CGTase and 10 mg of each α-amylase preparations were incubated with 5 mM β-CD in a total reaction volume of 1.1 ml at 25^0C overnight.

3.8 Effect of bioimprinting on dextrinizing activity of a-amylase

To investigate the influence of bioimprinting process on dextrinizing activity of α-amylase, the derivatized enzyme (30 mg/ml) and β-CD (25 mg ml) were dissolved in 1 ml of buffer. The mixture was incubated at 25 ^{0}C for 30 minutes. The resulting α-amylase-β-CD complex was precipitated and washed. The precipitated enzyme was then crosslinked. The dextrinizing activity of CLIP-α-amylase was determined as described in Section 2.7.1 and compare to that of the native, derivatized and crosslinked α-amylase. The results are shown in Table 10. The dextrinizing activities of native and derivatized α-amylase (1 mg/ml) were 3.25 and 2.08 U/mg, respectively. This implies that the derivatization process resulted in a decrease in dextrinizing activity of 36%. After immobilization step, the dextrinizing activity of the crosslinked (immobilization without imprinting) and CLIP-α-amylase (immobilization with imprinting) were decreased to 0.22 and 0.09, respectively. This markedly decrease in dextrinizing activity was due to the solubility and the rigid conformation of the immobilized enzymes (effect of the immobilization on the activity of the enzyme). However, the dextrinizing activity of CLIP-α-amylase was 42.4% in comparison with that of the crosslinked form (without the template molecule). This could be due to the imprinting effect on the dextrinizing activity of the enzyme. And thus, it may be concluded that the substrate specificity of CLIP-α-amylase has been altered although CDase activity could not be measured.

Table 10 Effect of bioimprinting on dextrinizing activity of derivatized, immobilized and CLIP-a-amylases

Enzyme Samples	Specific activity[a] (U/mg)	Relative specific activity (%)
Native α-amylase	3.25	100
Derivatized α-amylase	2.08	64
Crosslinked α-amylase	0.22	6.8
CLIP-α-amylase	0.09	2.8

[a] The data represent the mean values of three independent experiments

One hundred microliters of native and derivatized α-amylase (1 mg/ml) and five milligrams of each α-amylase preparations were incubated with 0.2 g% soluble potato starch in a total reaction volume of 0.3 ml for 3 minutes at 25^0C.

3.9 Analysis of the products of CLIP activity by TLC and HPLC

As can be seen in previous section that the ability to degrade starch by CLIP-α-amylase was remarkably reduced in comparison to that of the immobilized enzyme (crosslinked α-amylase without the imprinter). Nevertheless, the activity of the CLIP enzyme to hydrolyze β-CD could not be detected. In this experiment, TLC and HPLC methods were then used to check if there were any CDase reaction products produced by CLIP-α-amylase (as described in section 2.13.1 and 2.13.2, respectively). The dextrinizing products catalyzed by native, immobilized and CLIP-α-amylase were also examined by TLC and HPLC. The result (Figure 15) shows that the native (lane q), immobilized (lane k and m) and CLIP-α-amylase (lane l and o) could not synthesize oligosaccharide product by using β-CD as substrate (no CDase activity) under this condition. In the case of dextrinizing activity products, both immobilized (lane i and n) and CLIP-α-amylase (lane j and p) produced the same product pattern (maltotriose) while the native α-amylase (lane r) produced maltose and maltotriose.

For separation by HPLC, the chromatogram of reaction mixture catalyzed by CDase activity of native, immobilized and CLIP-α-amylase is shown in Figure 16. The retention time of β-CD was 17.6 minutes. The peak at 3.1 minutes was the solvent peak. The HPLC profiles were similar in all enzyme preparations. This result supports the results obtained by spectrophotometric method using DNS reagent in that all α-amylase preparations could not use β-CD as its substrate (no CDase activity) at the certain condition. In the case of the analysis of the reaction mixture catalyzed by dextrinizing activity of native, immobilized and CLIP-α-amylase (Figure 17), it can clearly be seen that less products were observed with CLIP-α-amylase, indicating that CLIP-α-amylase has lower dextrinizing activity than that of the immobilized and native α-amylase, respectively. This investigation also supports the results obtained by DNS method that the dextrinizing activity of CLIP-α-amylase was 42.4% in comparison with that of immobilized form.

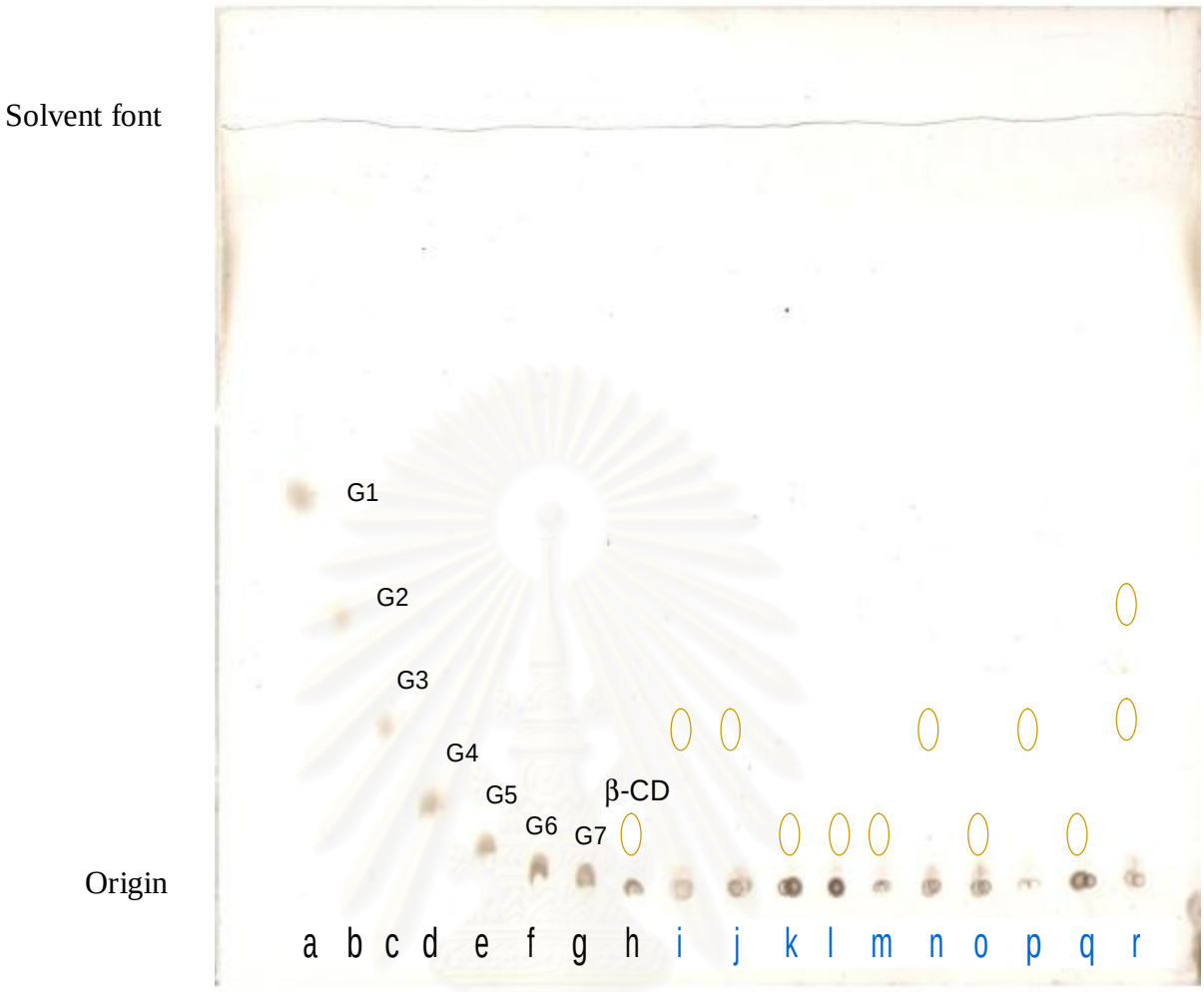


Figure 15. TLC analysis of the products catalyzed by native, immobilized and CLIP a-amylase. Spots were visualized with a dipping solution at 110^{0}C for 15 minutes for carbohydrate detection.

a) - g) Standard glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, respectively

h) Standard β-CD

i), n) Reaction mixture catalyzed by dextrinizing activity of immobilized α-amylase

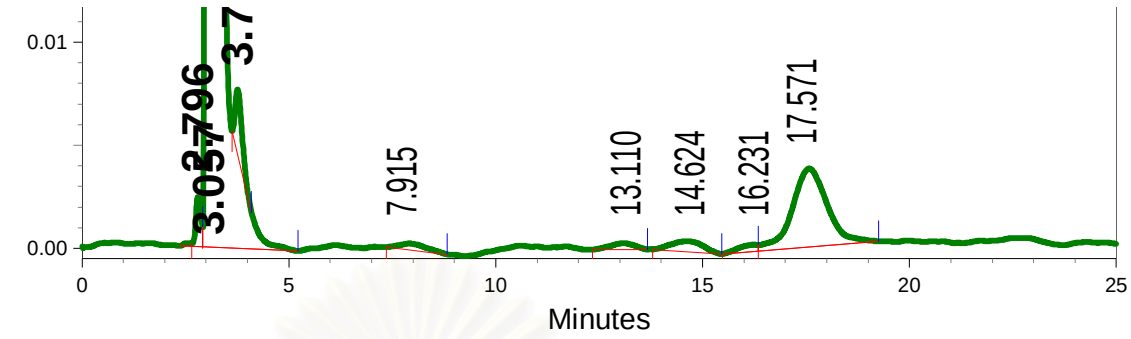
j), p) Reaction mixture catalyzed by dextrinizing activity of CLIP-α-amylase

k), m) Reaction mixture catalyzed by CDase activity of Immobilized α-amylase

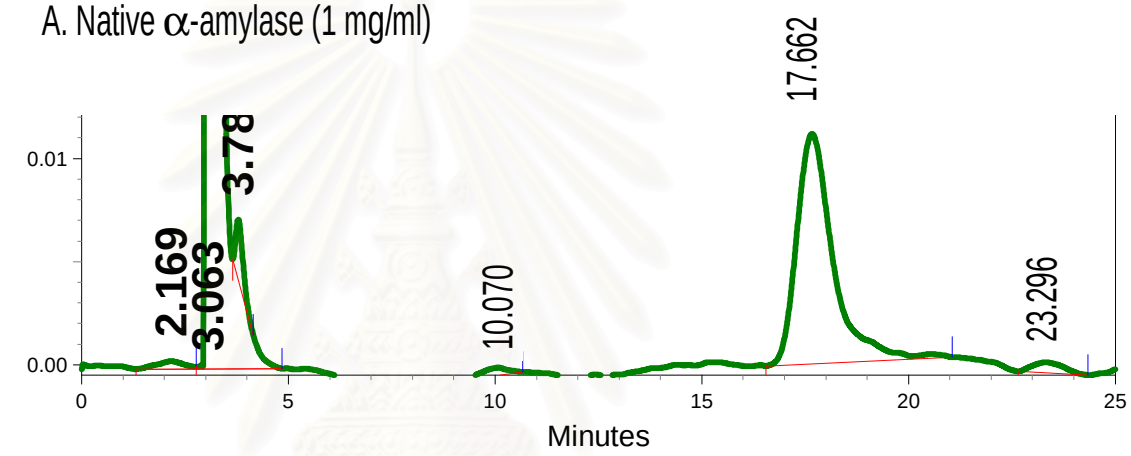
l), o) Reaction mixture catalyzed by CDase activity of CLIP-α-amylase

q) Reaction mixture catalyzed by CDase activity of Native α-amylase

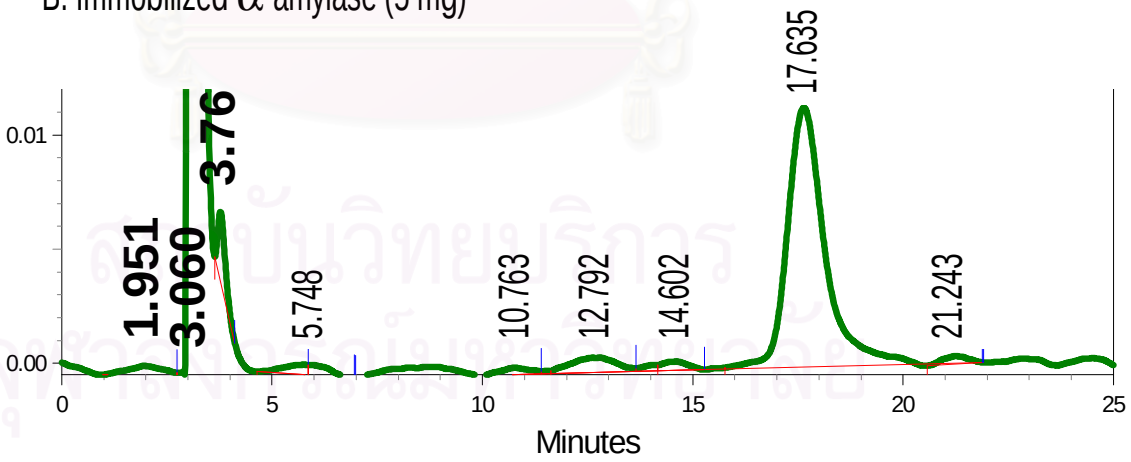
r) Reaction mixture catalyzed by dextrinizing activity of Native α-amylase



A. Native α-amylase (1 mg/ml)

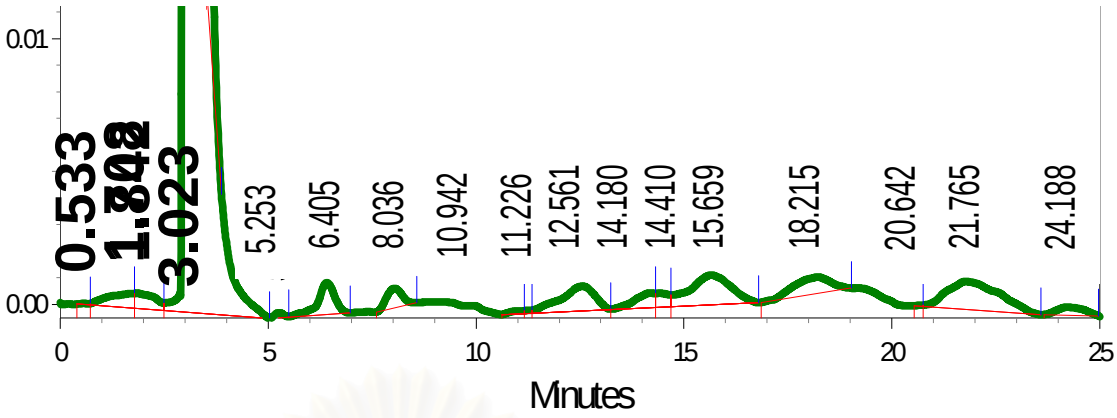


B. Immobilized α-amylase (5 mg)

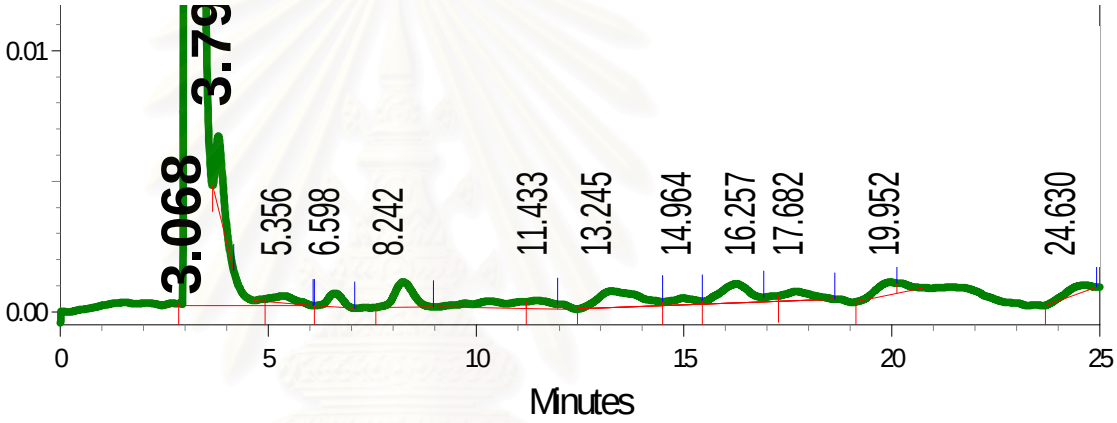


C. CLIP α-amylase (5 mg)

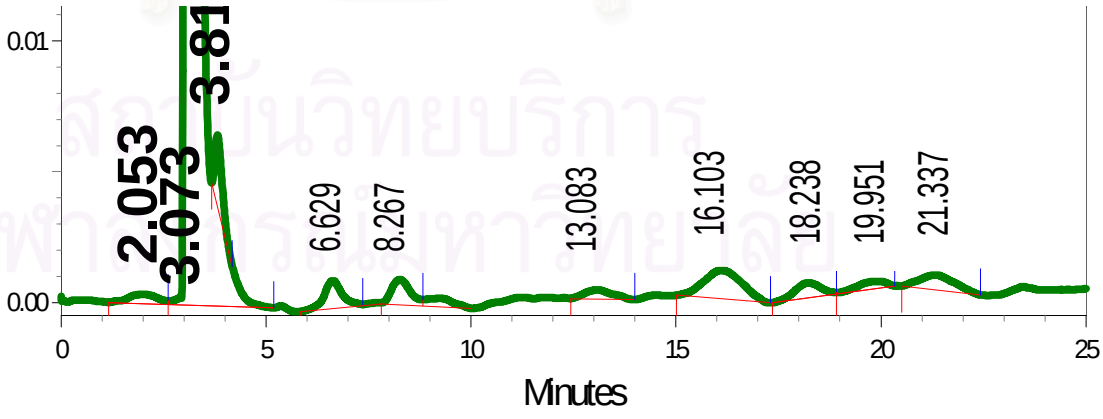
Figure 16. HPLC chromatogram of the CDase reaction products catalyzed by native, immobilized and CLIP a-amylase.



A. Native α-amylase (1 mg/ml)



B. Immobilized α-amylase (5 mg)



C. CLIP α-amylase (5 mg)

Figure 17. HPLC chromatogram of the dextrinizing products catalyzed by native, immobilized and CLIP a-amylase.

CHAPTER IV

DISCUSSIONS

4.1 Enzyme derivatization

The coupling of a cyclic anhydride with the primary amino groups on the surface of the protein has been reported (Atassi and Habeeb, 1972). This offers the possibility of the introduction of polymerizable vinyl groups into the protein, under very mild condition, when itaconic anhydride is used as the cyclic anhydride. Free amino groups of lysines, hydroxyl groups of tyrosines or sulfhydryl groups of cysteines of proteins were able to covalently couple with itaconic anhydride (Fischer *et al*., 1998). The side-chains that were able to react with the itaconic anhydride in aqueous solution, at ambient temperature and pH 6.0, were not only amino groups, as described by Kennedy and Chabral (1987), but also hydroxy and thiol groups. Also Meighen and Schachman (1972) reported on the acylation of tyrosine and thiol residues in proteins when using succinic anhydride as acylating reagent. However, the priority of this acylation reaction is clearly towards primary amino groups of lysines.

From previous reports with two-step immobilization technique, the optimum derivatization degrees of various proteins which resulted in optimum crosslinking process are shown in Table 11. Kronenburg *et al*. (2001) succeeded to manipulate an enantioselective of epoxide hydrolase (EH) using CLIP-EH at derivatization degree of 70% while Peiβker *et al*. (1999) reported that 60% derivatization degree was optimum considering the activity of resulting derivatized protease. Vaidya *et al*. (2004) found that an alteration of the substrate specificity of glucose oxidase could be achieved by using derivatization degree at 70%. Kaulpiboon *et al*. (2007) succeeded to manipulate the product specificity of the *Paenibacillus* sp. A11 and *Bacillus macerans* cyclodextrin glycosyltransferases towards the preferential formation of gamma-cyclodextrin by using derivatization degree of 61.7% and 64.6%, respectively. This shows that the optimum

Table 11 Optimum derivatization degrees of various proteins

Researchers	Proteins	Optimum derivatization degree (%)
Peißker *et al*. (1999)	Protease	60
Kronenburg *et al*. (2001)	Epoxide hydrolase	70
Vaidya *et al*. (2004)	Glucose oxidase	70
Kaulpiboon *et al*. (2007)	*Paenibacillus* CGTase	61.7
	B.macerans CGTase	64.6

derivatization degrees of various proteins which result in optimum crosslinking process are ranging from 60 to 70%.

In the case of the α-amylase from *Bacillus subtilis*, nearly all of the amino acid side-chains (78%) were able to react with the itaconic anhydride at pH 6.0 when the ratio of enzyme per itaconic anhydride was 1:10 (w/w). However, the remaining activity was only 45%. The loss of the α-amylase activity after co-polymerization could possibly be due to the very high level of enzyme acylation with itaconic anhydride. From the results obtained, the optimum ratio (w/w) of protein to itaconic anhydride was 1:5. The resulting degree of α-amylase derivatization determined by the TNBS assay was 60.7%. The remaining activity of the derivatized enzyme was 93%.

2,4,6-Trinitrobenzene sulfonic acid (TNBS) is a rapid and sensitive assay reagent for the determination of free amino groups (Habeeb *et al*., 1966). Primary amines, upon reaction with TNBS, form a highly chromogenic derivative, which can be measured at 335 nm. The reaction of TNBS with a primary amine containing molecules is shown in Figure 18.

4.2 Optimization of the bioimprinting procedure

In order to investigate the optimum condition for bioimprinting of α-amylase, the CDase and dextrinizing activity of CLIP-α-amylase should be determined by iodine method (Fuwa, 1954) and dinitrosalicylic acid (DNS) method, respectively. DNS method tests for the presence of free carboxyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose and ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions and the absorbance at 540 nm can be read.

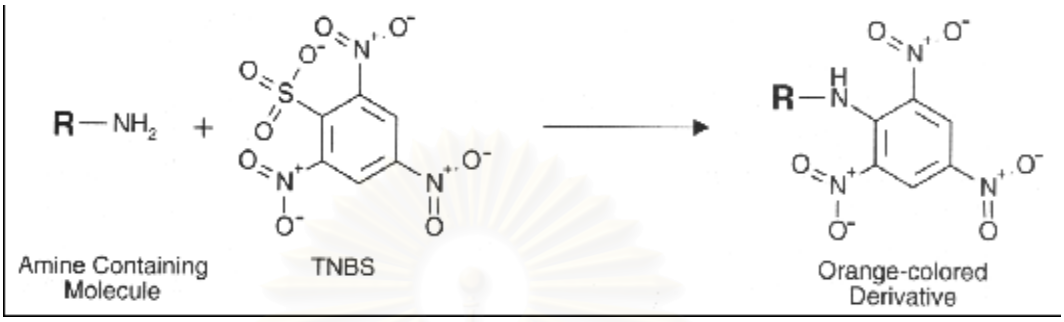


Figure 18. Reaction of TNBS with a primary amine-containing molecule to produce a chromogenic derivative.

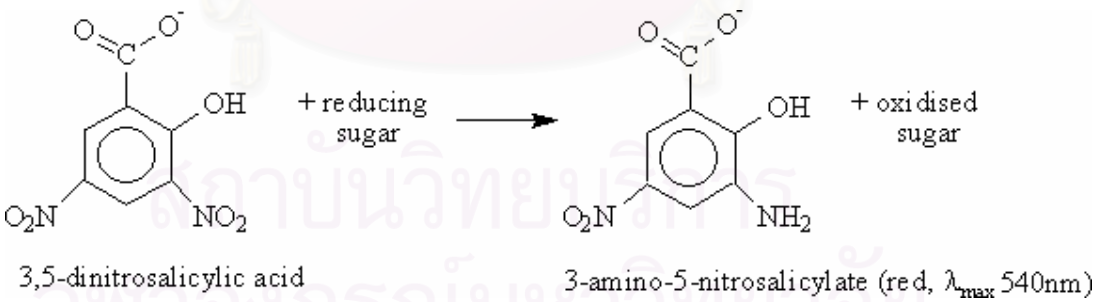
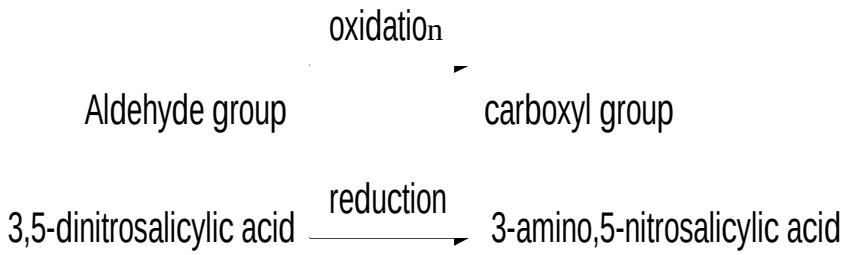


Figure 19. Reaction of DNS with reducing sugars.



However, the CDase activity of CLIP-α-amylase could not be measured under all investigated conditions. Thus, only dextrinizing activity of CLIP-α-amylase was investigated to study the effect of bioimprinting process on the activity of CLIP enzyme. The criteria used for the selection of an appropriate bioimprinting condition was to find the condition which resulted in the conformational change of the active site of α-amylase. The high reduction in dextrinizing activity of CLIP α-amylase (in comparison with non-imprinted enzyme) was defined as the optimum condition for bioimprinting of α-amylase.

First, the bioimprinting procedure of α-amylase was determined using various concentration of α-amylase. The results (Figure 10) demonstrated that at 20-50 mg/ml of α-amylase used, the dextrinizing activity of CLIP-α-amylases were indifferent. Joseph *et al*. (1997) has found that the activity and substrate specificity of subtilisin-catalyzed acylation of nucleosides in organic solvents can be controlled by lyophilizing the 10 mg/ml enzyme from an aqueous solution containing the substrate. Kronenburg *et al*. (2001) has been successful in the bioimprinting of membrane-associated epoxide hydrolase (EH) when the enzyme concentration of 30 mg/ml was used. Fishman *et al*. (2003) has reported that bioimprinting of lipases with fatty acids was shown to be feasible when imprinted 10 mg/ml of enzyme with caprylic acid. Vaidya *et al.* (2004) has reported that alteration of the substrate specificity of glucose oxidase could be achieved by using 30 mg/ml of enzyme in bioimprinting procedure. Kaulpiboon *et al.* (2007) succeeded to manipulate the product specificity of the *Paenibacillus* sp. A11 and *Bacillus macerans* cyclodextrin glycosyltransferases towards the preferential formation of gamma-cyclodextrin (CD8) by imprinting 30 mg/ml enzyme with 54

mg γ-CD. Thus, from previous studies and our investigated results, the amount of 30 mg of α-amylase was considered to be suitable for bioimprinting procedure to ensure enough amount of imprinted enzyme to be used in further steps of CLIP technique (immobilization).

Then, the effect of β-CD (imprinter) on bioimprinting procedure was investigated. The result is shown in Figure 11 which demonstrated that at 10, 25 and 50 mg/ml of β-CD used, there were not significantly different in CLIP dextrinizing activity. In previous studies, Joseph *et al*. (1997) has found that the activity and substrate specificity of subtilisin-catalyzed acylation of nucleosides in organic solvents can be controlled by lyophilizing the enzyme from an aqueous solution containing 20 mg/ml of substrate. Kronenburg *et al*. (2001) has successfully bioimprinted the membrane-associated epoxide hydrolase (EH) when the imprinter concentration of 5 mM was used. Fishman *et al*. (2003) has reported that bioimprinting of lipases with fatty acids was shown to be feasible when imprinted the enzyme with 1.67 mg/ml of caprylic acid. Vaidya *et al.* (2004) has reported that alteration of the substrate specificity of glucose oxidase could be achieved by using 54 mg/ml of imprinter in bioimprinting procedure. Kaulpiboon *et al.* (2007) succeeded to manipulate the product specificity of the *Paenibacillus* sp. A11 and *Bacillus macerans* cyclodextrin glycosyltransferases towards the preferential formation of gamma-cyclodextrin (CD8) by imprinting 30 mg/ml enzyme with 54 mg γ-CD. In this study, the amount of 25 mg/ml of β-CD was considered to be suitable for bioimprinting procedure since it exceeded the maximum solubility of β-CD in water (18 mg/ml). Thus, gave the maximum soluble imprinter molecules.

To investigate the effect of imprinting time on dextrinizing activity, the imprinting time was varied from 5 to 60 minutes. The result shown in Figure 12 demonstrated that there were no effects of imprinting time on the dextrinizing activity of CLIP-α-amylase. However, the imprinting time of 30 minutes was considered to be suitable for bioimprinting procedure to ensure the interaction between enzyme and crosslinker. From previous studies, the imprinting times

were 20 minutes (Kronenburg *et al*., 2001; Fishman *et al*., 2003) and 30 minutes (Vaidya *et al.*, 2004; Kaulpiboon *et al.*, 2007).

Since lyophilization itself causes unwanted structural changes on proteins (Mishra *et al*., 1996; Prestrelski *et al*., 1993), alcohol precipitation (Stahl *et al*., 1991) seems to be the more advantageous method to dehydrate proteins for imprinting purposes.

4.3 Optimization of the immobilization procedure

The main propose of this immobilization step was to stabilize imprinted protein properties, which were usually stable only in organic solvents (Klibanov *et al*., 1995), also in aqueous surroundings. The results of the previous experiments clearly demonstrated that crosslinking the acylated and imprinted enzymes with ethylene glycol dimethacrylate in an organic solvent prevented the enzymes from losing their ligand induced activities (Peiβker *et al*., 1999; Kronenburg *et al*., 2001; Vaidya *et al*., 2004; Kaulpiboon *et al*., 2007). Most covalent immobilization methods use an activated support with a given amount of reactive groups arranged at a suitable distance apart for the attachment of the functional groups of the proteins (Bruèmmer et al.1992; Lang et al. 1972; Rosevear 1984). Any undesired changes in the conformation of the protein are most likely due to random reactions with the active groups of the carrier. The advanced two-step immobilization technique, which uses itaconic anhydride and an arbitrary vinyl-group-bearing crosslinker, differs from this because the enzyme is first ``activated'' on the surface with nearly no loss of activity and then immobilized and stabilized through co-crosslinking of the introduced vinyl groups. Ideally, under optimum conditions, this procedure should allow the enzyme to keep its catalytically active conformation during and after the immobilization procedure. Only the introduced vinyl groups should react with the crosslinker, which can be varied in length and concentration, to maintain the active protein conformation in the resulting polymer.

For co-polymerization of the derivatized α-amylase, ethyleneglycol dimethacrylate (EGDMA) was used, but, in general, the crosslinking reagent can be varied. In experiments with other proteins, bisacrylamide or other divinylated agents could be used (Fischer *et al*., 1996). The crosslinking procedure of α-amylase was determined in various concentration of EGDMA. The results (Figure 13) demonstrated that at 0.44, 0.82 and 1.63 M of EGDMA used, the dextrinizing activity of CLIP-α-amylases were decreased. When the amount of crosslinker used was high, this caused mass transfer limitations and the rather low specific surface area of the polymer (Fischer *et al*., 1991). However, the EGDMA concentration of 0.82 M was considered to be suitable for crosslinking procedure. Since it ensured to maintain an imprinted property of the enzymes and did not result in too rigid conformation of the enzyme molecules. When the concentration of the crosslinker was too high (1.63 M), the enzyme activity was markedly decreased.

The effect of immobilization time on dextrinizing activity of CLIP-α-amylase is shown in Figure 14. By varying the UV irradiation time (λ=240 nm) from 0.5 to 4 hours, it was found that the immobilization time of 0.5 hour was not enough to initiate the polymerization of the crosslinker and thus, no CLIP-α-amylase could be collected. At 1-4 hours of UV irradiation time, the dextrinizing activities of CLIP-α-amylase were comparable. But, when the UV irradiation time was 5 hours, the resulting CLIP-α-amylase was markedly decreased in its dextrinizing activity. This could be due to the fact that the enzyme activity was significantly destroyed by UV irradiation at 240 nm for 5 hours or more. From the previous studies, the UV irradiation times were 5 hours at 366 nm (Peiβker *et al*., 1998), 5 hours at 335 nm (Vaidya *et al*., 2004; Kaulpiboon *et al*., 2007) and 16 hours at 366 nm (Kronenburg *et al*., 2001). The shorter optimum UV irradiation time of our study (3 hours) may be due to the lower wavelength (240 nm) used in our experiment. The comparison of UV irradiation time and wavelength used in immobilization step of CLIP technique are shown in Table 12.

Table 12 Optimum UV irradiation time in immobilization step of various proteins

Researchers	Proteins	wavelength (nm)	Optimum UV irradiation time (hrs)
Peiβker *et al*. (1999)	Protease	366	5
Kronenburg *et al*. (2001)	Epoxide hydrolase	366	16
Vaidya *et al*. (2004)	Glucose oxidase	335	5
Kaulpiboon *et al*. (2007)	*B. macerans* CGTase	335	5
Our study	α-Amylase	240	3

A comparison of the catalytic efficiency of optimized CLIP with the glutaraldehyde crosslinked enzyme aggregates (CLEAs) (Cao *et al*., 2000) would be interesting since both methods make use of crosslinking enzymes in their conformational stable forms (CLIP: enzymes are ``rigid'' due to non-aqueous environment; CLEAs: enzymes are ``rigid'' due to precipitation in aqueous solution). The CLEAs were added to the family of carrier-free immobilised enzymes (Cao *et al*., 2000). By changing properties that effect the proximity of soluble enzyme molecules, they can be made to form physical aggregates which after cross-linking are termed CLEAs. For example, it is possible to form aggregates by changing the hydration state of enzyme molecules or by altering the electrostatic constant of the solution by adding appropriate aggregation agents, such as those widely used in non-denaturing protein separation (Rothstein *et al*., 1994). Under these conditions, the originally highly solvated enzyme molecules will associate together to the extent that they precipitate as insoluble aggregates with native enzyme conformation. Analogous to the cross-linking of enzyme crystals, these insoluble physical aggregates can be subsequently cross-linked by the addition of bifunctional cross-linkers. It would be advantageous to produce CLIP-α-amylase by using glutaraldehyde as a crosslinker to crosslink the imprinted protein instead of using EGDMA which was polymerized with AIBN. AIBN is explosive chemical and at present is not commercially available in Thailand. Unfortunately, the immobilized form of the imprinted α-amylase prepared by glutaraldehyde could not be achieved. It was difficult to control the aggregation step using glutaraldehyde in non-aqueous environment. The resulting polymer was too sticky and stuck to the side of the test tube. However, Liu and his co-workers (1999) succeeded in producing a biocatalyst with glutathione peroxidase activity by imprinted denatured egg albumin with substrate derivative of glutathione peroxidase. Apart from using only the imprinter molecule, the chemical mutation of the binding site was also performed. The serine residues in the binding site were converted into selenocysteines by chemical mutation. The interesting point was that they used glutaraldehyde as a crosslinking agent and handled a procedure in aqueous environment. However, in other bioimprinting procedures where the enzyme was imprinted with a template only (without a

chemical mutation step), it was necessary to do a crosslinking step in non-aqueous organic solvent in order to maintain their imprinted memory. Thus, this approach needs further study to be able to use glutaraldehyde as a crosslinking agent in water-free organic solvent.

4.4 CLIP-α-amylase activity

It was concluded from the results obtained (Table 8 and 9) that the CDase and cyclization activity of α-amylase could not be generated by imprinting with β-CD under the investigated conditions. But, the dextrinizing activity of CLIP-α-amylase can be altered by this procedure. It was 42.4% decreased in dextrinizing activity in comparison with that of the immobilized form (immobilization without imprinting). This could be due to the imprinting effect on the dextrinizing activity of the CLIP enzyme. The results obtained by TLC and HPLC method support this conclusion that CLIP-α-amylase could not use β-CD as substrate but its dextrinizing activity was altered by this bioimprinting process. This could be caused by the structure of the ligand used. In our study, the ligand that we used was β-CD which is cyclic oligosaccharide while the natural substrates of α-amylase are linear oligosaccharides. Thus, we suggested that the ligand to be used in bioimprinting process should be more similar to the natural substrate of the enzyme to be modeled as described in the following successful cases. Peißker *et al*. (1999) has been successful in tailoring a substrate selectivity of α-chymotrypsin (CT) by imprinting with *N*-acetyl-D-tryptophan (*N*-Ac-D-Trp) (competitive inhibitor), the resulting CLIP-CT was able to accept both *N*-Ac-D-Trp and *N*-Ac-L-Trp as the substrate in ester synthesis while the native CT accept only *N*-Ac-L-Trp. Kronenburg *et al*. (2001) has found that the enantioselective conversion of (±)-1,2-epoxyoctane was reversed from a preference for (*R*)-1,2 epoxyoctane to (*S*)-1,2-epoxyoctane when the enzyme had been imprinted with (*S*)-1,2-epoxyoctane prior to co-polymerization. Vaidya *et al.* (2004) has reported that an alteration of the substrate specificity of glucose oxidase could be achieved (the enzyme can accept galactose as its substrate) by imprinting with galactose which only differs from glucose at C-4 position of hydroxyl group. Kaulpiboon *et*

al. (2007) succeeded to manipulate the product specificity of the *Paenibacillus* sp. A11 and *Bacillus macerans* cyclodextrin glycosyltransferases from β- and α-CD, respectively towards the preferential formation of γ-CD (CD8) by imprinting the enzyme with γ-CD which was one of its products.

However, there has been a report on the new types of enzymatic activity which could be obtained although the starting proteins had no activity. For example, as previously mentioned, Liu *et al.* (1999) succeeded in producing a biocatalyst with glutathione peroxidase activity by imprinting denatured egg albumin with substrate analogue of glutathione peroxidase and then changed the binding site residues by chemical mutation. However, in order to generate a new enzymatic activity of this non-enzyme protein, it should be necessary to use the chemical mutation steps which are more complicated and difficult than the bioimprinting of the natural enzymes.

Although the natural substrate of α-amylase is starch or linear oligosaccharides whose structures are different from CD, there are some α-amylases such as Taka-amylase A (TAA) which can hydrolyze CD. Moreover, α-amylase, CGTsae and CDase are the enzymes in the same family (α-amylase family) and contain 4 identical conserved regions. Thus, the chance to alter the substrate specificity of bacterial α-amylases from using starch to CD could be possible. However, the results obtained from this study showed that the substrate specificity of *B. subtilis* α-amylase could not be altered, i.e. the enzyme could not use β-CD as its substrate, by bioimprinting with β-CD. This could be caused by the difference in domain structure. The α-amylase has 3 major domains (A, B, C) while CGTase has 5 domains (with 2 additional domains, D and E) and CDase has only 2 domains. This difference may play an important role in the control of substrate specificity of the enzyme. In addition, since TAA could hydrolyze γ-CD better than β- and α-CD, respectively (Table 3), it would be interesting to use γ-CD as an imprinter to see whether γ-CD could alter the substrate specificity of *B. subtilis* α-amylase better than β-CD or not.

CHAPTER V

CONCLUSIONS

1. CGTase from thermotolerant *Paenibacillus* sp. RB01 was partially purified by starch adsorption. The specific activity was 3244.9 U/mg with the purification fold of 28.3 and the obtained yield was 78%.
2. α-Amylase from *Bacillus subtilis* had no CDase activity at 25^0C, overnight whereas the α-amylase from *Aspergillus oryzae* had strong CDase activities under the same condition.
3. The optimum ratio (w/w) of protein to itaconic anhydride was 1:5. The resulting degree of α-amylase derivatization determined by the TNBS assay was 60.7%. And the remaining activity of the derivatized enzyme was 93%.
4. The optimal condition for bioimprinting α-amylase was 30 mg/ml of α-amylase incubated with 25 mg/ml β-CD in 50 mM sodium acetate buffer pH 6.0 for 30 minutes.
5. The optimal condition for crosslinking imprinted α-amylase was to use 10 mg/ml of α-amylase suspended in cyclohexane with the addition of 4 mg AIBN and EGDMA at the final concentration of 0.82 M.
6. The CDase and cyclization activity of α-amylase could not be generated by imprinting with β-CD under the investigation conditions.
7. The dextrinizing activity of CLIP-α-amylase could be altered by imprinting with β-CD. It was 42.4% in comparison with that of crosslinked form, and was 2.8% in comparison with that of native form.

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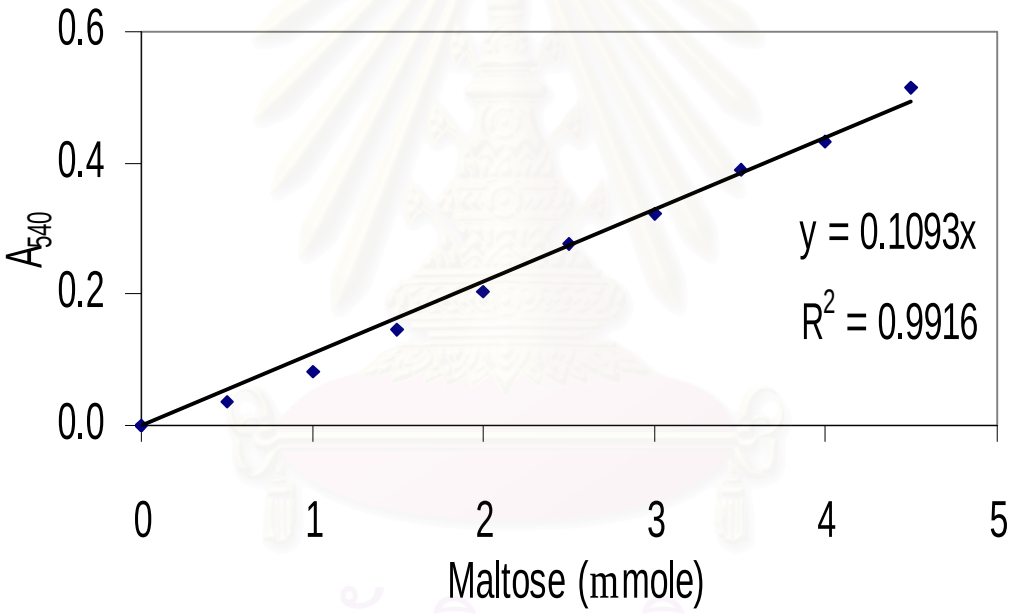
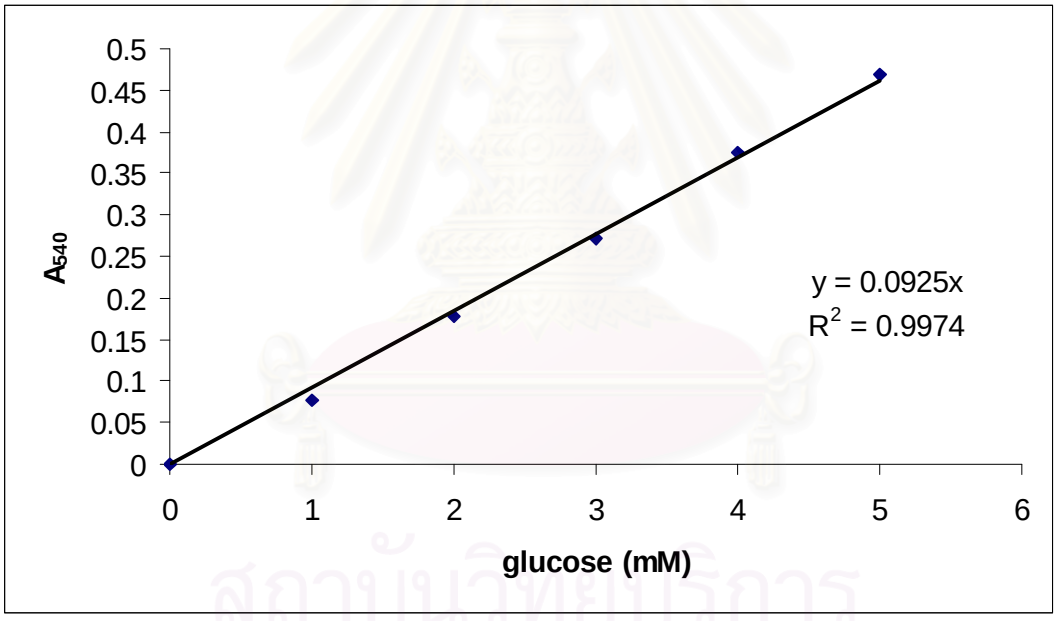
APPENDICES

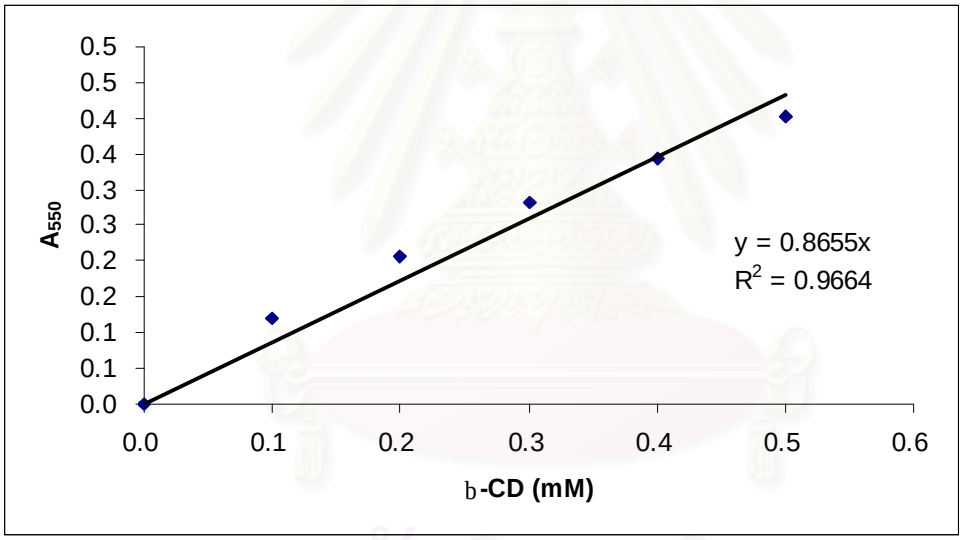
APPENDIX A: Standard curve of maltose by DNS method



APPENDIX B: Standard curve of glucose by DNS method



APPENDIX C: Standard curve of b-cyclodextrin by phenolphthalein method



APPENDIX D: Raw Data

Table E1 Effect of bioimprinting on Dextrinizing activity of a-amylase

(0.02% I_2 in 0.2% KI, 5 mg prepared enzyme, 0.2% soluble starch, 25^0C 3 min)

Enz. Sample	A_{600}				%blue	U/mg/min	Activity (%)
	1	2	3	average			
Control	0.2484	0.2483	0.2486	0.2484	100	0	0
Crosslinked	0.2019	0.2020	0.2012	0.2017	81.2	0.13	100
CLIP	0.2261	0.2253	0.2244	0.2253	90.7	0.06	46

1. (0.02% I_2 in 0.2% KI, 5 mg prepared enzyme, 0.2% soluble starch, 25^0C 3 min)

Enz. Sample	A_{600}				%blue	U/mg/min	Activity (%)
	1	2	3	average			
Control	0.3421	0.3444	0.3425	0.3430	100	0	0
Crosslinked	0.2288	0.2290	0.2296	0.2291	66.8	0.21	100
CLIP	0.2970	0.2961	0.2961	0.2964	86.3	0.09	**42.8**

2. (0.02% I_2 in 0.2% KI, 5 mg prepared enzyme, 0.2% soluble starch, 25^0C 3 min)

Enz. Sample	A_{600}				%blue	U/mg/min	Activity (%)
	1	2	3	average			
Control	0.3392	0.3393	0.3397	0.3394	100	0	0
Crosslinked	0.2224	0.2219	0.2226	0.2223	65.5	0.23	100
CLIP	0.2882	0.2886	0.2887	0.2885	85.0	0.10	**43.5**

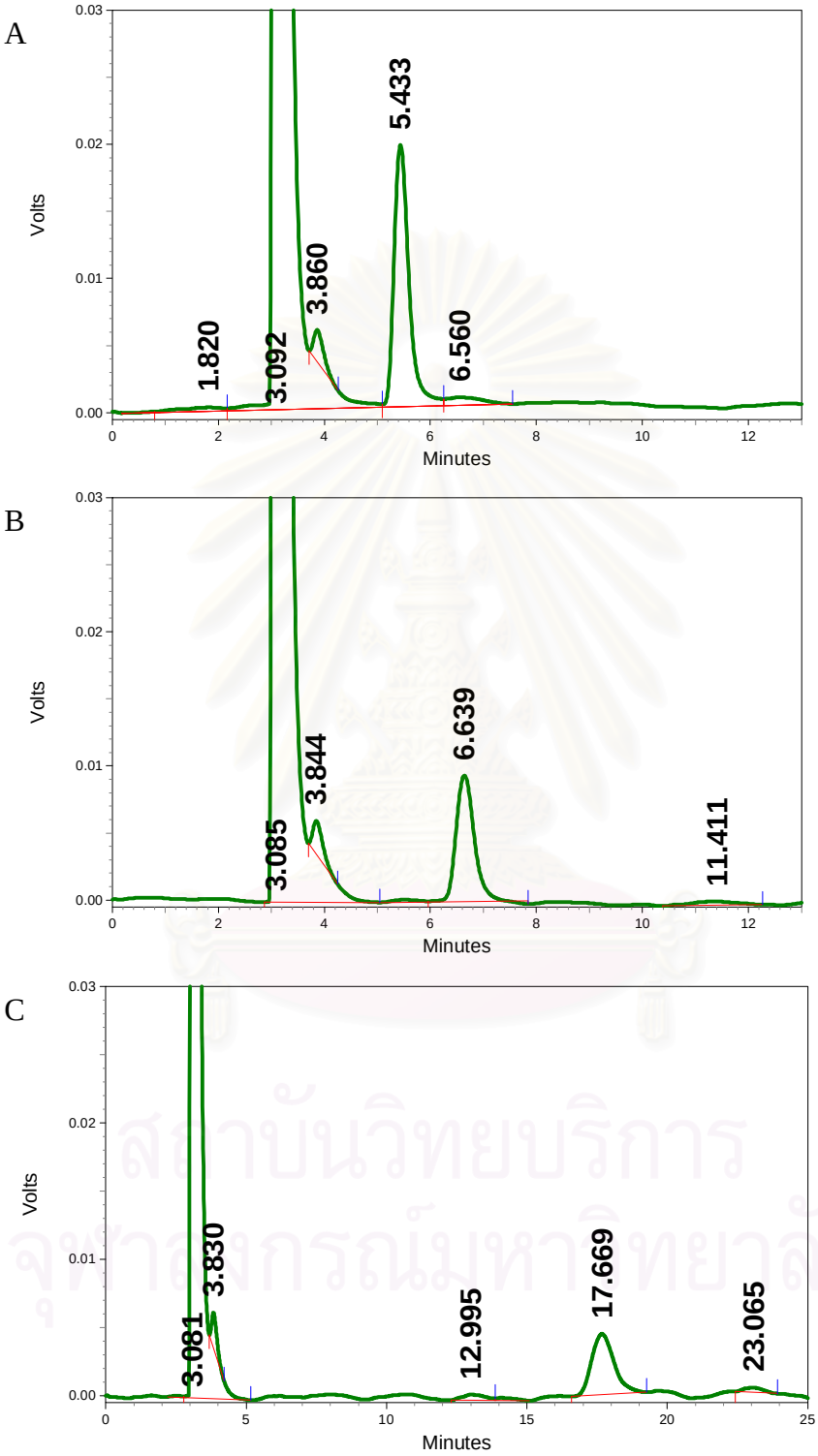
3. (0.02% I_2 in 0.2% KI, 5 mg prepared enzyme, 0.2% soluble starch, 25^0C 3 min)

Enz. Sample	A_{600}				%blue	U/mg/min	Activity (%)
	1	2	3	average			
Control	0.3406	0.3411	0.3402	0.3406	100	0	0
Crosslinked	0.2298	0.2303	0.2303	0.2301	67.6	0.22	100
CLIP	0.2962	0.2971	0.2970	0.2968	87.1	0.09	**40.9**

Average (0.02% I_2 in 0.2% KI, 5 mg prepared enzyme, 0.2% soluble starch, 25^0C 3 min)

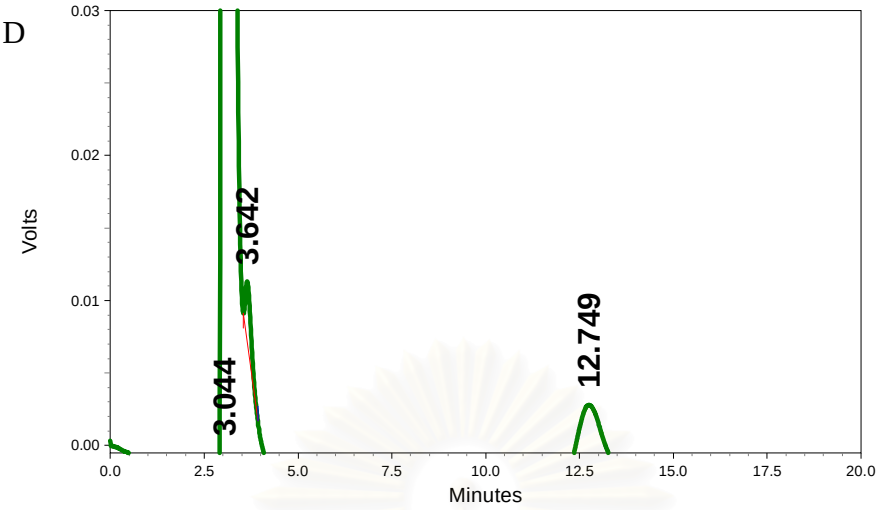
Enz. Sample	%blue	U/mg/min	Activity (%)
Control	100	0	0
Crosslinked	66.7	0.22	100
CLIP	**86.1**	**0.09**	**42.4**

APPENDIX E: HPLC chromatograms of standard oligosaccharides

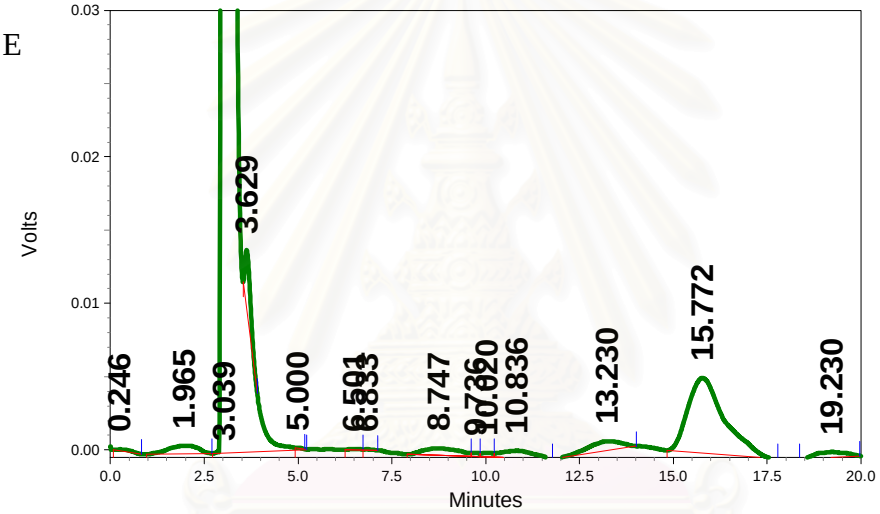


A = Glucose, B = maltose, C = β-CD

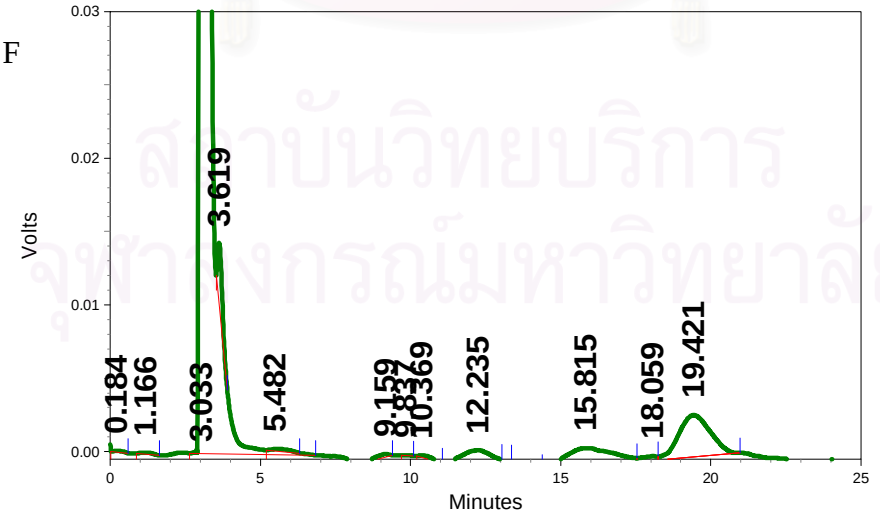
D



E



F



D = Maltopentaose, E = Maltohexaose, F = Maltoheptaose

BIOGRAPHY

Mr. Pisit Sirisuwan was born on March 27, 1981. He graduated with the Bachelor Degree of Science in Medical Technology from Chulalongkorn University in 2004 and studying for Master in Biochemistry Program, Faculty of Science, Chulalongkorn University.