STUDIES ON IMMOBILISATION OF β-GLUCOSIDASE/ β-FUCOSIDASE FROM *Dalbergia Cochinchinensis* PIERRE (THAI ROSEWOOD) SEEDS

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ชื่อวิทยานิพนธ์	การศึกษาการตรึงเอนไซม์เบต้า-กลูโคไซเดสและเอนไซม์เบต้า-ฟิวโคไซเดสจากเมล็ดพยุง
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บทคัดย่อ

เอนไซม์เบต้า-กลูโคไซเดส และเอนไซม์เบต้า-ฟิวโคไซเดสจากเมล็ดพยุง (*Dalbergia cochinchinensis* Pierre) ถูกทำให้บริสุทธิ์บางส่วนด้วยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตที่ ๓๕ - ๗๕ เปอร์เซนต์อิ่มตัว ซึ่งเป็นขั้นตอนที่ดีในการกำจัดโปรตีนที่ไม่ต้องการออกไปได้จำนวนมาก โดยสูญเสียแอคทิวิตี้น้อยของเอนไซม์ทั้งเบต้า-กลูโคไซเดสและเบต้า-ฟิวโคไซเดส เมื่อนำเอนไซม์มาตรึงบนถ่านและซีไลท์พบว่าเอนไซม์ เบต้า-กลูโคไซเดส ๐.๔ ยูนิตถูกตรึงได้ด้วยถ่าน ๑๒.๕ มิลลิกรัม และเอนไซม์คงเหลือ แอคทิวิตี้ ๕๐ เปอร์เซนต์เมื่อเทียบกับแอคทิวิตี้ตั้งต้น ส่วนซีไลท์ ๑๒.๕ มิลลิกรัม สามารถใช้ตรึงเอนไซม์เบต้า-กลูโคไซเดสได้ได้ ๐.๑ ยูนิตและเอนไซม์เหลือแอคทิวิตี้ ๖๐ เปอร์เซนต์ เอนไซม์เบต้า-กลูโคไซเดสนี้ยังได้นำไปเชื่อมเข้ากับ bovine serum albumin (BSA) ๕ เปอร์เซนต์ด้วยกลูตาราลดีไฮด์ ๐.๓ เปอร์เซนต์ และพบว่ามีแอคทิวิตี้คงเหลือเพียง ๓ เปอร์เซนต์เทียบกับแอคทิวิตี้ตั้งต้น

จากการศึกษาผลของพีเอช ต่อปฏิกิริยาการย่อยสลายพาราไนโตรฟีนิล-เบต้า-ดีกลูโคไพราโนไซด์ของ เอนไซม์เบต้า-กลูโคไซเดส และพารา-ไนโตรฟีนิล-เบต้า-ดี-ฟิวโค-

ไพราโนไซด์ของเอนไซม์เบต้า-ฟิวโคไซเดส พบว่าเกิดขึ้นได้ดีที่ช่วง พีเอช ๔.๐ ถึง ๕.๐ ซึ่งตรงกันกับเอนไซม์อิสระ จากการศึกษาด้านจลศาสตร์ของเอนไซม์ที่ถูกตรึงบนถ่านต่อสับเสตรทพาราไนโตรฟีนิล-เบต้า-ดี-กลูโคไพรา-โนไซด์ และเบต้า-ดี-ฟิวโคไพราโนไซด์ ได้ค่า K_m เท่ากับ ๕.๔๗ และ ๐.๗๓ มิลลิโมสาร์ ตามลำดับ ซึ่งไม่แตกต่างจากเอนไซม์อิสระ ซึ่งมีค่าเท่ากับ ๕.๔๗ มิลลิโมลาร์ สำหรับพาราไนโตรฟีนิล-เบต้า-ดี-กลูโคไพราโนไซด์ และ ๐.๗๓ มิลลิโมลาร์ สำหรับพาราไนโตรฟีนิล-เบต้า-ดี-ฟิวโคไพราโนไซด์

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จากการศึกษาการนำเอนไซม์ที่ถูกตรึงบนถ่านกลับมาใช้ใหม่ พบว่าสามารถนำกลับมาใช้ได้อย่างน้อย ๒ ครั้ง โดยทำการสังเคราะห์ครั้งละ ๕ วัน พบว่าในแต่ละครั้งของการนำกลับมาใช้ใหม่นั้นโอลิโกแซคคาไรด์ที่ได้มีปริมาณเท่าๆกันทุกครั้ง เมื่อเทียบปริมาณโอลิโกแซคคาไรด์ทั้งหมดที่ได้จากการนำเอนไซม์กลับมาใช้ใหม่กับเอนไซม์อิสระที่ทำการสังเคราะห์เป็นเวลา ๑๕ วัน พบว่าเอนไซม์ที่ถูกตรึงให้ปริมาณโอลิโกแซคคาไรด์ทั้งหมดมากกว่าเอนไซม์อิสระ แต่ให้ปริมาณไตรแซคคาไรด์ในปริมาณทั้งหมดที่พอๆกัน เมื่อทำการล้างถ่านที่มีเอนไซม์ติดอยู่ด้วยน้ำกลั่น ๒ ครั้งตามด้วย ๕ เปอร์เซนต์ และ ๑๐ เปอร์เซนต์ เอธานอล ผลปรากฏว่ามีไดแซคคาไรด์ และน้ำตาลที่มีโมเลกุลใหญ่กว่าไดแซคคาไรด์หลุดออกมาด้วย

Thesis Title	Studies on Immobilisation of β-Glucosidase/β-Fucosidase from *Dalbergia cochinchinensis* Pierre (Thai Rosewood) Seeds
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ABSTRACT

A crude preparation of β-glucosidase/β-fucosidase from the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre) was obtained by ammonium sulphate precipitation, a good step in removing a large amount of proteins, with a good recovery of β-glucosidase and β-fucosidase. The fold purification of β-glucosidase and β-fucosidase in the 35-75% ammonium sulphate fraction was 4.73 and 4.33 with yield of 60.85% and 55.75% respectively.

The supports used for immobilisation were activated carbon (Merck) and celite (Fluka). The enzyme was also cross-linked with bovine serum albumin (BSA) by 0.3% (w/v) glutaraldehyde. Four-hundred milliunits of crude β-glucosidase could be immobilised on 12.5 mg of activated carbon with about 50 % immobilisation. For celite, only 100 mU of crude β-glucosidase could be immobilised on 12.5 mg support with 60%

immobilisation. The percent immobilisation of crude enzyme cross-linked with 5% (w/v) bovine serum albumin (BSA) using glutaraldehyde was about 3%.

Enzyme immobilised on activated carbon and on celite were less stable at 50°C than free enzyme, and this may be due to the alterations of structure upon immobilisation. The pH optimum of crude enzyme immobilised on activated carbon was similar to that of free enzyme, namely about pH 4-5. K_m of crude enzyme immobilised on charcoal for pNP-β-D-glucopyranoside was 4.57 ± 0.03 mM and for pNP-β-D-fucopyranoside was 0.94 ± 0.08 mM. The K_m of free enzyme for both substrates were similar to immobilised enzyme (5.47 ± 0.4 mM for pNP-β-D-glucopyranoside and 0.73 ± 0.05 mM for pNP-β-D-fucopyranoside).

Enzyme concentrations were varied (0.1, 0.2 and 0.4 U) for both free and immobilised enzyme for use in oligosacchaide synthesis. For free enzyme, 0.2 U β-glucosidase was the most appropriate concentration since it gave a moderate rate of synthesis and with highest yield of oligosaccharide at equilibrium. For immobilised enzyme, 0.2 U enzyme was also the most appropriate concentration, since it gave the highest yield per unit enzyme. From the time course of synthesis in the first 8 h for both free and immobilised enzyme, gentiobiose (glucose-β-1-6-glucose) was the first and major product being synthesised. The results from thin-layer chromatography show that gentiobiose can be readily seen at day 5 of synthesis for both free and immobilised enzymes.

Immobilised enzyme of 0.2 U was re-used twice to synthesise oligosaccharide over periods of 5 days. The results show higher total disaccharide products than free enzyme that has been used to synthesise oligosaccharide for 15 days but the amount of trisaccharides were the same for both free and immobilised enzyme. Each usage of immobilised enzyme

showed no dramatic difference in oligosaccharide products, which were almost the same as free enzyme. After two re-uses, immobilised enzyme was washed with distilled water twice, followed by 5% and 10% ethanol. The elution profile of oligosaccharide from activated carbon shows a detectable quantity of disaccharide and other higher oligosaccharides eluted out.

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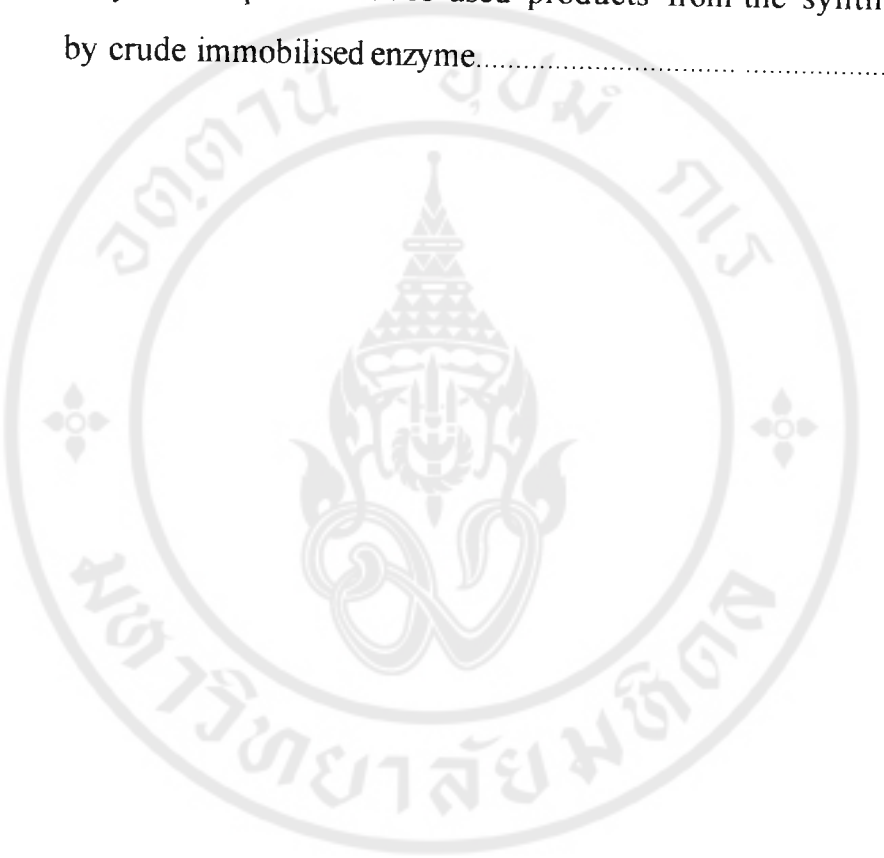

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LIST OF ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
F	β-fucosidase
G	β-glucosidase
g	gravitational force
gal	galactopyranoside
glc	glucopyranoside
GlcNAc	N-acetyl-glucosamine
GalNAc	N-acetyl-galactosamine
ara	arabinopyranoside
fuc	fucupyranoside
man	mannopyranoside
xyl	xylopyranoside
h.p.l.c.	high performance liquid chromatography
t.l.c.	thin-layer chromatography
$AmSO_4$	ammonium sulphate
4-MU-Gal	4-methylumbelliferyl-β-D-galactopyranoside
4-MU-Glc	4-methylumbelliferyl-β-D-glucopyranoside
4-MU-Fuc	4-methylumbelliferyl-β-D-fucopyranoside
MW	molecular weight
PMSF	phenylmethylsulfonylfluoride
PAGE	polyacrylamide gel electrophoresis
PVPP	polyvinylpolypyrrolidone
pNP	p-nitrophenol
oNP	o-nitrophenol

SDS	sodium dodecyl sulphate
U	unit

CHAPTER I

INTRODUCTION

1.1 β-Glucosidase

β-Glucosidases are a group of glycosidase enzymes that catalyse the hydrolysis of aryl- and alkyl-β-D-glycosides as well as glycosides with only carbohydrate moiety (e.g., cellobiose). They occur widely in prokaryotes and eukaryotes and have significant scientific, medical, and economic implications.

β-Glucosidases and β-glucosides are ubiquitous in the living world, and β-glucosidases appear to share some common structural and catalytic properties. β-Glucosidases from widely different sources show remarkable similarity in substrate specificity for glycone (e.g., glucose) and some non-physiological aglycones (e.g., nitrophenols and umbelliferone), although they may have widely different physiological glucosidic substrates with different aglycone moieties. In general, β-glucosidases from different orders and kingdoms appear to differ in their specificities for the aglycone (an aryl or alkyl group) linked to glucosyl group by a β-glycosidic bond.

β-Glucosidases of fungi, bacteria, humans and dicotylodenous plants (e.g., Thai Rosewood and mango) have been shown to be glycosylated, while that of monocotyledons (maize and sorghum) are not. In plants, β-glucosidases of dicotyledons are localised to the cell wall or protein bodies while the β-glucosidases of monocotyledons are localised to the plastids. In mammals (e.g., humans and mice), acid β-glucosidase is localised to the lysosome while its neutral counterpart is a soluble cytosolic protein (1).

It would be expected that an enzyme such as β-glucosidase that is not involved in a mainstream metabolic pathway would differ from organism to organism due to divergent or convergent evolution. This would be especially true among members of different kingdoms as well as among members of higher taxonomic groups in the same kingdoms.

1.2 β-Glucosidase specificity (2)

One of the controversial issues related to β-glucosidases is their substrate specificity. Many questions were raised whether β-glucosidases catalyse the hydrolysis of β-glucosidic linkages in all mono- and disaccharides or they are specialists in catalysing only the hydrolysis of β-glucosidic linkages between glucose and specific aryl and alkyl aglycones, or whether the enzymes are specific for the glycone or the aglycone or both. For example, maize β-glucosidase purified to homogeneity showed 5 times greater activity towards p-nitrophenyl-β-D-fucoside than p-nitrophenyl-β-glucoside (the difference is due to higher V_{max}, the K_ms are the same). Similarly, its relative activity towards pNP-β-D-galactoside is about 10% of that towards pNP-β-D-glucoside. It may be asked whether the maize enzyme is a β-fucosidase, a β-glucosidase or a β-glycosidase?

The wide-spread and frequently exclusive use of artificial substrates such as benzyl, nitrophenyl and 4-methylumbelliferyl glycosides during purification and characterisation of glycosidases also has contributed to this commonly held but inaccurate view. The following examples of glycosidases demonstrate that these enzymes show specificity towards naturally occurring substrates.

1) Two homogeneous forms of amygdalin hydrolase (AH I and AH II) that occur in cherry seed hydrolyse the terminal glucose of amygdalin and form the monoglucoside known as prunasin. These enzymes also hydrolyse p- and o-nitrophenyl-β-D-glucosides (pNP-Glc and oNP-Glc) at somewhat reduced rates, and pNP-β-D-galactoside (pNP-Gal) more slowly. AH I hydrolysed oPN-Glc, pNP-Glc and pNP-Gal at the rate of 84%, 68% and 16% respectively of the rate of amygdalin hydrolysis. While 4-methylumbelliferyl-β-D-glucoside (MU-Glu) was also rapidly hydrolysed (relative rate 110 compared to amygdalin, 100). AH I does slowly catalyse the hydrolysis of the two cyanogenic glycosides found in flax seed, linustatin and neolinustatin; their relative rates of hydrolysis were 10% and 27% of that of amygdalin. Both isoforms were inactive toward the following glycosides: (S)-dhurrin, linamarin, laminarin, cellobiose, sucrose, lactose, maltose, methyl-β-D-glucoside and phenyl-β-D-glucoside. Gentiobiose was also not hydrolysed by AH I or AH II, which is interesting, since amygdalin is a β-gentiobioside. (3)

2) Linustatinase, which catalyses the removal of the terminal glucose and the related monoglucoside linamarin, was purified to homogeneity from flax seed and characterised. Linamarase was also purified to homogeneity from the same source. Linustatinase hydrolysed linustatin (relative velocity, 100) and also hydrolysed neolinustatin, a second cyanogenic diglucoside found in flax seed, with a relative rate of 59. This diglucosidase also hydrolysed amygdalin, β-gentiobiose and β-1,4-cellobiose with relative rates of 66, 13 and 14 but, as expected only slowly hydrolysed linamarin and was inactive on (R)-prunasin. Linustatinase surprisingly hydrolyses the monoglucoside dhurrin (relative rate, 167) possibly because the aromatic aglycone bears some slight similarity to the p-nitrophenyl group; pNP-Glc

was also rapidly hydrolysed by linustatinase (relative rate, 242), perhaps for the same reason (4).

3) β-Glucosidases from some tropical plants (*Manihot glaziovii*, *Passiflora foetida* and *Prunus polystachya*) (5) that are taxanomically and economically diverse were tested for their specificity for their substrates, by comparing their ability to hydrolyse different pNP-β-D-monoglucosides and pNP-β-D-diglucosides. Although the enzyme preparations might contain other glycosidases as impurities, they showed better hydrolysis of the β-linkage of pNP-β-D-glucoside and pNP-β-D-fucoside than that of pNP-β-D-galactoside, pNP-β-D-xyloside and pNP-β-D-mannoside. None of the enzyme preparations showed detectable hydrolysis of pNP-β-D-diglucosides, such as pNP-β-D-cellobiose, pNP-β-D-lactose, pNP-β-D-maltose and pNP-β-D-mellibiose. The relatively higher activity towards pNP-β-D-fucoside rather than pNP-β-D-glucoside of *Passiflora* and *Prunus* could be explained by the greater affinity of these enzymes for the compound, with the K_m (pNP-β-D-fucoside) values being 0.94 mM and 0.99 mM, respectively. The *Passiflora* enzyme was not able to hydrolyse linamarin or prunasin, while the *Manihot* β-glucosidase was specific for linamarin and the *Prunus* enzyme for prunasin.

4) Both activities of β-glucosidase and β-fucosidase could be purified to homogeneity from the seeds of *Dalbergia cochinchinensis* Pierre (6). The enzyme showed a higher relative activity towards pNP-β-D- fucoside than pNP-β-D-glucoside (relative activity, 124 and 100, respectively) but a higher K_m (5.4 mM and 0.54 mM at 5 mM, respectively). pNP-β-D-galactoside, pNP-β-D-xyloside, phenyl-β-D-glucoside and pNP-α-L-arabinoside at 5 mM were hydrolysed much more slowly (relative activity, 8.95, 3.91, 5.00, and 4.89,

respectively). The ability to hydrolyse pNP-β-D-galactoside, pNP-β-D-xyloside, and pNP-α-L-arabinoside probably resulted from the fact that these three glycosides have the same *trans*-equatorial configuration to the oxygen at C_1, C_2 and C_3 as pNP-D-β-fucoside and pNP-β-D-glucoside. The enzyme had no activity towards pNP-α-L-glycosides, indicating its specificity to the β-glycosidic linkage. The study of natural substrate hydrolysis at 5 mM showed very low relative activities toward sophorose, laminaribiose, and gentiobiose, and activity towards cellobiose was even smaller (percent activity, 0.39, 0.34, 0.29 and 0.06, respectively). No hydrolysis of the cyanogenic glucosides linamarin or prunasin was observed, but some hydrolysis of amygdalin and salicin was found.

Differences in kinetic properties and substrate specificity of the plant β-glucosidases are evident. From the viewpoint of protein evolution, it is indeed interesting that the plant β-glucosidases and even fungal cellobiase (a β-glucosidase) which is different in many ways from the plant enzymes, share similar catalytic mechanisms.

1.3 Sources of β-glucosidase

1.3.1. Plant β-glucosidase

Plant β-glucosidases have been implicated in a variety of key metabolic events: growth-related responses, productivity, and food and feed toxicity-related reactions. They range from defense against some pathogens to herbivores. In plants, one of the functions of β-glucosidase is thought to be in cyanogenesis, resulting in the release of HCN upon the hydrolysis of

cyanogenic glucosides. Cyanogenesis has been shown to occur in over 3,000 plant species belonging to 110 different families (7).

It was shown that the enzyme (β-glucosidase) and the substrate (cyanogenic glucosides) are present in different cellular compartments (1). The compartmentalisation has to be disrupted, as would be the case after injury to cells and tissues by herbivores leading to the hydrolysis of cyanogenic glucosides and the release of HCN. This would suggest that cyanogenesis is a chemical defense response to organisms feeding on intact plant parts or attacking the plant through a site of injury.

In addition to cyanogenesis, plant β-glucosidases have been implicated in the hydrolysis of glucosides of phytohormones and thus activation of these hormones. Plant development has been shown to be influenced by a set of 5 phytohormones (auxins, cytokinins, gibberellins, abscisic acid and ethylene), each of which can elicit a remarkable variety of responses. Auxins promote cell division, cell elongation, differentiation of vascular tissues and root initiation. They have been proposed to influence apical dominance and tropisms. Cytokinins are thought to promote shoot initiation, cell division and, together with auxins, regulate plant growth and differentiation.

An attractive model explaining plant growth control may be based on the action of β-glucosidases. Transcriptional regulation of their genes could be linked to environmental cues and thus allow various developmental adaptations. Auxin and cytokinin conjugates have been found to be broadly distributed in plants. Thus, phytohormone-specific β-glucosidases might represent a link between environmental stimuli and the release of active phytohormones from precise locations in plant. Although these ideas are far

from being proven, they open a promising area of research in plant development (8).

β-Glucosidase is also responsible for aroma enhancement in grape where Gunata (9) reported the occurrence of monoterpenylglycosides as a quantitatively important part of the aroma fraction of fruit.

1.3.2. Human β-glucosidase

Mammals contain two β-glucosidases: lysosomal glucocerebrosidase, and a cytosolic β-glucosidase which has a very broad specificity and is most active at neutral pH. The physiologic function of the cytosolic β-glucosidase is obscure, but it does hydrolyse toxic plant glycosides found in the diet of man. The lysosomal β-glucosidase (glucocerebrosidase) has a pH optimum in the acidic range. It is now well established that the lysosomal β-glucosidase is the enzyme responsible for the hydrolysis of the glycosphingolipid, glucocerebroside, to glucose and ceramide. The deficiency of glucocerebrosidase is the biochemical basis for the sphingolipidosis called Gaucher disease. Cytosolic β-glucosidase, that has a broad substrate specificity, catalyses the hydrolysis of β-D-glucosides, β-D-galactosides, α-L-arabinosides and β-D-xylosides conjugated to the p-nitrophenol or 4-methyumbelliferone. The function and pathological significance of the neutral pH optimum glycohydrolase remain uncertain (10).

1.3.3. Microbial β-glucosidase

Cellulose, an abundant biopolymer, is composed of repeating glucose units linked by β-1,4-glycosidic bonds. Cellulases, produced by a wide variety of microorganisms, degrade such polymers and play a major role in recycling the biomass. Cellobiases, while specific for cellobiose, belong to the very diverse family of enzyme β-glucosidase. Cellulase components are thought to act in a stepwise process and can act synergistically to achieve more efficient degradation. The major end product of concerted endoglucanase and cellobiohydrolase activity is cellobiose. Cellobiose is then hydrolysed to glucose by β-glucosidase with the fermentative synthesis of ethanol (11). The synergistic action of cellulase and β-glucosidase is the so-called simultaneous saccharification (SSF) process. This biochemical conversion of cellulosic biomass to ethanol is a promising alternative fuel and can be carried out efficiently and economically (12).

Cellulose hydrolysis primarily depends on at least 3 enzymes. These include several endo- and exo-cellulases and β-glucosidases or cellobiase. The former 2 enzymes can degrade cellulose synergistically to generate cellobiose which is a product inhibitor for these enzymes. Cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21)plays an important role of scavenging the end product cellobiose by cleaving the β (1→4) linkage to generate D-glucose and also in the regulation of exo- and endo β(1→4) glucanase synthesis. Cellobiases purified from different sources, were reported to have molecular weight ranging from 40,000 to 300,000or more with broad substrate specificity. The presence of isoenzymes have also been indicated (13).

The microorganisms found to be responsible for the degradation of cellulose are those of fungi e.g., *Termitomyces clypcatus*, *Aspergillus niger* and *Trichoderma reesei* (13, 14, 15) Enzymes from the 3 fungi share similar properties as shown in Table 1.

Table 1-1 Comparison of β-glucosidase properties among 3 microorganisms, *Trichoderma reesei*, *Aspergillus niger* and *Termitomyces clypeatus* (13, 14, 15).

Microorganism / Parameter	*Trichoderma reesei* β-glucosidase I	*Trichoderma reesei* β-glucosidase II	*Aspergillus niger*	*Termitomyces clypeatus*
MW (SDS-PAGE)	71 kDa	114 kDa	120 kDa	110 kDa
pI	8.7	4.8	4.0	4.5
K_m (pNPG)	182 μM	135 μM	0.9 mM	0.5 mM
K_m (cellobiose)	2.1 mM	11.1 mM	2.3 mM	1.25 mM
Optimum temperature	65-70°C	60°C	55°C	65°C
Optimum pH	4.6	4.0	4.5	5.0

1.4 β-Glucosidase/β-fucosidase from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) seeds (6)

In our laboratory, two glycosidase activities, β-D-glucosidase and β-D-fucosidase could be purified from the seeds of *Dalbergia cochinchinensis* Pierre. These two activities were found in the same peak in both isoelectric focusing and in gel filtration, corresponding to pI of 5.6 and molecular weight of 330,000 respectively. They gave a single band for both activity and protein in non-denaturing gels and also gave one band with molecular weight 66,000 on SDS-PAGE. These results suggest that both β-D-glucosidase and β-D-fucosidase activities are due to the same enzyme.

The kinetic properties of the Thai Rosewood enzyme were also studied and it was found that β-glucosidase has both a higher K_m and a higher V_{max} compared to β-fucosidase. However, the substantially higher V_{max}/K_m ratio for β-fucosidase compared to β-glucosidase suggests that the enzyme may be more properly designated as a β-D-fucosidase.

Surarit *et al* (16) modified the enzyme with condural epoxides (epoxyalkyl glycosides), the chemical that attaches to the active sites of both α-D-glucosidase and β-D-glucosidase, and showed that both β-D-glucosidase and β-D-fucosidase activities were diminished. This result suggested that the enzyme has a single active site. Tris was also employed to confirm the single active site of the enzyme. The study showed that Tris, an inhibitor of glycosidases, has equal effects on the rate of hydrolysis of both β-glucosidase and β-fucosidase by the enzyme by means of competitively inhibiting the hydrolysis of both substrates. Hereafter, this thesis will be emphasised on β-D-glucosidase activity rather than β-D-fucosidase activity.

1.5 β-Fucosidase

β-D-Fucosidase isolated from various sources were found to be associated with a high activity of β-D-galactosidase and/or β-D-glucosidase. The β-D-fucosidase listed in the latest Enzyme Commission Nomenclature are all associated with high β-D-galactosidase and/or β-D-glucosidase activity. The β-D-fucosidase from pig kidney possesses a β-D-galactosidase activity which was even higher than its β-D-fucosidase activity. Two enzymes isolated from the digestive juice of the giant African snail *Achatina balteata* can be described as β-D-fucosidases but their activities toward β-D-galactosidase and β-D-glucosidase are still quite significant (16).

On the other hand, β-D-fucosidase purified to electrophoretic homogeneity from *Aspergillus phoenicis* was said to be strictly a β-D-fucosidase due to its high specificity toward p-nitrophenyl-β-D-fucoside. The enzyme was inhibited by D-fucose and D-galactono-γ-lactone but not by D-galactose, D-galactono-γ-lactone, D-glucose, or D-glucono-γ-lactone; the latter compounds are specific inhibitors of β-D-galactosidase and β-D-glucosidase, respectively (17).

1.6 Mechanism of glycosidase action

The generally accepted mechanism of action of glycosidases which cleave the glycosidic linkage with overall retention of configuration is originally proposed by Koshland (18). The mechanism involves an initial binding of the substrate to the enzyme, by protonation of the anomeric oxygen atom by an acidic group of the enzyme to give the aglycone moiety of the substrate, followed by a general acid-catalysed attack of an enzymic

nucleophile upon the anomeric centre to form a glycosyl-enzyme intermediate. This intermediate is then hydrolysed by general base catalysis, and a hydroxyl group is added to the glycosyl moiety of the substrate. Water, alcohol or some other hydroxyl compound can be involved as hydroxy-group donor (19). The β-glycose product is then formed and enzyme returns to its original protonation state. Both the formation and the hydrolysis of the glycosyl-enzyme intermediate likely proceed through transition states with substantial oxocarbonium ion character. A covalent structure is formed and hydrolysed via oxocarbonium ion-like transition state.

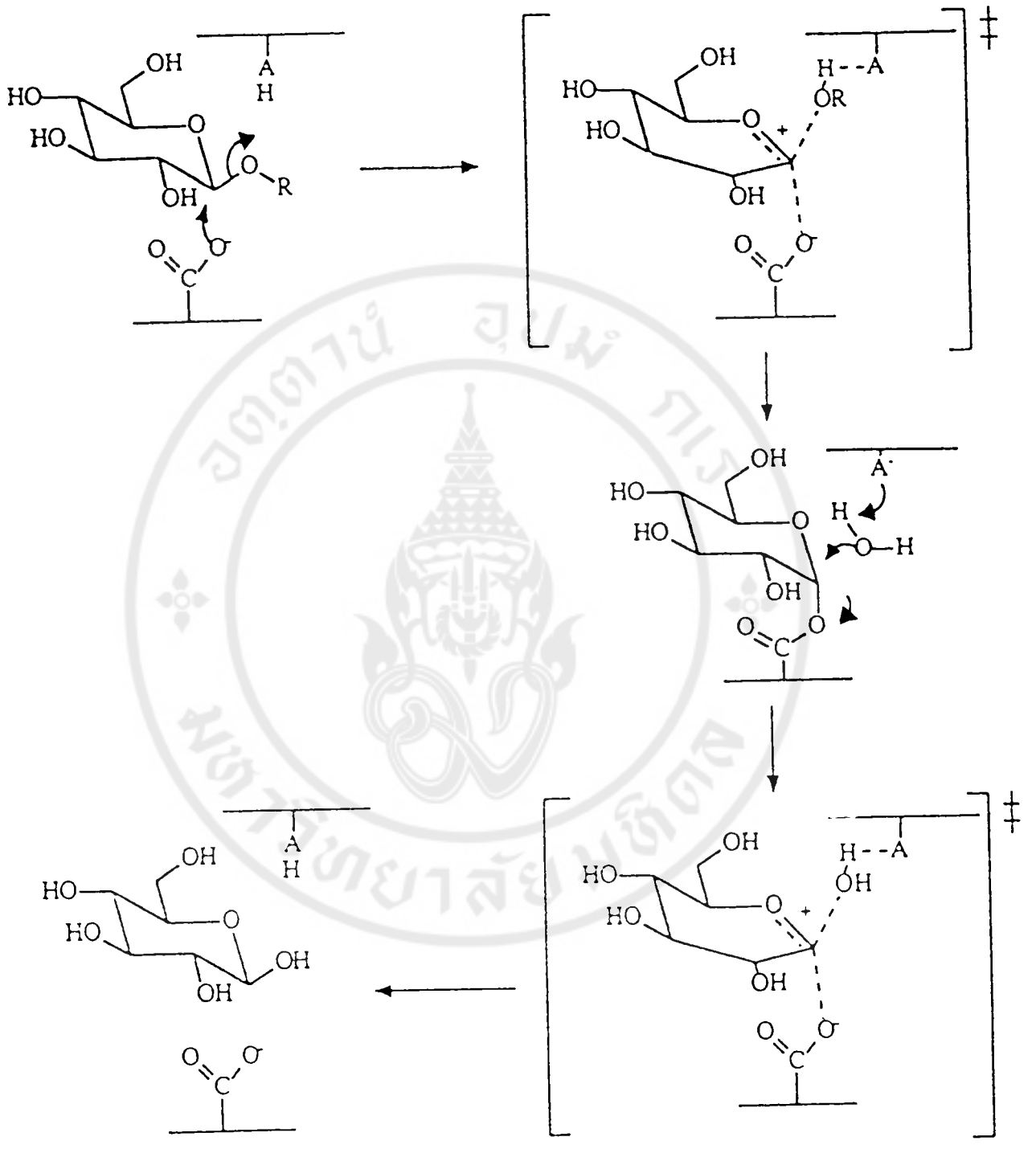

Figure 1-1 Mechanism of action of a retaining glycosidase enzyme (19).
‡ = transition state

With respect to the amino acid residues essential for the enzyme complex catalysed by β-glucosidase, several authors (19) have pointed to the presence of two essential carboxy groups at the active site of the enzyme, suggesting a mechanism that resembles that described by lysozyme. Kinetic and chemical modification studies of the enzyme from *Botryodiplodia theobromae* have shown the participation of a carboxyl group and a histidine residue at the catalytic centre (19,20). Both types of amino acid residue have also been suggested to play a catalytic role in β-glucosidase from almonds. Figure 1 shows the mechanism of action of glycosidase enzyme (18).

1.7 Application of glycosidases

Glycosidases have been used as excellent tools for many studies such as:

1) The elucidation of the primary structure of glycans by sequential degradation (21). Since exoglycosidases cleave only monosaccharide residues which are located at the non-reducing terminal, they can be useful tools for sequencing the sugar chain. An example of utilising exoglycosidases to analyse the structure of oligosaccharide is shown in Figure 1-2 (22).

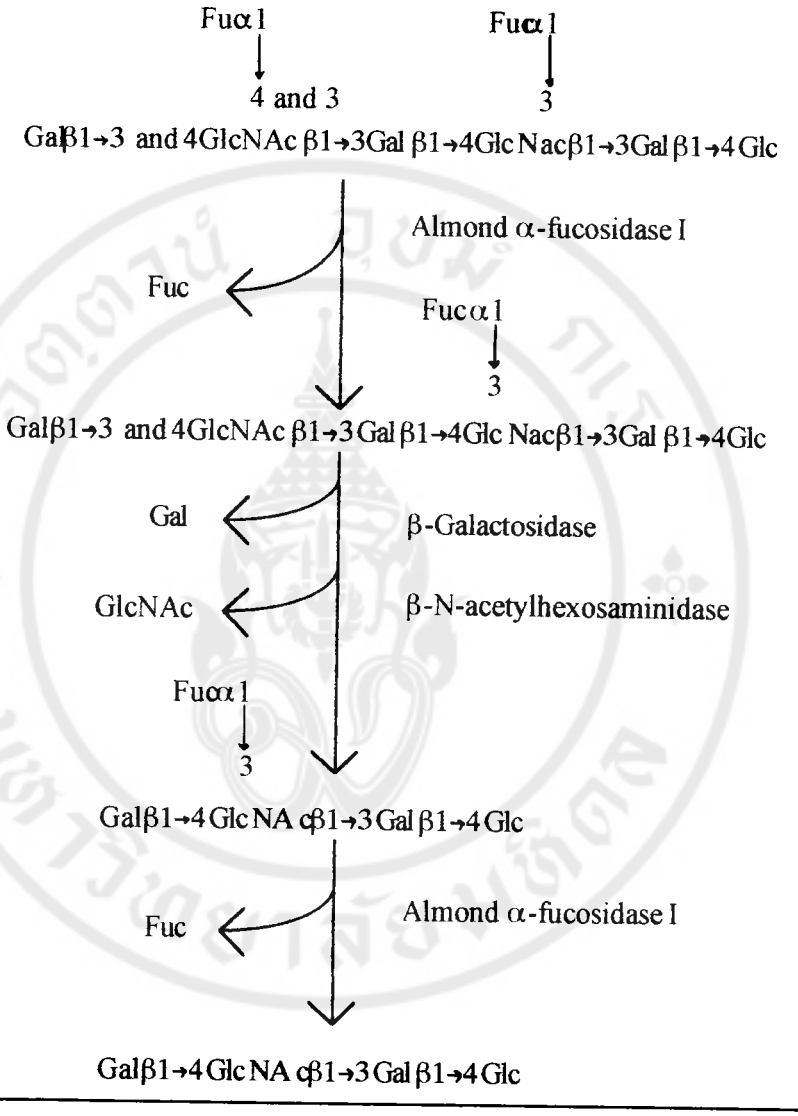

Figure 1-2 Sequential degradation of a milk oligosaccharide fraction by exoglycosidases (22).

For endoglycosidases, three kinds of the enzyme have proved to be useful thus far, namely endo-N-acetyl-β-D-glucosaminidase, endo-α-N-acetyl-galactosaminidase, and endo-β-D-galactosidase. These enzymes have been shown as useful tools for structural studies of glycoconjugates, because they can release oligosaccharides amenable to further analysis.

2) The determination of anomeric linkage of each conjugated monosaccharide. Most of glycosidases from various sources have different specificities, such as β-galactosidase purified from *Diplococcus pneumoniae* cleaves Gal-β1,4-GlcNac but not Gal-β1,3-GlcNac and Gal-β1,6-GlcNac (23), while jack bean α-D-mannosidase cleaves Man-α1,2-Man and Man-α1,6-Man linkages almost at the same rate, but cleaves Man-α1,3 at very slow rate (about 7% of Man-α1,2-Man) (24). Such enzyme can be used to discriminate the positional isomers in sugar chain structure. For example, a study of the sugar chains of ovalbumin by Kobata *et al* in 1975 confirmed the structure of a radioactive tetrasaccharide liberated from an ovalbumin glycopeptide as being Man-α1,2-Man-α1,2-Man-β1,4-N-acetyl [^{3}H] glycosaminitol and not as Man-α1,3-Man-α1,2-Man-β1,4-N-acetyl [^{3}H] glycosaminitol by measuring its hydrolytic rate using jack bean α-D-mannosidase.

3) Other uses of glycosidases have also been reported. For instance, they are tools for the controlled modification of glycoprotein glycans, including membrane glycoproteins, for the exploration of their biological role and for the preparation of specific acceptors for glycosyl transferase activity.

4) Recently, the use of glycosidases to synthesise oligosaccharide using the reversal of the hydrolytic reaction have become of interest. Efforts to synthesise oligosaccharides have expanded due to the increasing recognition of the importance of oligosaccharide moieties of glycoproteins and glycolipids, while their biological functions are still less well understood.

Since the oligosaccharide moieties of glycoproteins and glycolipids play a key role in cell-cell and cell-virus recognition, their importance is being increasingly recognised and the efforts to synthesise them are expanding. The number of functional groups of carbohydrate monomers and the variety of configurations that oligomers can adopt is greater than with nucleotides/nucleic acids or amino acids/peptides. By reversing the hydrolytic action of glycosidases and by using highly regiospecific glycosyltransferases, enzymatic oligosaccharide synthesis can be performed. The chemical synthesis of simple disaccharide is possible, but it requires 5 to 7 steps with the use of selective protection of hydroxyl group (25).

1.8 Roles of oligosaccharides

It is clear that the complex oligosaccharides chains (glycans) play important roles in a variety of biological processes, such as the following (25, 26, 27):

I) They serve as cell surface receptors for influenza viruses, bacteria, bacterial toxins and some lectins.

ii) They involve in intracellular migration and secretion of glycoproteins.

iii) They serve as modulators of cell growth and cellular differentiation.

iv) They determine blood group activities, which differ in oligosaccharide structure.

v) The nature and extent of glycosylation of protein determines the half-life of their survival in the blood stream.

vi) Since they are the primary markers for cell recognition so the contact between oligosaccharides will initiate the invasion of cells by viruses and also initiates the production of antibodies to the viruses.

vii) The glycoprotein Galβ1-3GalNAc imparts substantial anti-freeze properties on antarctic fish serum (28).

The functions of free, simple oligosaccharides, are still less well known. In plants, they have, however, potent biological activities involved in the production of secondary products which are important for the development of inflorescenses and auxillary shoots. In addition, oligosaccharides can elicit phytoalexins, which are antimicrobial or antibiotic molecules synthesised in plants in response to various stimuli, such as during the invasion of plant tissues by fungi. Clearly, if oligosaccharides, either as free or complex molecules, can be identified, they might be useful and appropriate subjects for medical, biochemical and agricultural research. Some examples of important oligasaccharide fragments which are sufficient for biological specificity are shown in Table 1-2 and some possible conversions and application of oligosaccharides are shown in Table 1-3 (25).

Table 1-2 Examples of short bioactive structures (25).

Blood group active structures	
Fuc(α1,2)Gal(β1,3)GlcNAc...	H-structure
GalNAc(α1,3)[Fuc(α1,2)]Gal(β1,3)GlcNAc...	A-structure
Gal(α1,3)[Fuc(α1,2)]Gal(β1,3)GlcNAc...	B-structure
Receptors for pathogens	
Gal(α1,4)Gal(1)-R	P-fimbriated *E. coli*
Tumour-associated antigens	
NeuAc(α2,8)Neu(α2,3)Gal(1,4)Glc(β1)-R	melanoma
NeuAc(α2,3)Gal(β1,3)GlcNAc(1)-R	various cancers
NeuAc(α2,3)Gal(β1,3)[Fuc(α1,4)]GlcNAc(1β)-R	pancreas, colon cancer
Gal(β1,4)[Fuc(α1,3)]GlcNAc(1β)-R	gastrointestinal adenocarcinoma

Table 1-3 Some conversions and applications of various glycosides (25).

Allyl glycosides
Inhibitors
Temporary anomeric protection
Polysaccharide copolymer
Affinity supports
Affinity adsorbents
Affinity labels
Neoglycoproteins
Benzyl glycosides
Inhibition studies
Enzyme substrates
2-Bromomethyl glycosides
Glycosides for affinity labeling or coupling
Neoglycoproteins
Neoglycolipids
Methyl glycosides
Inhibition studies
Temporary anomeric protection
p-Nitrophenyl glycosides
Temporary anomeric protection
Inhibition studies
Coupling to proteins, affinity adsorbents
Affinity labeling

1.9 Sources of oligosaccharides

There are a number of methods for obtaining oligosaccharides

i) Extracting from the natural sources such as human milk, the colostrum of various animals, the faeces of unweaned infants and other obscure sources (26).

ii) Synthesis by chemical method. Carbohydrates contain multiple hydroxyl groups of similar reactivity and chemical methods are complicated by the many protection and deprotection steps that are necessary for regioselective synthesis and the total number of steps increases with the size of the oligosaccharide. The total yields are often low and large scale synthesis is difficult.

iii) Synthesis by enzymatic methods. These methods offer several advantages over chemical methods. A wide variety of regiospecific and often highly regioselective reactions can be catalysed very efficiently without protection and deprotection steps, so that the reaction takes place under mild conditions, often at room temperature and neutral pH and harzadous chemicals can be avoided.

Two types of enzyme have been used for preparation of complex oligosaccharides, the glycosyltransferases (Transglycosidases, EC 2.4) and the glycosidases (EC 3.2).

1.10 Enzymatic synthesis of oligosaccharides

The glycosyltransferases catalyse the stereo- and regiospecific transfer of monosaccharide from a donor substrate (glycosyl nucleotide) to an acceptor substrate. They are classified by the sugar transferred from donor and acceptor specificity as shown in the examples of Table 1-4. However, the use of glycosyltransferases may not be an attractive prospect because they are, in general, unstable and present in natural sources in very small amounts. The enzymes also require expensive sugar nucleotides as substrates, so that the use of glycosyltransferases is often time consuming and costly.

Another alternative method to synthesise oligosaccharides is to use glycosidase enzymes. The advantage of glycosidases over glycosyltransferase as catalysts for glycosyl transfer is that expensive sugar nucleotide donors are not required to achieve reaction. Additionally, the glycosidases are generally more readily available than glycosyltransferases and simple substrates can be used.

Table 1-4 Examples of reaction catalysed by glycosyltransferases (25).

Reactions	Products
β-D-galactoside(α2,6)sialyltransferase	
CMP-Neu5Ac+Gal(β1,4)GlcNAc	→Neu5Ac(α2,6)Gal(1,4)GlcNAc+CMP
β-D-galactoside(1,2)fucosyltransferase	
GDP-Fuc + Gal(β)-R	→Fuc(α1,2)Gal(β)-R + GDP
N-acetylglucosaminide(β1,4)galactosyltransferase	
UDP-Gal + GlcNAc	→Gal(β1,4)GlcNAc + UDP
UDP-Gal + Fuc(α1,6)GlcNAc(β)-OR	→Gal(β1,4)[Fuc(α1,6)]GlcNAc(β)-OR
CMP-Neu5Ac + Neu5Ac(α2,3)-Gal(β1,3)GalNAc(β)-OPh	→Neu5Ac(α2,3)Gal(β1,3)-[Neu5Ac(α2,6)]GalNAc(β)-OPh
CMP-Neu5Ac + Gal(β1,3)-GlcNAc(β)-OMe	→Neu5Ac(α2,3)Gal(β1,3)

Synthesis of oligosaccharides using glycosidases may be carried out by using equilibrium-controlled or kinetically-controlled approaches. The equilibrium approach reverses the glycosidase hydrolysis reaction by combining a free monosaccharide and a nucleophile. This method was demonstrated by Bourquelot and Bridel in 1912(29).

However, the equilibrium constant for this reaction greatly favours hydrolysis over glycoside formation. So that, high concentration of substrate, increased temperature and molecular traps have been utilised to shift the unfavourable equilibrium to product formation. For example, oligomannosides

(mainly α1,6 linked) have been obtained in good yield (37% yield) using a high initial concentration of mannose (up to 80% w/w) and increased temperature. Wallenfels (29) used a carbon celite column as a molecular trap to enrich the yields of Gal →GlcNAc (mainly β1,6 linked) produced by β-galactosidase from 3% to 15% by circulation of the reaction mixture through an active carbon column. After multiple cycles of Gal and GlcNac circulation, the yield of products increased to over 30%.

The kinetic approach was reported in 1935 by Rabate (29). In this approach, a glycoside with an aliphatic or aromatic aglycone is used as glycosyl donor. Such a technique can accumulate a higher concentration of the oligosaccharide products than the equilibrium approach. However, an efficient donor and acceptor used in a high concentration are required by this method to favour the transglycosidation reaction.

Figure 1-3 Equilibrium-controlled synthesis of glycosidases. a) General reaction. b) High concentration of substrate and molecular traps are used to facilitate the synthetic reaction (25).

a) General reaction

$$\text{Monosaccharide} \overset{-H_2O}{\Leftrightarrow} \text{Disaccharide} \overset{-H_2O}{\Leftrightarrow} \text{Higher oligosaccharide}$$

b) Driving mechanism for synthesis

High substrate concentration

$$\text{Man (83\% w/w)} \overset{\alpha\text{-mannosidase}}{\Leftrightarrow} \alpha\text{-Man-Man (37\% yield after 12 h)} + \text{higher oligomannoside}$$

Molecular trap

$$\text{Gal (10\% w/w)} + \text{GlcNAc (30\%w/w)} \overset{\beta\text{-galactosidase}}{\Leftrightarrow} \text{N-acetylallolactosamine}$$

$$\downarrow$$

Molecular trap (active carbon)

(91% yield)

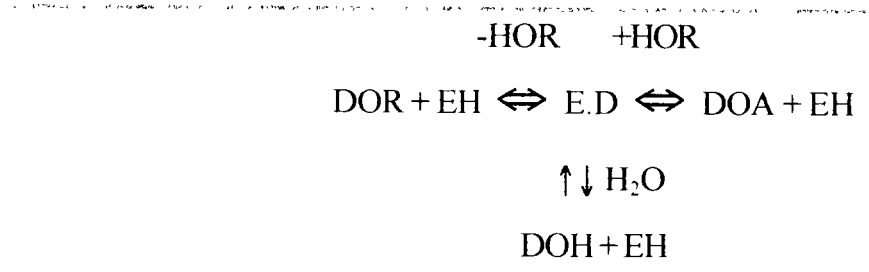

Figure 1-4 Kinetically-controlled synthesis of oligosaccharide by glycosidase. The process is facilitated by efficient glycoside donor (DOR) and high concentration of efficient acceptor (25).

In our laboratory, the hydrolytic activity of nine glycosidase enzymes were tested in the crude extracts from the seeds of 50 Thai plant species from 17 families (30). The results showed 6 major enzymes were found: α-mannosidase, β-D-N-acetylglucosaminidase, α-D-galactosidase, β-D-galactosidase, β-D-fucosidase and β-D-glucosidase α-D-Mannosidase was the most frequently found enzyme, present in most species of plants, and good sources of this enzyme included *Albizzia procera* Benth, *Hibiscus sabdariffa spp.* and *Acacia catechu* Willd. However, most interestingly, the levels of β-D-glucosidase and β-D-fucosidase found in *Dalbergia cochinchinensis* Pierre were more than ten-fold higher than those of any other glycohydrolases

1.11 Applications of β-glucosidase

1.11.1 Enzyme immobilisation

The main purpose of enzyme immobilisation is to stabilise enzyme against denaturation and utilise them efficiently. To be utilised efficiently, the enzyme must be able to be recovered for re-use. To achieve this, the product must be separated from the enzyme during the reaction using a 2-phase system: one phase contains the enzyme and the other contains the product. The enzyme is captured within the phase, allowing its re-use or continuous use but preventing it from contaminating the product; other molecules, including the reactants, are able to move freely between the 2 phases. The productivity of an enzyme, so immobilised, is greatly increased, as it may be more fully used at high substrate concentration for longer periods than is the free enzyme (3).

There are 2 broad principal methods available for immobilising enzymes: (1) immobilisation by noncovalent, and (2) immobilisation by covalent and coordination procedures.

1.11.1.1. Immobilisation of enzyme by noncovalent method

1.11.1.1.1 Immobilisation of enzyme by adsorption

Adsorption, the longest established technique for the noncovalent immobilisation of the enzymes, is based on the physical adsorption or ionic binding, or both, of the enzyme to the surface of the support. The forces responsible for immobilisation by physical adsorption include hydrogen bonding, Van der Waals forces and, importantly, hydrophobic interactions. Immobilisation via ionic bindings, as the term implies, involves salt-linkage formation between charged groups on the protein and opposite charges on the surface. Figure 6 shows immobilisation of enzyme by adsorption system (31).

A major advantage of adsorption as a general method of making enzymes insoluble is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap and easily carried out, and tends to be less disruptive to the enzymic protein than chemical means of attachment (32).

Because of the nature of the forces involved in the noncovalent immobilisation of proteins, it is relatively simple to reverse the immobilisation process as so to effect desorption from the support. Change in parameters such as pH, ionic strength and temperature influence both desorption and adsorption. Another disadvantage is that the adsorption surface is non-specific, and will therefore adsorb further protein or other substances to which it is exposed in the course of its use. This may alter the properties of immobilised enzyme or, if the substance adsorbed is a substrate for the

enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate (33).

Immobilisation by adsorption is less specific than the covalent techniques and the decrease in activity will ensure if adsorption involves groups at active site. Although immobilisation by adsorption is mild compared with the chemical interactions required for covalent binding, the final environment of covalently immobilised preparations can be engineered to be essentially neutral by the use of minimally charged base media. By contrast, the microenvironment of an adsorbed protein will almost certainly be different than the macroenvironment. This, in association with the gross interactions that can take place between a charged support and the charged protein, may lead to alterations in quaternary structure and a destabilising environment (34).

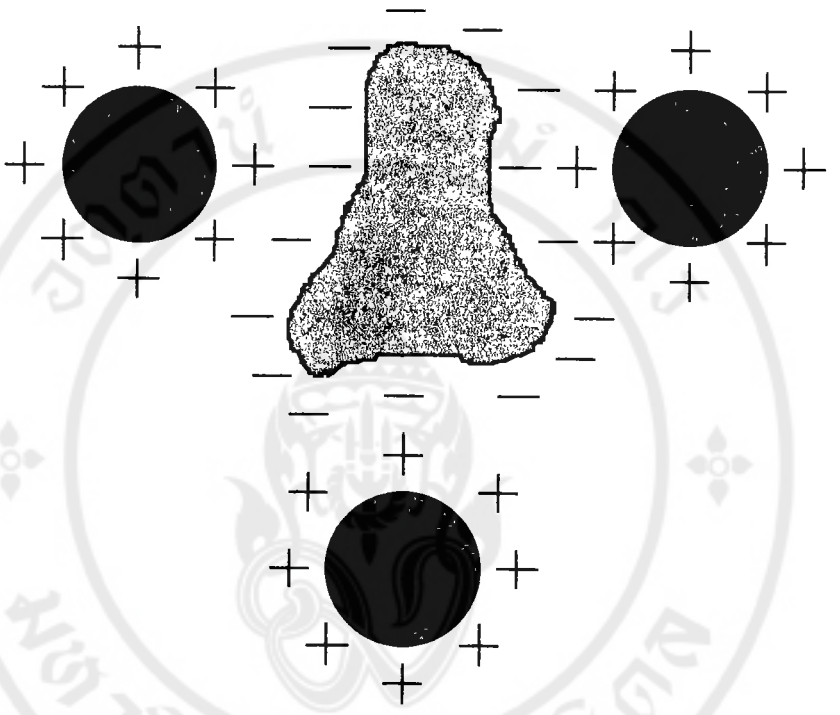

Figure 1-5 Immobilisation of enzyme onto the support by adsorption (31).

The followings are the examples of supports to prepare adsorbed enzymes.

i) DEAE-Sephadex: This technique involves electrostatic bonding of the enzyme to the polymer. Yields as low as 1% of soluble activity have been reported. However, the DEAE-Sephadex immobilised amylocyclase exhibited 86% of the original activity. Immobilisation on DEAE-Sephadex would change the environment of the enzyme with respect to pH with a consequence shift in the apparent pH activity curve towards acid pH values (34).

ii) Charcoal: The properties of charcoal with respect to adsorption of protein have been exploited in industrial processes. Activated carbon is a highly porous carbonaceous material with large internal pore surface. It is prepared by dehydration and carbonisation, followed by activation, of organic substances of mainly vegetable origin (34).

1.11.1.1.2 Immobilisation of enzyme by entrapment (32)

Enzymes have been immobilised by confining them within the lattices of polymerised gels, which allow the free diffusion of low molecular weight substrates and reaction products, but have a small enough pore size to prevent leakage of the high molecular weight enzyme. The usual method is to polymerise the hydrophilic matrix in an aqueous solution of the enzymes, and break up the polymeric mass to the desired particle size. Figure 7 shows enzyme imobilisation by entrapment (31).

In most of the cases of this method of immobilisation the monomer used was acrylamide, and the cross-linking was effected by means of N,N-methylene-bis-(acrylamide).

As there is no bond formation between the enzyme and the polymer matrix, entrapment provides a method which is generally applicable to any enzyme and, in theory, involves no disruption of the protein molecules. However, free radicals generated in the course of the polymerisation may affect the activity of the entrapped enzyme. Another disadvantage is that only low molecular weight substrates can diffuse into the vicinity of the enzyme, thus making the method unsuitable for enzymes which act on macromolecular substrates, such as ribonuclease, trypsin and dextranase.

This technique is limited to the applications for immobilising macromolecules. The matrices commonly used have lattice dimensions that allow molecular migration relatively easily. Thus, to maximise enzyme entrapment and minimise leakage, a high degree of polymer cross-linking is required. This extensive cross-linking limits the diffusion of substrates and products.

The formation of calcium alginate gels allow immobilisation of biological materials under mild conditions. This technology has been used extensively for the immobilisation of living cells, of nonviable cells in which the activity of a single enzyme is desired, and of enzymes. Entrapment within alginate gels may also be used for the co-immobilisation of living cells and enzymes (35, 36).

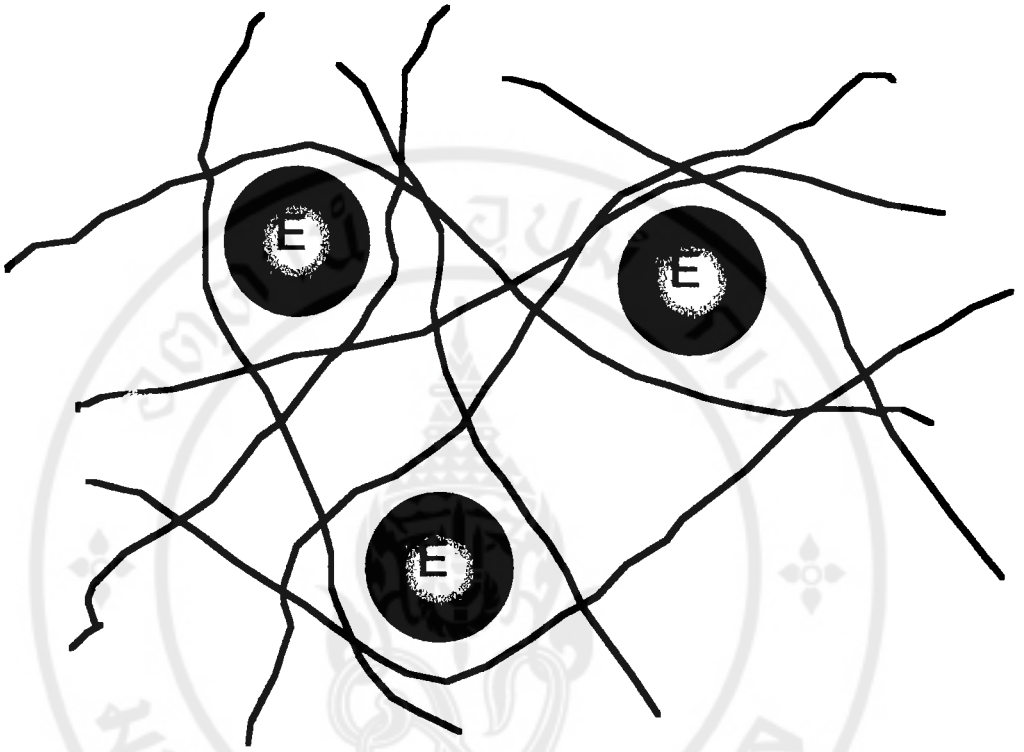

Figure 1-6 Immobilisation of enzyme by entrapment system (31).

1.11.1.2. Immobilisation of enzyme by covalent coupling (37)

The immobilisation of enzymes on solid supports by covalent coupling usually leads to very stable preparations with extended active life when compared with immobilised enzyme preparations obtained with other coupling methods, namely, physical adsorption and ionic binding. The most intensely studied of the insolubilisation techniques is the formation of covalent bonds between the functional groups of enzyme and the support matrix. The choice of method is limited by the fact that the binding reaction must be performed under conditions which do not cause loss of enzymatic activity, and the active site must be unaffected by the reagents used.

The functional groups of proteins suitable for covalent binding under mild condition include (I) amino groups, the ε-amino groups of lysine and the α-amino groups of the N-termini of the polypeptide chains; (ii) carboxyl groups, the β- and γ-carboxyl groups of aspartic and glutamic acid, respectively, and terminal α-carboxyls; (iii) the phenol ring of tyrosine, (iv) the sulfhydryl group of cysteine; (v) the hydroxyl groups of serine, threonine, and tyrosine; (vi) the imidazole group of histidine and (vii) the indole groups of tryptophan (37). In practice, most of the common covalent coupling reactions involve amino groups, carboxyl, or the aromatic rings of tyrosine and histidine.

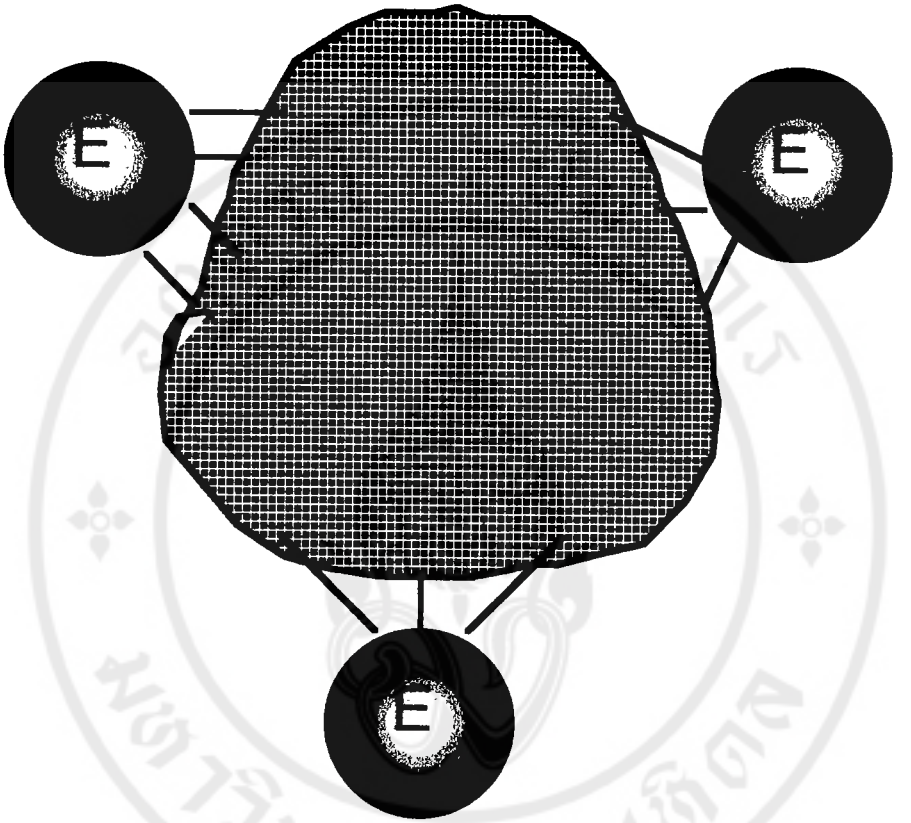

Figure 1-7 Immobilisation of enzyme by covalent coupling (31).

Covalent bonding provides stable, insolubilised enzyme derivatives which do not leach enzyme into the surrounding solution. The wide variety of binding reactions, and insoluble carriers with functional groups capable of covalent coupling, or being activated to give such groups, makes this a generally applicable method of insolubilisation.

There are many ways to immobilise enzyme by covalent coupling but 2 methods will be noted here: (1) cross-linking with bi- or multifunctional reagents and (2) coupling to polymeric supports

1.11.1.1.2.1 Cross-linking with bi- or multifunctional supports (38)

Intermolecular cross-linking by bi- or multifunctional reagents has been used routinely for the fixation of enzyme crystals and similar macromolecular aggregates of well defined structure and in histochemistry for the preservation of cellular ultrastructure, e.g., in the preparation of specimens for electron microscopy.

Immobilisation of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. The cross-linking was brought about by the use of bi- or multifunctional reagents, which may either possess two identical functional groups; such as glutaraldehyde; bisdiazobenzidine-2,2'-disulphonic acid and 1,5-difluoro-2,4-dinitrobenzene, or two different functional groups of different reactivities, such as toluene-2-isocyanate-4-isothiocyanate; 3-methoxy-diphenylmethane-4,4'diisocyanate and trichloro-s-triazine (38).

Glutaraldehyde, which is by far the most commonly used cross-linking reagent, has been employed for the fixation of crystals and histochemical preparations. It was first applied to the cross-linking of enzyme crystals by Quiocho and Richards (39). This dialdehyde appeared to give the most satisfactory and reproducible results. It is known to react with amino groups of lysine through double bonds of its oligomer. Lysine-rich proteins are readily insolubilised. In order to insolubilised low concentrations of an active protein, such as enzyme, it is often necessary to add an inert lysine-rich protein to the enzyme solution. When glutaraldehyde is added, cross-linking between the enzyme and the inert protein is observed. If the concentration of the auxiliary protein is high enough, it can be considered as the insoluble support matrix of the enzyme. Often bovine serum albumin was chosen as the auxiliary soluble protein to the enzyme solution because of its lysyl residue content. When employed at concentrations higher than 50 mg/ml, it is easily insolubilised at any pH between 5 and 7 (39).

1.11.1.2.2 Immobilisation of enzymes by covalent coupling to polymeric supports (38)

Covalent binding to polymeric supports has been the most thoroughly investigated approach to enzyme immobilisation. Nevertheless, the compositional and structural complexity of proteins has not allowed, except in a very limited number of cases, the application of general rules by means of which the method best suited for a specific task could be predicted.

Two main factors that have to be considered when coupling enzymes to the polymeric supports are: (I) type of functional groups on the protein through which the covalent bonds with the support material are formed and

hence the chemical reaction to be employed; (ii) the physical and chemical characteristics of the support material onto which chemically reactive groups are to be grafted.

The type of functional groups on the protein through which the covalent bond with the support is to be formed should naturally be nonessential for the catalytic activity of the enzyme; moreover, binding reactions should be carried out under relatively mild conditions and in essentially aqueous media conditions, and have relatively high specificity toward one type of functional group on the protein and minimal side reactions with other functional groups or with the aqueous medium.

Table 1-5 Methods of covalent coupling to polymeric support of mainly interest (38).

Parent polymer	**Modification of polymer**	**Method of coupling**	**Capacity (mg protein/gm conjugate)**
Dextran; cross-linked beads (Sephadex)	3-(4-Isothiocyanatophenoxy)-2-hydroxypropyl ether	Thiocarbamylation of amino groups of protein	-
Chitin	-	Activation with transition metal	-
VINYL POLYMERS			
Polyacrylamide; cross-linked beads	-	Activation with glutaraldehyde	30-100
Polyacrylamide; cross-linked beads (Bio-Gel)	p-Aminaobenzamidoethyl derivative	Activation by diazotization; azo bond formation mainly with tyrosine residue	300
Poly(p-amino-styrene)	-	Activation by diazotization; azo bond formation mainly with tyrosine residue	2-14

Parent polymer	Modification of polymer	Method of coupling	Capacity (mg protein/gm conjugate)
Polypropylene	Radiation grafted poly-(p-nitrosty-rene), reduced and converted to isothiocyanate derivative	Coupling by thiocarbamylation of amino group of protein	-
POLYAMINO ACIDS AND PROTEINS			
Polyglutamic acid	-	Activation of support with N-ethyl-5-phenylisoxazolium 3'-sulfonate (Woodward's Reagent K); peptide bond formation with amino groups of protein	600-800
Collagen	Acid hydrazide derivative	Activation of support hydrazide groups by conversion to azides; peptide bond formation with amino groups of protein	-

Parent polymer	Modification of polymer	Method of coupling	Capacity (mg protein/gm conjugate)
POLYAMIDES			
Polyamides (nylon-6-nylon-6,6)	Acid hydrazide derivative of partially hydrolysed nylon	Activation of support hydrazide groups by conversion to azide; peptide bond formation with amino groups of protein	-
Polyamides (nylon-6-nylon-6,6)	O-Alkylation of peptide bonds with dimethyl sulphate to form polymeric imidate salts	Coupling via formation of amidines with amino groups of proteins	-
INORGANIC SUPPORTS			
Porous glass	Alkylamino derivatives	Activation with glutaraldehyde	12-16
Iron oxide powder (magnelite. Fe_2O_3)	Particle coated with cellulose	Cyanogen bromide activation	4

1.12 Immobilised β-glucosidases and their use

C. Ravet *et al* (40) synthesised gluco-oligosaccharides through the enzymatic condensation of D-glucose at high concentration using a commercial β-glucosidase. The synthesis reactions were carried out with both free and immobilised enzymes. The use of 5 M glucose solution permitted only disaccharides to be obtained, whereas with a glucose concentration of 7.5 M, di- tri-, and tetrasaccharides were produced. The synthesis yield (oligomers mg/ml mg of enzyme) after immobilisation, by a cross-linking method using serum albumin and glutaraldehyde at a subzero temperature, was 573% with 33% recovery yield for hydrolytic activity compared to that of free enzyme, when a 7.5 M glucose solution was tested. When 5 M glucose was used the synthesis recovery yield was 147%.

Jose Agaudo *et al* (41) immobilised β-glucosidase from *Penicillium funiculosum* on nylon powder previously activated with triethyloxonium tetrafluoroborate, 1,2-diaminoethane and glutaraldehyde. The activation of the nylon powder and the immobilisation processes were studied and optimised for the enzyme and the matrix. A high activity retention (67%) was obtained using the activation and immobilisation conditions finally selected.

Broun *et al* (42) used glucose oxidase to impregnate onto cellophane. Cellophane sheet were impregnated with 6 mg/ml protein solution containing 78 IU glucose oxidase. Glucose oxidase bound in a membrane retained 100% of its original activity after 48 h of incubation with trypsin, chymotrypsin and bacterial pronase. This resistance can be explained by steric hindrance.

Soluble enzyme is rapidly denatured at 55°C. Addition of albumin slows down the process while cellophane-bound enzyme is even more slowly denatured. Moreover, the bound enzyme can be kept at 37°C under dry

conditions and in solution for one month. The dry prepration retained all its initial activity while enzyme in solution retained 60%. Under the same conditions, free enzyme lost all of its activity.

Baker *et al* (43) have been purified *Aspergillus niger* β-glucosidase and cross-linked with glutaraldehyde followed by borohydride reduction. The resulting enzyme retained at least 75-80 % of the total initial activity of the free enzyme and is substantially thermostable. Whereas the free enzyme has a half-life on the order of 30 min at pH 5.0 and 65°C, the cross-linked enzyme retained half of its activity even after 3 h. at 65°C. The enzyme possessed a biphasic process in which approximately half the activity is lost rapidly with the remainder of the activity being lost at a much slower rate. This is due to the existence in the treated enzyme sample of two distinct populations of enzyme molecules with dramatically different thermal stabilities, rather than to the stepwise loss of activity from a single population.

The acylation of amino groups of protein by pyromellitic dianhydride (PMDA), which leads to an increase in negative charges on the protein by four units for every amino acid modified, has been used to modify enzymes for the purpose of adsorption on DEAE-cellulose (44). The 49% amino groups of amyloglucosidase were modified with 90% residual enzyme activity. Whereas the free enzyme bound to DEAE-cellulose and could be eluted out with 0.1 M sodium chloride, the modified enzyme exhibited stronger binding and could only be eluted with 0.25 M sodium chloride. In the case of adsorbed free enzyme, heating at 60°C for 1 h led to desorption of 97% protein. In the case of modified enzyme, the immobilised preparation retained full activity under similar conditions. β-Glucosidase (which did not bind to DEAE-cellulose) upon modification with PMDA was found to bind to DEAE-cellulose and could be eluted out with 97% protein recovery by washing with

0.2 M sodium chloride. This may be a useful strategy for obtaining enzyme derivatives for reversible adsorption on anion exchanger (44).

Immobilised enzymes have also been employed as specific biosorbents in affinity chromatography, as heterogeneous catalysis in enzyme reactors and as model systems in studying microenvironmental effects on enzyme action. In many applications, they are utilised in packed beds such as a chromatographic columns or packed reactors (45).

Immobilised enzymes can also served as experimental and theoretical models for bound enzymes in living systems. It is increasingly recognised that in the cellular environment, most enzymes are localised to various cell compartments and the catalytic properties can be quite different from those of the same enzymes in the free solution (46).

1.13 Aim of Thesis

1. To find suitable support to immobilise enzyme for the purpose of oligosaccharide synthesis.

2. To study the properties of immobilised enzyme compared with free enzyme.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials

Thai Rosewood seeds (*Dalbergia cochinchinensis* Pierre) were kindly provided by the ASEAN-Canada Forest Tree Seed Centre, Muaklek, Saraburi, Thailand and Department of Forestry, Kasertsart University, Bangkok, Thailand.

2.1.2 Chemicals

Phenylmethysulfonyl fluoride (PMSF), Dowex 2X-8, p-nitrophenol, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-fucopyranoside, 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-fucopyranoside, δ-gluconolactone, Tris(hydroxymethyl)aminomethane, sodium dodecyl sulphate, n-butanol, Coomassie brilliant blue R, bromophenol blue, β-mercaptoethanol, polyvinyl-polypyrrolidone (PVPP), molecular weight standard kit for denaturing-PAGE (bovine serum albumin, egg albumin, carbonic anhydrase from bovine erythrocytes, phosphorylase b from rabbit muscle, β-galactosidase from *E. coli* and myosin from rabbit muscle), glycine, bovine serum albumin were products of Sigma Chemical Company, St. Louis, Mo., U.S.A.

Acetic acid, sodium hydroxide, sulphuric acid, 2-propanol, methanol, activated carbon and t.l.c. aluminum sheet silica 60 were purchased from E.

Merck, Darmstadt, Germany. Sodium carbonate, citric acid, hydrochloric acid, sodium hydrogen phosphate, sodium acetate and sodium dihydrogen phosphate were from Riedel-de Haen AG., Seelze, Germany. D-Glucose was from BDH Chemicals Ltd., Poole, England. Serva blue G and ammonium sulphate were from Farmitalia Carlo Erba, S.P.A. Milan, Italy. Biolyte 3/10, biolyte 4/6, acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulphate and N,N,N'N' tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratory Division, Richmond, Ca., U.S.A. Sephadex G-150 was product of Pharmacia Fine Chemicals, Uppsala, Sweden. Celite 535 was from Fluka. Pure activated charcoal (Cat. no. 4127403) was from Merck, Damstadt, Germany. Aminex HPX-87Ch.p.l.c. column was from Bio-Rad, USA.

2.2 Methods

2.2.1 Enzyme purification

2.2.1.1 Preparation of submerged seeds

Thai Rosewood seeds were surface-sterilised with 0.1% hypochlorite (diluted from Clorox) for 30 sec and then washed thoroughly with distilled water. The washed seeds were softened for easy homogenisation by submerging the cleansed seeds in distilled water overnight at room temperature.

2.2.1.2 Preparation of crude enzyme

The crude enzyme was obtained by homogenising the imbibed seeds in Waring blender with ice cold 100 mM sodium acetate buffer pH 5.0 containing 1 mM PMSF (phenylmethylsulfonyl fluoride) and 5% PVPP (polyvinylpolypyrrolidone) in a ratio of 2 ml buffer solution to 1 gram seed.

The homogenisation was performed at low speed for 30 sec for 5 times with an 1-min intermission in an ice bath to minimise heat generation. After passing through 2-3 layers of gauze cloth, the homogenate was centrifuged at 10,000 x g for 30 min at 4°C to remove the insoluble materials. The pellet was discarded and the supernatant solution was then stirred with Dowex 2X-8 for 1 h at 4°C to remove phenolic compounds. The Dowex 2X-8 was removed by suction, and the crude extract was obtained after centrifugation at 10,000 x g for 30 min at 4°C.

2.2.1.3 Ammonium sulphate precipitation

The crude extract was fractionated with ammonium sulphate. At 4°C, solid ammonium sulphate was gradually added to the stirred crude extract to make 35% saturation. The precipitate formed after 4 h of stirring was collected and the supernatant was raised to 75% saturation by the further addition of solid ammonium sulphate. The slurry was left stirring overnight at 4°C and then the precipitate was collected by centrifugation at 10,000 x g for 30 min at 4°C. Both of the precipitate fractions were re-dissolved in a small volume of 100 mM sodium acetate buffer pH 5.0 and dialysed overnight against a surplus volume of the same buffer.

2.2.2 Free β-glucosidase/β-fucosidase assay using synthetic substrates, p-nitrophenol-β-D-glucopyranoside and p-nitrophenol-β-D-fucopyranoside

The β-glucosidase/β-fucosidase activity was assayed according to the methods of Evans (47). A one-ml reaction mixture contained 100 mM sodium acetate buffer pH 5.0, 1 mM p-nitrophenol-β-D-glucoside or p-nitrophenol-β-D-fucoside and 50 µl of appropriately diluted enzyme. The reaction was initiated by the addition of 100 µl of 10 mM substrate and incubated at 30°C for 10 min in a shaking thermostated water bath. The reaction was stopped by the addition of 2 ml of 2 M sodium carbonate. The absorbance of p-nitrophenol released at 400 nm was measured using with a spectrophotometer. The autolysis of substrate was determined by the same manner as described above except for the addition of the enzyme. The enzyme blank contained 100 mM sodium acetate buffer pH 5.0 instead of substrate.

2.2.3 Immobilised enzyme assays

The reaction mixture was the same as for free enzyme except that after the reaction was stopped, the immobilising support was spun down by centrifugation at 10,000 x g for 5 min before measuring the p-nitrophenol absorbance at 400 nm.

2.2.4 Assay of crude enzyme cross-linked with BSA by glutaraldehyde

The product of enzyme cross-linking was in the form of sponge. The sponge had to be weighed before putting in a test tube and then 900 µl of 100 mM sodium acetate buffer, pH 5.0 was added followed by the addition of 100 µl of 10 mM sodium acetate buffer, pH 5.0 to start the reaction. After 10 min of incubation at 50°C, the reaction was stopped by adding 2 ml of 2 M sodium carbonate. The sponge was filtered out before measuring the p-nitrophenol released at 400 nm.

2.2.5 Preparation of p-nitrophenol standard curve

One ml of varying concentrations (5-200 µM) of p-nitrophenol were prepared from a stock solution of 10 mM p-nitrophenol in 100 mM sodium acetate buffer pH 5.0. A graph was drawn relating the absorbance at 400 nm to the concentrations of p-nitrophenol.

2.2.6 Protein determination by Bradford's method

Protein concentrations from 0.1-1 mg/ml were determined according to the method of Bradford (48).

20 µl of appropriately diluted sample was mixed with 50 µl of 0.1 M NaOH prior to the addition of 1 ml of Serva Blue G solution. The mixture was left at room temperature for 5 min before measuring the absorbance at 595 nm. The Serva Blue G solution comprised of 100 mg Serva Blue G (Serva, Westbury, N.Y.), 100 ml of 85% phosphoric acid and 50 ml of 95% ethanol.

After the dye was completely dissolved, the volume was brought up to 1 litre with distilled water.

Crystalline BSA (2-200 µg) was used as the standard protein and was treated in the same manner as described above to construct the standard curve.

2.2.7 Enzyme immobilisation

2.2.7.1 Immobilisation of enzyme on activated carbon and celite

An accurately weighed amount (25 mg) of activated carbon or celite was placed into a tube. A 2 ml volume of 100 mM sodium acetate buffer, pH 5.0 containing known activity units of enzyme was added into the tube. Another 200 µl volume of enzyme solution was taken prior to the addition and was designated as pre-immobilised enzyme. The tube was then tightly closed and placed horizontally on a shaker at room temperature and shaken for 20 min.

The support to which the enzyme was already coupled was then centrifuged at 10,000 x g for 5 min at 4°C to separate the coupled enzyme from the unbound one. The resulting residue was then washed with 2 ml of 100 mM sodium acetate buffer, pH 5.0 and then centrifuged at 10,000 x g for 5 min at 4°C. The washed fraction was thus obtained. The unbound and washed fraction were kept for measurement of enzyme activity and protein concentration. The immobilised enzyme was kept in 2 ml of 100 mM sodium acetate buffer, pH 5.0 at 4°C.

2.2.7.2 Maximum loading of enzyme on the support

Two parallel experiments were performed in order to determine the maximum loading of enzyme on support.

In the first experiment, concentrations of enzyme were varied from 0.6 U β-glucosidase to 1.2 U β-glucosidase while the amount of the support was kept constant at 25 mg. The maximum concentration of enzyme that could be bound onto 25 mg of support was hence obtained.

The second experiment was performed by varying the weights of the support from 10 to 25 mg to determine the least amount of support that could bind the enzyme.

Percent immobilisation was determined by measuring the enzyme activity and protein concentration. The measured activity and protein concentration of immobilised enzyme were then compared to the initial concentrations in pre-immobilised enzyme.

2.2.7.3 Immobilisation of crude enzyme by cross-linking with BSA by glutaraldehyde

To the enzyme solution containing 20 units of β-glucosidase, was mixed various amounts (2.5%, 5% and 10% w/v) of BSA. The solution was gently stirred until BSA was completely dissolved, and then 0.3% (w/v) of glutaraldehyde was added, gently stirred and kept at -20°C for 4 h.

2.2.8 Properties of free and immobilised enzymes

2.2.8.1 Optimum pH of free and immobilised β-glucosidase/β-fucosidase

The reaction mixture was the same as for the assay for enzyme activity with synthetic substrate but 100 mM McIlvaine buffer pH 3-7 was used instead of 100 mM sodium acetate buffer, pH 5.0.

2.2.8.2 Temperature stability of free and immobilised enzyme

Enzyme solution, both free and immobilised enzyme, was incubated in a heating block at 50°C and aliquots were taken every 1 h to test for the enzyme activity by the method in section 2.6 (free enzyme) and section 2.7 (immobilised enzyme).

2.2.8.3 Kinetic studies of β-glucosidase/β-fucosidase

2.2.8.3.1 K_m determination

2.2.8.3.1.1 Michaelis-Menten kinetics with p-nitrophenyl-β-D-glucopyranoside

About 0.005 U of β-glucosidase was assayed using various concentrations of p-nitrophenyl-β-D-glucopyranoside (1.0, 2.0, 6.0, 8.0, 10.0 and 20.0 mM) in 100 mM sodium acetate buffer pH 5.0 at a final volume of 1.0

ml. The reaction was allowed to proceed for 10 min at 30°C before being stopped with 2 vol of 2 M Na_2CO_3.

2.2.8.3.1.2 Michaelis-Menten kinetics with p-nitrophenyl-β-D-fucopyranoside

About 0.01 U of β-fucosidase was assayed using various concentrations of p-nitrophenyl-β-D-fucopyranoside (0.1, 0.2, 0.4, 0.8, 1.0 and 2.0 mM) in 100 mM sodium acetate buffer pH 5.0 at a final volume of 1.0 ml. The reaction was allowed to proceed for 10 min at 30°C before being stopped with 2 vol of 2 M Na_2CO_3.

2.2.9 Oligosaccharide synthesis

2.2.9.1 Time course of oligosaccharide synthesis

In a synthesis reaction of a total of 100 mg, 0.1 U β-glucosidase was incubated with 50% (w/w) D-glucose in 10 mM sodium acetate buffer pH, 5.0 at 50°C. Aliquots of 30 µl were taken each day and the enzyme was heat-killed and products were analysed by t.l.c. and h.p.l.c.

2.2.9.2 Determination of suitable enzyme concentration in oligosaccharide synthesis

Varying amounts (0.1, 0.2 and 0.4 U) of both free and immobilised enzyme were used to determine the suitable concentration for use in the oligosaccharide synthesis. The reactions were allowed to proceed as described in section 2.15.

2.2.9.3 Re-usability of enzyme immobilised on activated carbon in oligosaccharide synthesis

The reaction mixture of 0.2 U/100 mg of immobilised enzyme in the presence of 50% (w/w) glucose was incubated at 50°C for 15 days. For every 5 days the re-utilisation of immobilised enzyme was performed by removing products from the enzyme by centrifugation at 10,000 x g for 5 min. The products in the supernatant solution were taken and the volume was measured. Then 50% (w/w) glucose was added into the pellet in the same volume as that taken out. The reaction mixture was then incubated for another 5 days and re-utilisation was performed again. The products and yield of synthesis were analysed by t.l.c. and h.p.l.c.

The 0.2 U/100 mg of free enzyme in the presence of 50% (w/w) glucose was incubated at 50°C for 15 days and analysis of the products and yield of synthesis were determined by t.l.c. and h.p.l.c.

After 15 days of incubation, the products were removed, and the pellet of immobilised enzyme was washed with 200 µl of distilled water twice followed by 200 µl of 5% ethanol and 200 µl of 10% ethanol to recover the

products adsorbed onto the activated carbon. The products in the washing were analysed by t.l.c. and h.p.l.c.

2.2.9.4 Identification of disaccharides on thin-layer chromatography

Since high concentration of glucose in the synthesis reaction shows movement of other disaccharides obtained in the reaction in t.l.c., so they do not correspond to markers, this experiment was performed in order to identify the products. On each lane of appropriately diluted synthesis products, glucose, sophorose, laminaribiose, and gentiobiose was loaded with the sample. The t.l.c. was run as mentioned in section 2.2.11.

2.2.10 Analysis of oligosaccharide products

2.2.10.1 Thin-layer chromatography

2.2.10.1.1 T.L.C. plate activation

T.l.c. aluminum sheet silica gel 60 (Merck) was activated by heating in an hot-air oven at 100°C for at least 1 h and allowing the plate to cool at room temperature.

2.2.10.1.2 Sample application

An appropriately diluted synthesis reaction was spotted onto the activated t.l.c. plate. The spot was then dried by a hair-blower.

2.2.10.1.3 Developing system

The t.l.c. solvent was comprised of n-butanol:2-propanol:distilled water (10:5:4). The t.l.c. tank was equilibrated with the solvent overnight before use.

The t.l.c. plate was left standing in the equilibrated tank in an upright position until solvent reached the top of the plate. A second run was performed in the same direction after drying the plate.

2.2.10.1.4 Sample detection

After the second run, the plate was dried and sprayed with concentrated sulphuric acid and placed in an hot-air oven at 100°C until the colour was fully developed.

2.2.10.2 High performance liquid chromatography

A 5-µl sample of synthesised oligosaccharide product was injected into an Aminex (Aminex HPX-87C, 300 x 7.8 mm) column on a hplc (Waters 625 LC system) with an RI detector (Waters). The conditions were: flow rate 0.5 ml/min, retention time 20 min, 85°C and degassed double-deionised water (sparge 50 ml/min)as mobile phase.

CHAPTER III

RESULTS

3.1 Purification of β-glucosidase/β-fucosidase from the seeds of *Dalbergia chochinchinensis* Pierre

3.1.1 Crude extract preparation

β-Glucosidase/β-fucosidase was purified from 10 g of seeds which were surface sterilised and submerged in distilled water overnight. The swollen seeds were homogenised in 0.05 M sodium acetate buffer, pH 5.0 containing 5% (w/v) PVPP and 1 mM PMSF. After centrifugation, 25% (w/v) Dowex 2X-8 was added into the supernatant. A second centrifugation step, after 1 h of stirring with Dowex, yielded the crude extract as the supernatant. After measuring the enzyme activity in the crude extract with 10 mM p-nitrophenol-β-D-glucoside and 10 mM p-nitrophenol-β-D-fucoside, the activities of the enzymes were found to be 24.6 U/g seed for β-glucosidase and 40.5 U/g seed for β-fucosidase This step was regarded as 100% yield and 100% fold purification.

3.1.2 Ammonium sulphate fractionation

The crude extract was subjected to 0-35% ammonium sulphate saturation, stirred for 4 h, centrifuged and the supernatant was raised to 75% ammonium sulphate saturation. The results showed that most of the activity was found in the 35-75% ammonium sulphate fraction with little enzyme activity found in the 0-35% ammonium sulphate fraction. The specific

activities of β-glucosidase/β-fucosidase were increased as the undesired proteins were removed. The fold purification of β-glucosidase and β-fucosidase in the 35-75% ammonium sulphate fraction were 4.73 and 4.33 respectively.

Table 3-1 Partial purification of β-glucosidase/β-fucosidase from 10 gm seeds. β-Glucosidase activity was designated as G and β-fucosidase as F.

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	% Yield
Crude extract	G 245.6 F 404.7	306.25	1.32 2.87	1 1	100 100
35-75% precipitate	G 12.9 F 17.5	45	6.25 12.44	4.73 4.33	60.85 55.74

3.2 Enzyme immobilisation

3.2.1 Maximum loading of crude β-glucosidase/β-fucosidase immobilised on activated carbon

Crude β-glucosidase activity of 0.4 U was immobilised on 6.25, 1.25, 2.5, 5.0 and 6.25 mg of activated carbon in 1 ml of 100 mM sodium acetate buffer, pH 5.0. The results in Table 3-2 show that 0.4 U of crude enzyme could be bound to 12.5 mg of activated carbon maximum with no activity or protein

Table 3-2 Maximum loading of crude enzyme immobilised on activated carbon when the enzyme concentration was fixed at 0.4 U β-glucosidase and the amounts of activated carbon were varied.

Carbon (mg)	% Bound activity recovered	% Activity in unbound fraction	Protein in unbound fraction (μg)
50	49	none	none
25	58	none	none
12.5	60	none	none
6.25	45	35	45

Table 3-3 Maximum loading of crude enzyme immobilised on activated carbon when fixing the amount of activated carbon at 12.5 mg and β-glucosidase concentrations were varied.

Starting enzyme (U)	% Bound activity recovered	% Activity in unbound fraction	Protein in unbound fraction (μg)
0.4	50	none	none
0.5	48	5	50
0.8	40	14	112
1.0	37	23	212

found in the unbound fraction. There were some detectable activities and proteins found in the unbound fraction when enzyme was immobilised on 6.25 mg of activated carbon.

When the amount of activated carbon was fixed at 12.5 mg and enzyme concentrations were varied (table 3-3), it was confirmed that the maximum loading of activated carbon used to immobilise enzyme was 0.4 U enzyme per 12.5 mg of activated carbon in 1 ml of 100 mM sodium acetate buffer, pH 5.0.

3.2.2 Maximum loading of crude enzyme immobilised on celite

The experiments were performed in the same manner as enzyme immobilised on activated carbon. The results show that 12.5 mg of celite can immobilise 0.1 U of enzyme with 70% immobilisation and no activity was found in the unbound fraction (Table 3-4). When 0.1 U of enzyme was immobilised on 6.25 mg of celite or immobilised 0.2 U of enzyme on 12.5 mg of celite (Table 3-5), some activities and protein were found in the unbound immobilised fraction.

Table 3-4 Maximum loading of crude enzyme immobilised on celite when fixing crude β-glucosidase concentration at 0.1 U and varying the amounts of celite.

Celite (mg)	% Bound activity recovered	% Activity in unbound fraction	Protein in unbound fraction (μg)
50	55	none	none
25	60	none	none
12.5	63	none	none
6.25	35	29	45

Table 3-5 Maximum loading of crude enzyme immobilised on celite when fixing the amount of celite at 12.5 mg and varying the concentration of crude β-glucosidase.

Starting enzyme (U)	% Bound activity recovered	% Activity in unbound fraction	Protein in unbound fraction (μg)
0.05	60	none	none
0 10	70	none	none
0.20	45	21	36
0.30	30	32	55

3.2.3 Cross-linking of enzyme and BSA by glutaraldehyde.

Crude β-glucosidase concentration of 20 U was cross-linked with various amounts of BSA (2.5%, 5% and 10%) using 0.3% glutaraldehyde. The sponge obtained from cross-linking enzyme with 0.25% BSA yielded a soft sponge while that with 10% BSA yielded the hardest. Cross-linking of enzyme with 5% BSA gave the highest percent immobilisation which was 13.5%. Percent immobilisation of cross-linked enzyme is shown in Table 3-6.

Table 3-6 Percent immobilisation (% bound activity recovered) of 20 U of crude β-glucosidase cross-linked with varying amounts of BSA by 0.3 % (w/v) glutaraldehyde.

BSA % (w/v)	Cross-linked enzyme recovered (U)	% Bound activity recovered	% Activity in unbound fraction
2.5	0.32	1.57	none
5	2.69	13.47	none
10	2.42	12.12	none

3.3 Properties of free and immobilised enzymes.

3.3.1 pH optimum of crude β-glucosidase/β-fucosidase and crude β-glucosidase/ β-fucosidase immobilised on activated carbon and celite

The activities of free and immobilised crude enzyme were assayed at 30°C in 0.1 M McIlvaine buffer at various pHs, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. The results as shown in Figure 3-3, 3-4 and 3-5 show that the maximum activities were at pH 5.5 for free β-glucosidase/ β-fucosidase, pH 5.0-5.5 for crude β-glucosidase/β-fucosidase immobilised on activated carbon and pH 5.0 for crude β-glucosidase/β-fucosidase immobilised on celite.

3.3.2 Temperature stability at 50°C of crude β-glucosidase/ β-fucosidase, crude β-glucosidase/β-fucosidase immobilised on activated carbon and celite.

Crude β-glucosidase/β-fucosidase, both free and immobilised forms, were incubated at 50°C in 100 mM sodium acetate buffer, pH 5.0. Aliquots were taken and the residual activity was measured. Results in Figure 3-6 show that free enzyme lost 40% of its starting activity after 1 h of incubation. Both activities of the enzyme were sharply declined after 5 h of incubation and then gradually declined until 5% residual activity remained at 72 h of incubation. The activities of enzyme immobilised on celite retained 40% of its original activity after first hour of incubation and gradually declined until 3% residual activity after 48 h of incubation. When immobilised on activated carbon, enzyme lost almost all of its activities after 3 h of incubation.

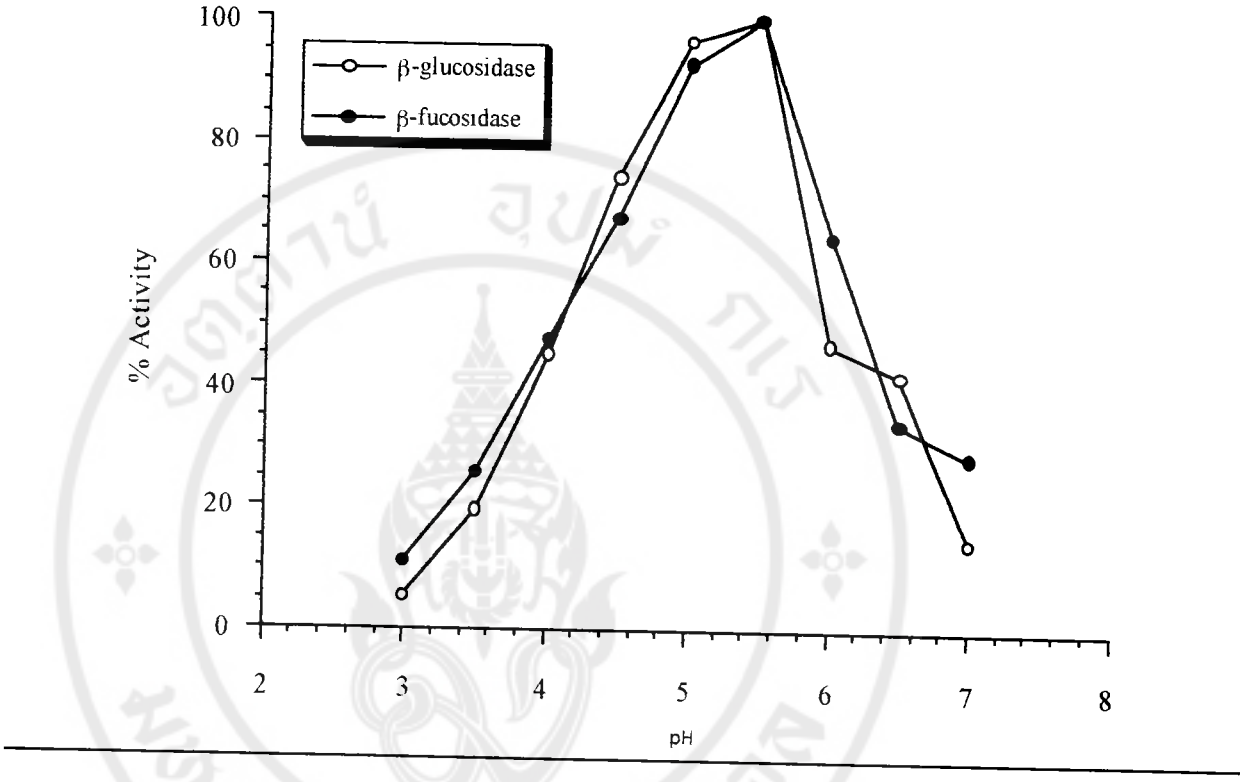

Figure 3-1 The pH profile of crude enzyme. The reaction mixture was incubated in 100 mM McIlvaine buffer at various pHs (3.5 - 7.0) and contained 1 mM pNP-β-glucopyranoside or pNP-β-fucopyranoside. The reaction was performed for 10 min at 30°C before stopping the reaction with 2 M sodium carbonate.

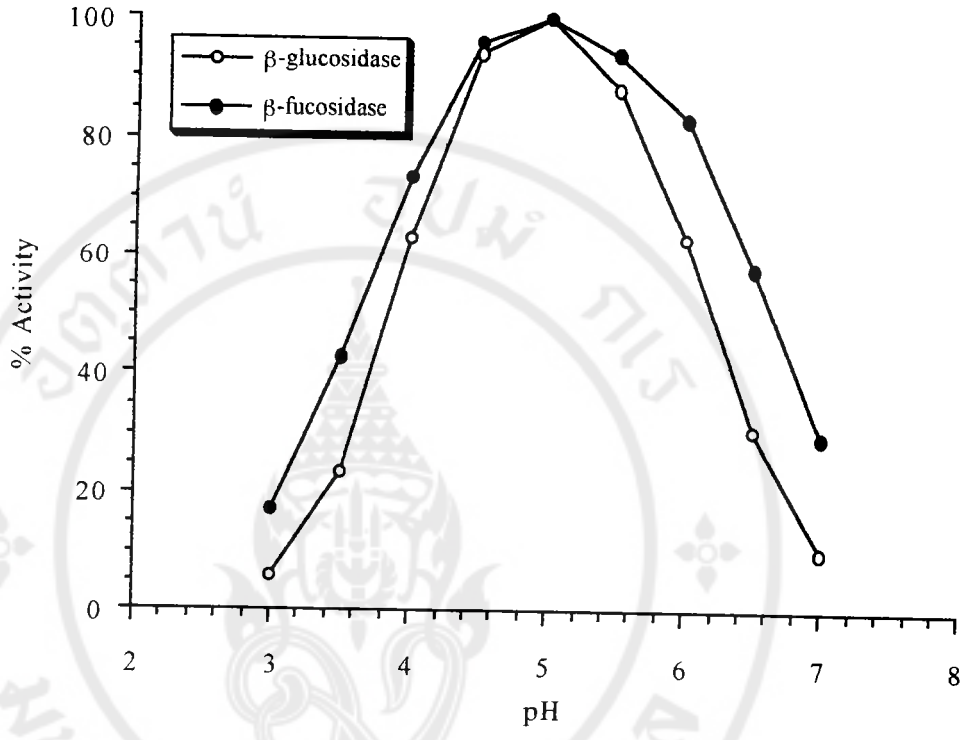

Figure 3-2 The pH profile of crude enzyme immobilised on activated carbon. The reaction mixture was incubated in 100 mM McIlvaine buffer at various pHs (3.5 - 7.0) and contained 1mM pNP-β-glucopyranoside or pNP-β-fucopyranoside. The reaction was performed for 10 min at 30°C before stopping the reaction with 2 M sodium carbonate.

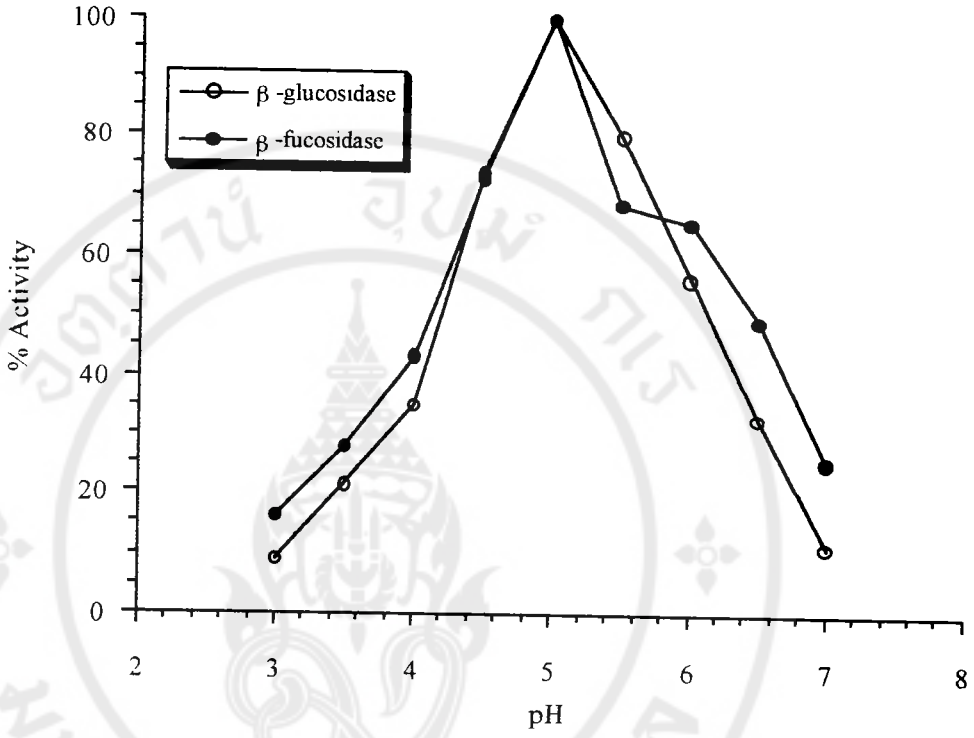

Figure 3-3 The pH profile of crude enzyme immobilised on celite. The reaction mixture was incubated in 100 mM McIlvaine buffer at various pHs (3.5 - 7.0) and contained 1 mM pNP-β-glucopyranoside and pNP-β-fucopyranoside. The reaction was performed for 10 min at 30°C before stopping the reaction with 2 M sodium carbonate.

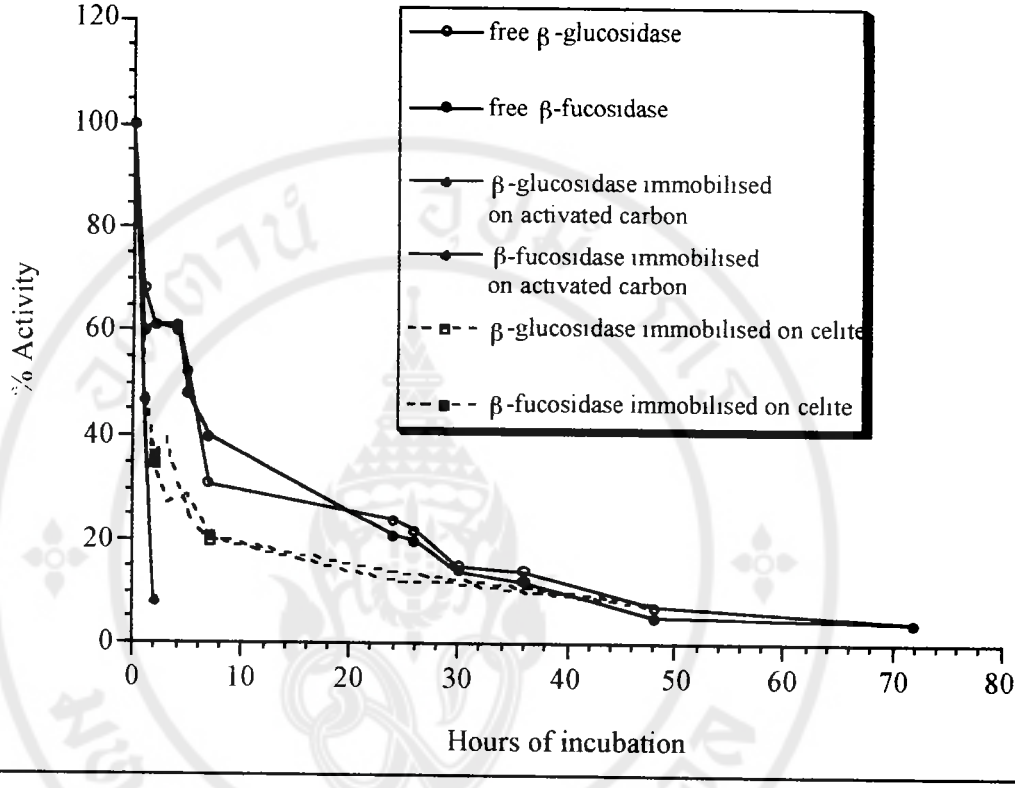

Figure 3-4 Heat stability of free and enzyme immobilised on activated carbon and celite. The enzymes were heated at 50°C for various times.

3.4 Kinetic studies

3.4.1 Michaelis-Menten kinetics of free crude enzyme and crude enzyme immobilised on activated carbon using p-nitrophenol-β-D-glucopyranoside as substrate

β-Glucosidase activities were determined at a range of 1.0 - 20.0 mM of pNP-β-D-glucopyranoside dissolved in distilled water, as described in section 2.2.9.3.1.1. The Michaelis-Menten curve and Lineweaver-Burk graphs drawn using the Enzfit program are shown in Figure 3-5 a, b and 3-6 a, b. The K_m calculated by non-linear regression was 5.47 ± 0.4 mM for free crude enzyme and 4.57 ± 0.03 mM for crude enzyme immobilised on activated carbon.

3.4.2 Michaelis-Menten kinetics of crude enzyme and crude enzyme immobilised on activated carbon with p-nitrophenol-β-D-fucopyranoside

β-Fucosidase activities were determined at a range of 0.1 - 2.0 mM of pNP-β-D-fucopyranoside dissolved in distilled water, as described in section 2.2.9.3.1.2. The Michaelis-Menten curve and Lineweaver-Burk graphs drawn using the Enzfit program are shown in Figure 3-7 a, b and 3-8 a, b. The K_m calculated by non-linear regression was 0.73 ± 0.05 mM for free crude enzyme and 0.94 ± 0.08 mM for crude enzyme immobilised on activated carbon.

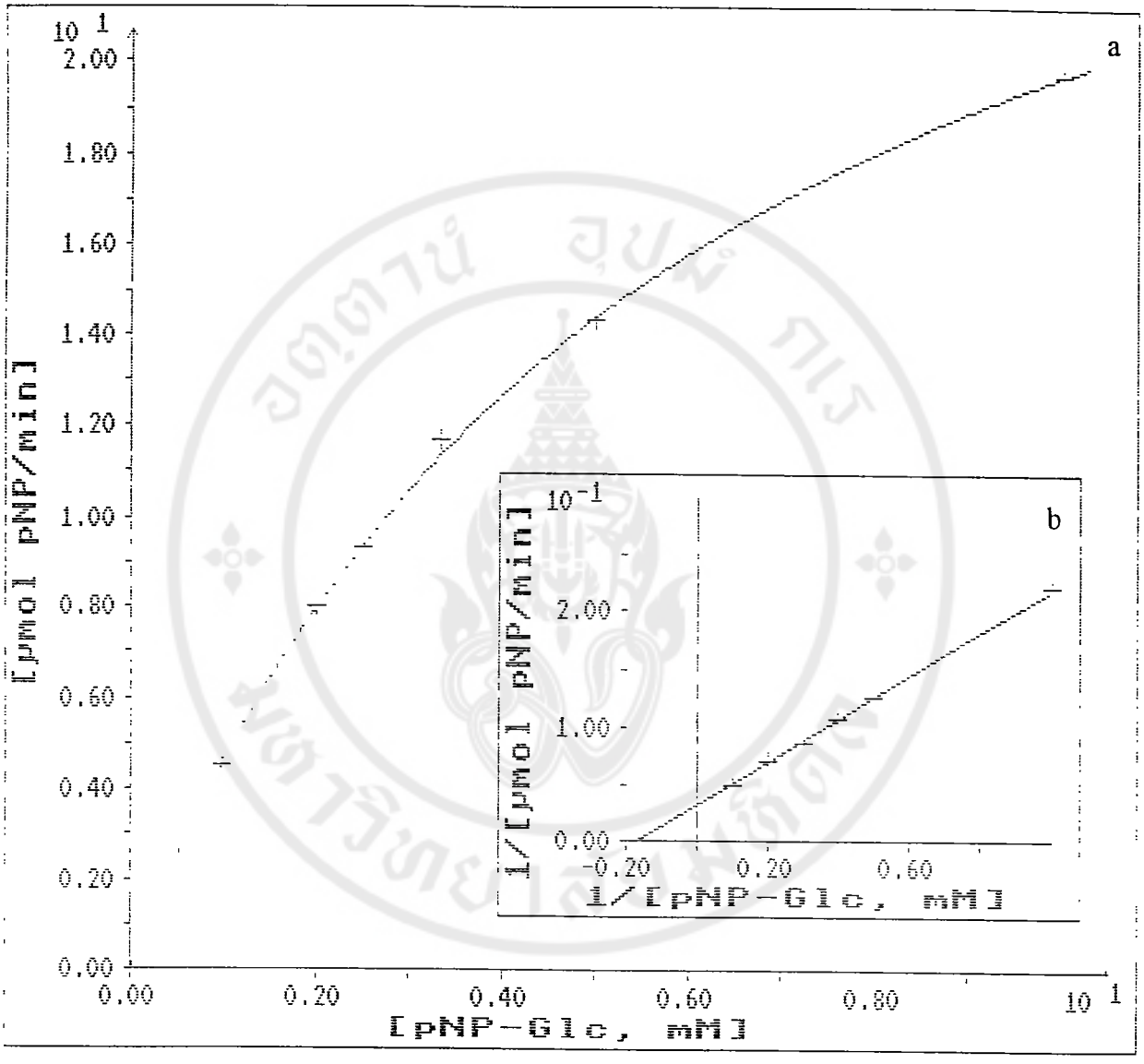

Figure 3-5 Kinetics studies of crude free β-glucosidase/β-fucosidase enzyme with pNP-β-D-glucopyranoside. a. Michaelis-Menten plot of β-glucosidase/β-fucosidase with pNP-β-D-glucopyranoside. b. Lineweaver-Burk plot of β-glucosidase/β-fucosidase with pNP-β-D-glucopyranoside.

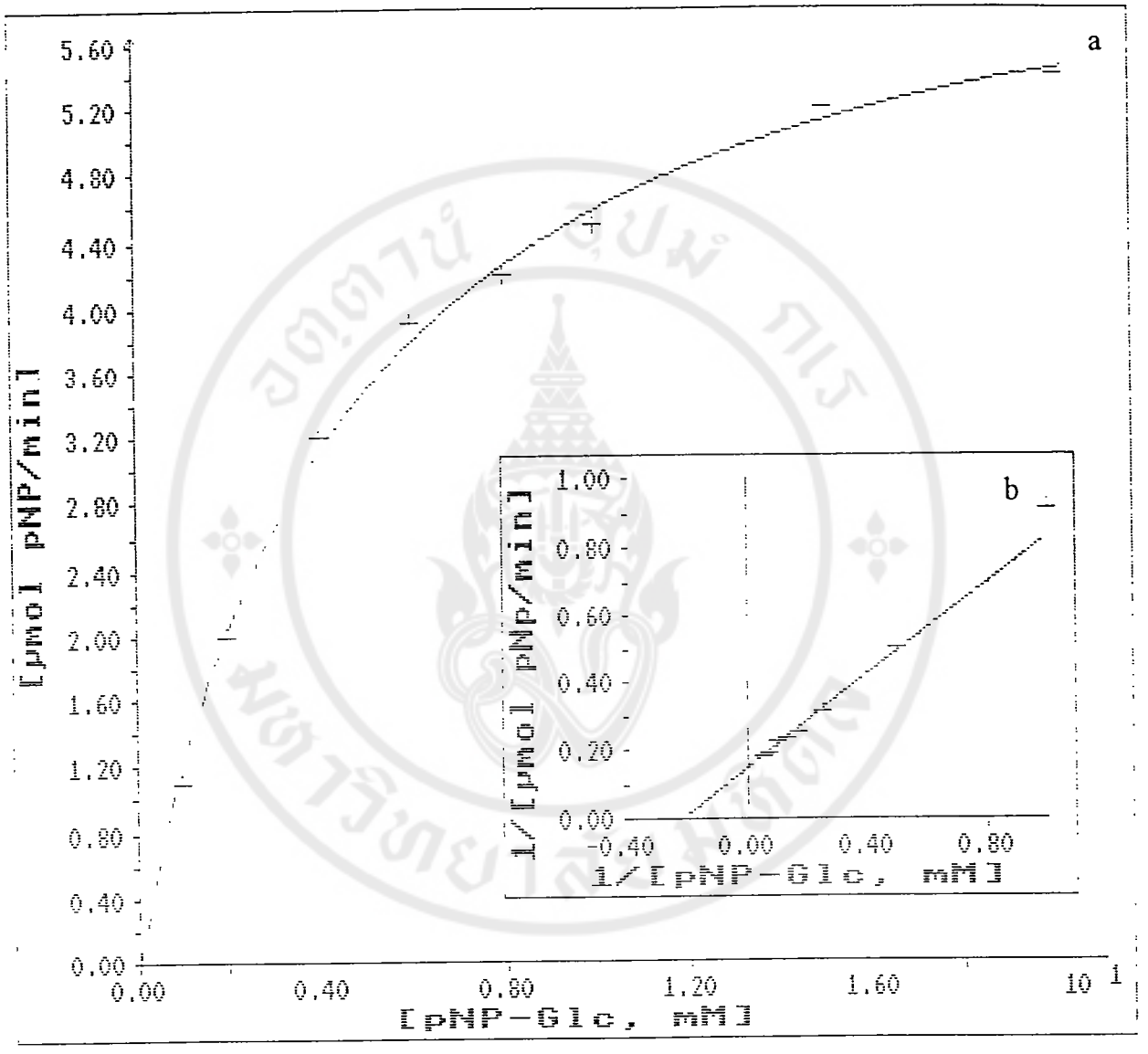

Figure 3-6 Kinetics studies of immobilised crude β-glucosidase/β-fucosidase with pNP-β-D-glucopyranoside. a. Michaelis-Menten plot of β-glucosidase/β-fucosidase with pNP-β-D-glucopyranoside. b Lineweaver-Burk plot of β-glucosidase/β-fucosidase with pNP-β-D-glucopyranoside.

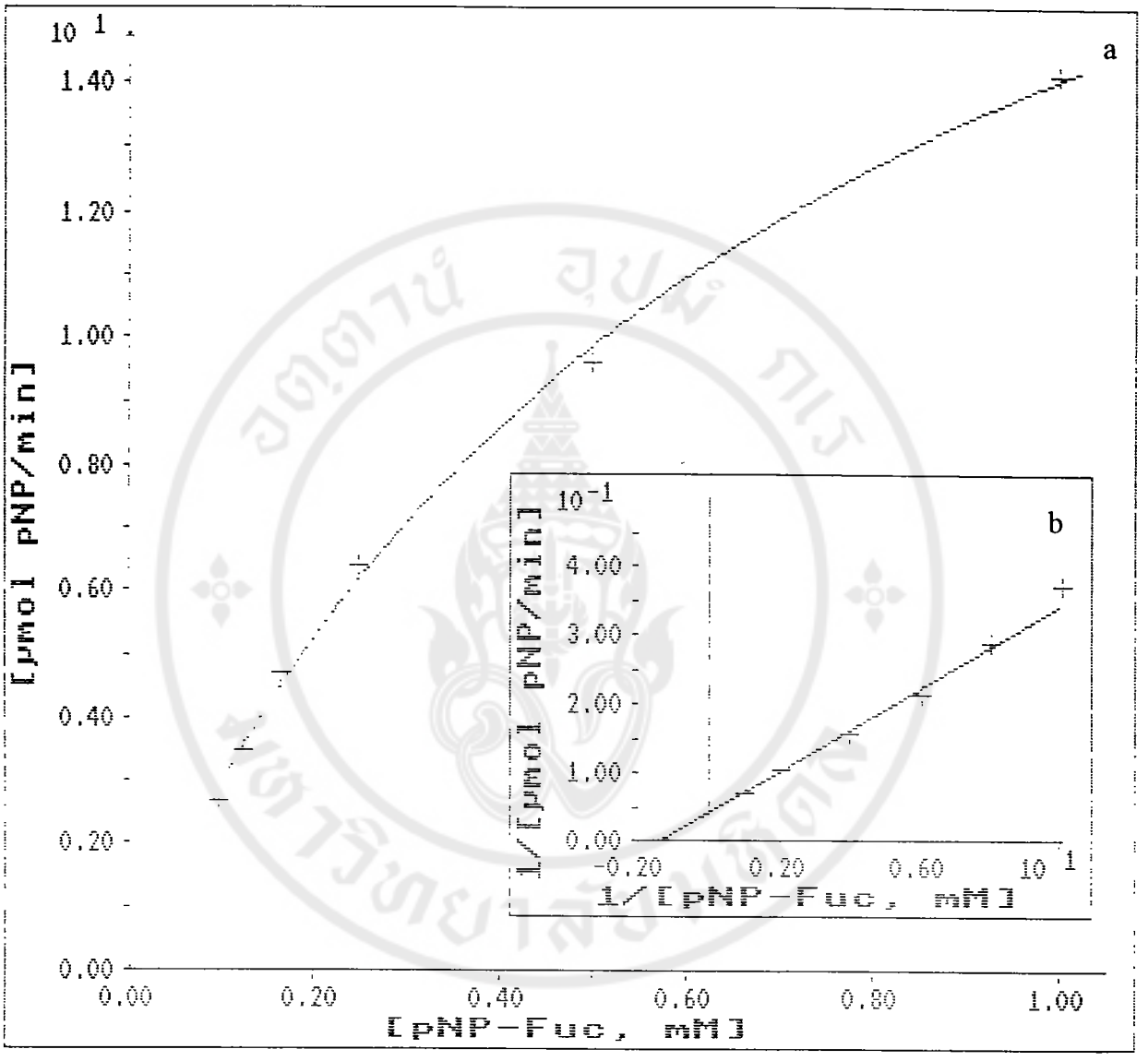

Figure 3-7 Kinetics studies of crude free β-glucosidase/β-fucosidase enzyme with pNP-β-D-fucopyranoside a. Michaelis-Menten plot of β-glucosidase/β-fucosidase with pNP-β-D-fucopyranoside b. Lineweaver-Burk plot of β-glucosidase/β-fucosidase with pNP-β-D-fucopyranoside.

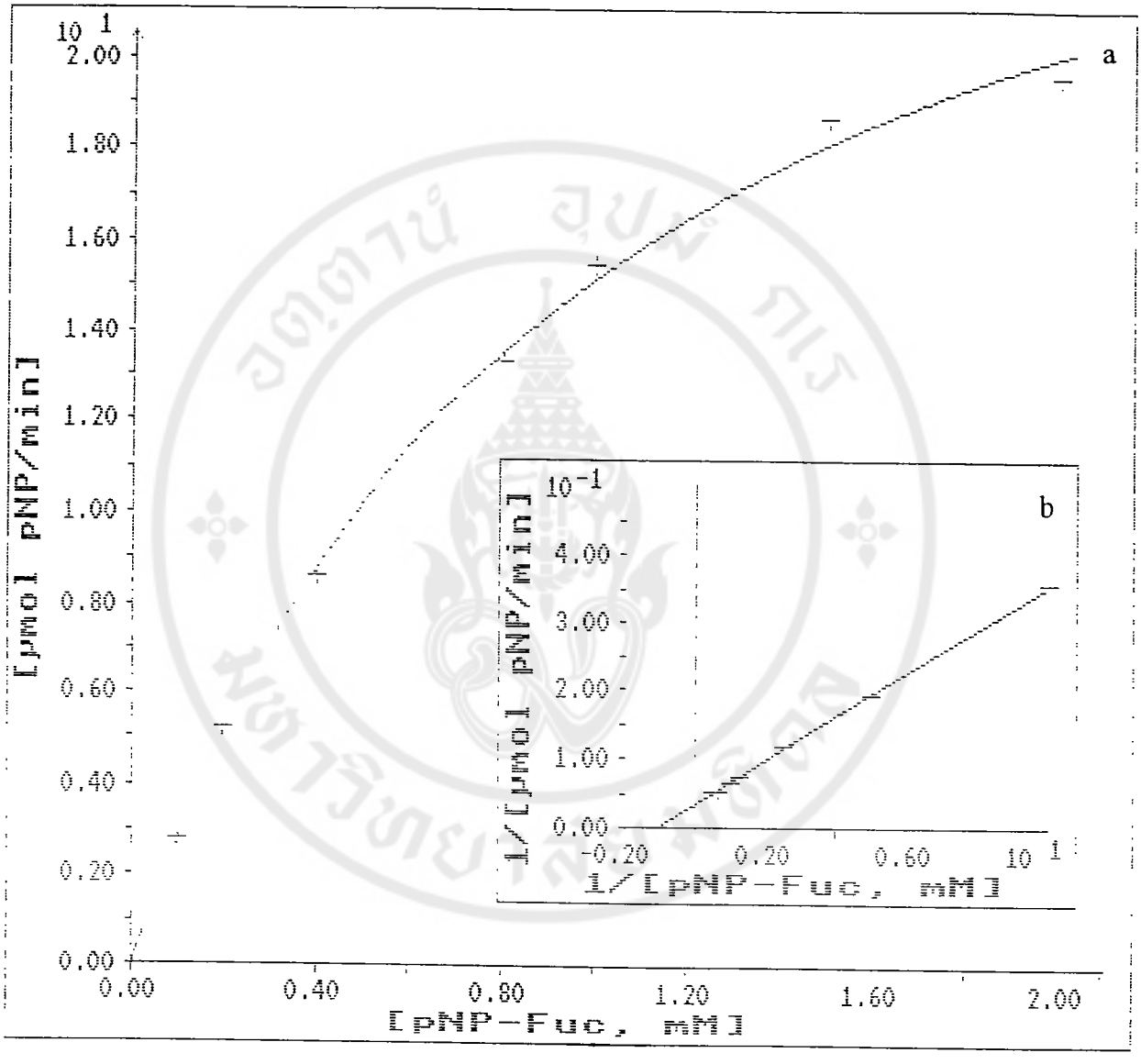

Figure 3-8 Kinetics studies of immobilised crude β-glucosidase/β-fucosidase with pNP-β-D-fucopyranoside. a Michaelis-Menten plot of β-glucosidase/β-fucosidase with pNP-β-D-fucopyranoside. b. Lineweaver-Burk plot of β-glucosidase/β-fucosidase with pNP-β-D-fucopyranoside.

3.5 Oligosaccharide synthesis

3.5.1 Time course of oligosaccharide synthesis by free and immobilised enzymes

Varying concentrations (0.1, 0.2 and 0.4 U) of β-glucosidase were incubated at 50°C with 50% (w/w) glucose with the total reaction weight of 100 mg for 8 days. Aliquots of 0.03 ml were taken everyday and enzyme was heat-killed. Five µl samples at each day of synthesis were subjected to h.p.l.c. analysis with the results shown in Figure 3-9 and 3-10.

The products of synthesis as analysed by h.p.l.c. showed that the major product was disaccharide for both free and immobilised enzymes. The fastest reaction rate was obtained when using 0.4 U of enzyme in the synthesis reaction with equilibrium being reached faster than using 0.1 and 0.2 U of enzyme. Oligosaccharide products synthesised by 0.1 U free enzyme were obtained in the lowest yield, while that by 0.2 U enzyme gave the highest yields. With immobilised enzyme, 0.4 U enzyme gave highest yield of products among the 3 concentrations used.

H.p.l.c. chromatogram of 3 concentrations of both free and immobilised enzyme being used are similar but showed differences in the amounts of products. Figure 3-13 is a representative chromatogram of all 3 concentrations of free enzyme, while Figure 3-15 is a representative chromatogram for immobilised enzyme.

The results from thin-layer chromatography show the main synthesised product was gentiobiose. The patterns of thin-layer chromatogram are the same for all concentrations of enzyme without noticeable changes in amount of products. Figure 3-12 is the thin-layer

chromatogram of a time course of synthesis by free enzyme and Figure 3-14 is the time course for immobilised enzyme.

Figure 3-16 is the thin-layer chromatogram of synthesis by 0.1 U crude free enzyme during the first 8 h of synthesis and Figure 3-17 is for 0.1 U immobilised enzyme. The results show that the first and major product being synthesised was gentiobiose for both free and immobilised enzyme. However, it is possible that small amounts of sophorose and laminaribiose were produced but were not detectable due to similarity in mobility to the large spot of glucose.

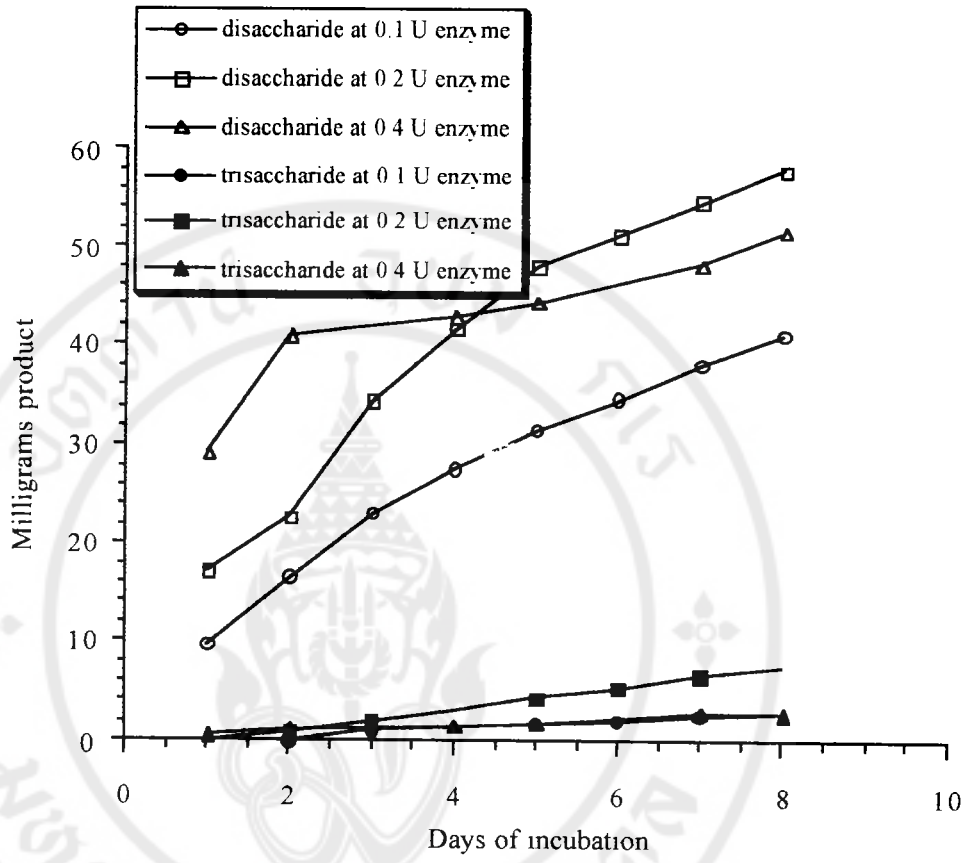

Figure 3-9 Time course of oligosaccharide synthesis at 50°C by various concentrations (0.1, 0.2 and 0.4 U/100 mg) of free enzyme with 50% (w/w) glucose as substrate.

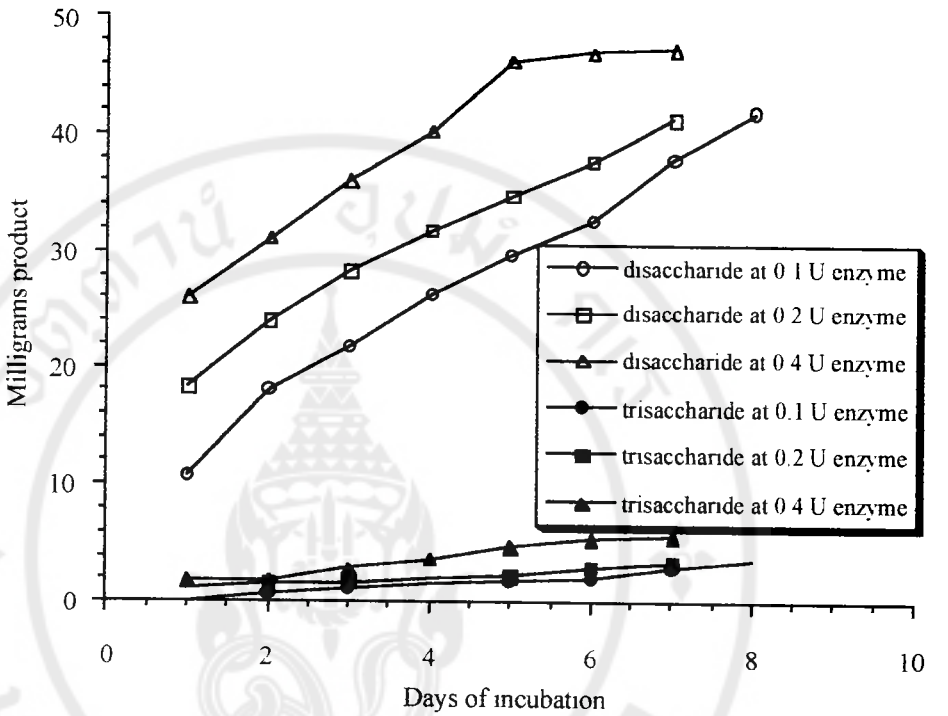

Figure 3-10 Time course of oligosaccharide synthesis by enzyme immobilised on activated carbon. Varying concentrations (0.1, 0.2 and 0.4 U/100 mg) of enzyme were used in the presence of 50% (w/w) glucose as substrate at 50°C.

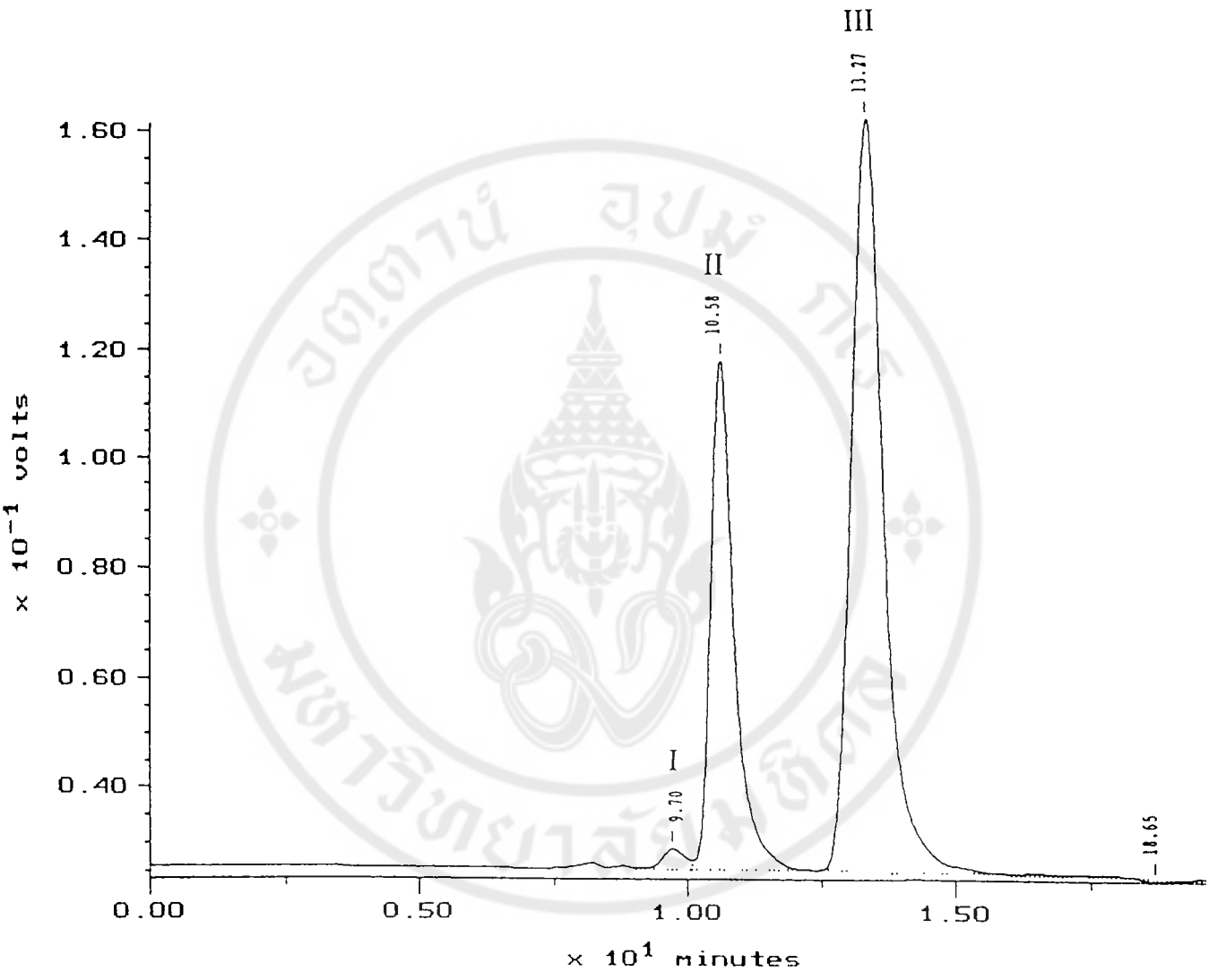

Figure 3-11 H.p.l.c. chromatogram of standards for oligosaccharide products from the synthesis reaction. (I = laminaritriose, II = gentiobiose, III = glucose)

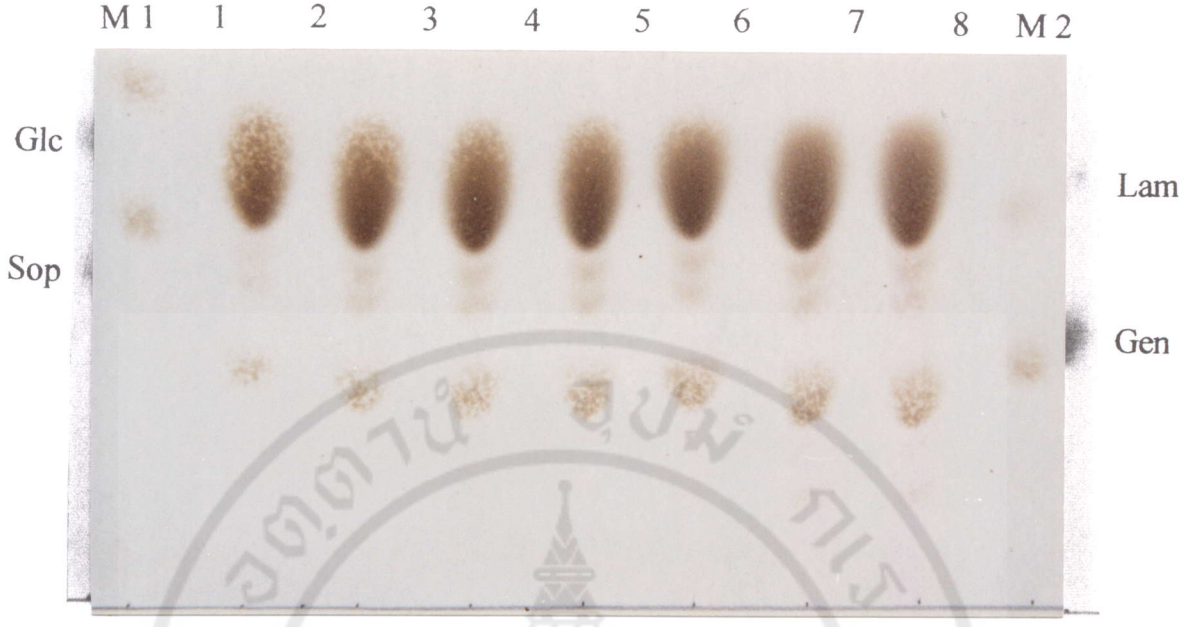

Figure 3-12 Chromatogram of thin-layer chromatography of oligosaccharide products from the synthesis by 0.2 U of crude free enzyme with 50% (w/w) glucose as substrate in the total weight of 100 mg. Each sample was diluted 20 times and loaded for 2 μl.

Lane M 1 = markers: 4 μg glucose (Glc)+ 4 μg sophorose (Sop)
1 = products at day 1 of synthesis
2 = products at day 2 of synthesis
3 = products at day 3 of synthesis
4 = products at day 4 of synthesis
5 = products at day 5 of synthesis
6 = products at day 6 of synthesis
7 = products at day 7 of synthesis
8 = products at day 8 of synthesis
M 2 = markers: 4 μg laminaribiose (Lam) + 4 μg gentiobiose (Gen)

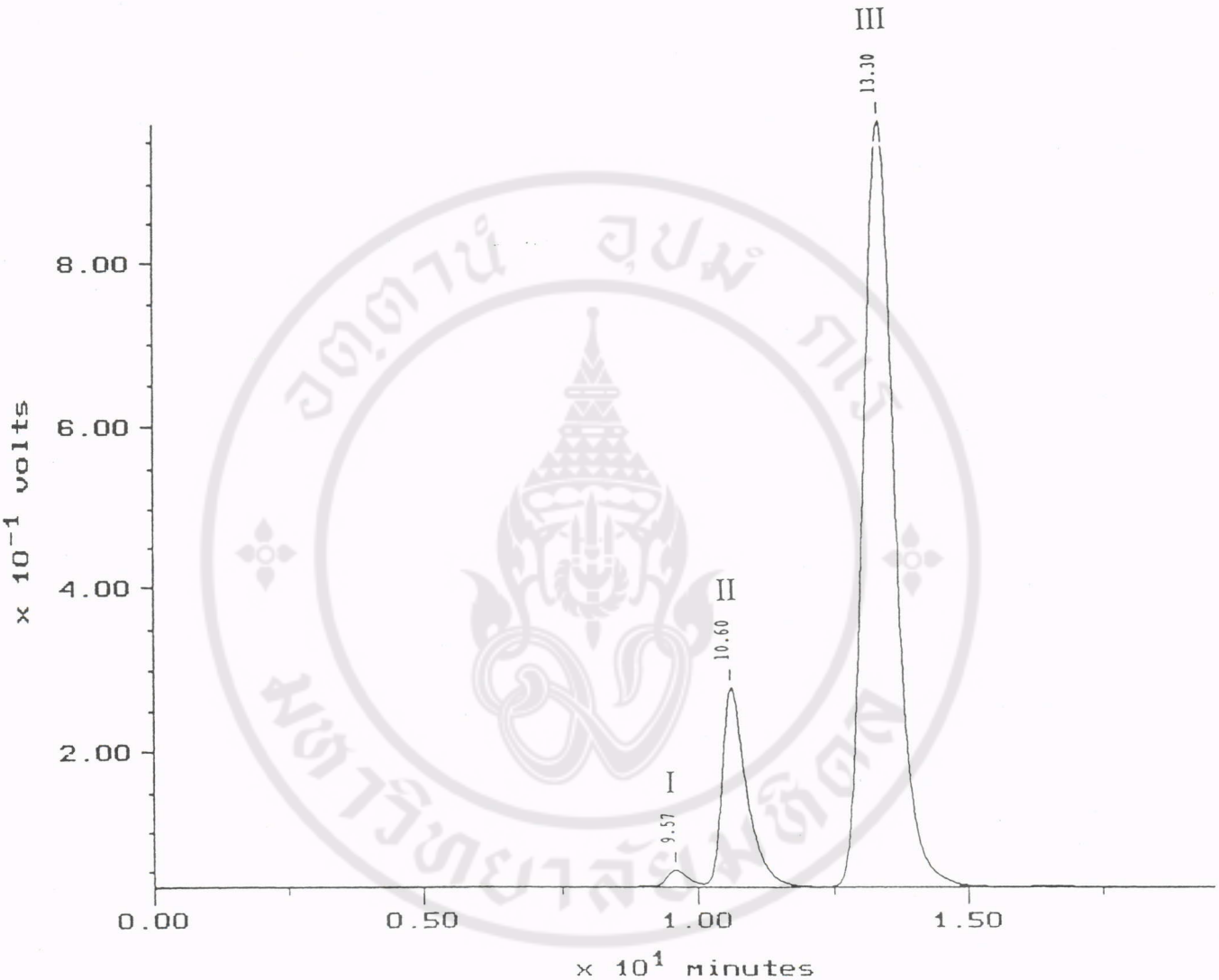

Figure 3-13 Representative h.p.l.c. chromatogram of time course of synthesis at day 4 by 0.2 U crude enzyme with 50% (w/w) glucose as substrate in the total weight of 100 mg. (I = trisaccharide, II = disaccharide, III = monosaccharide)

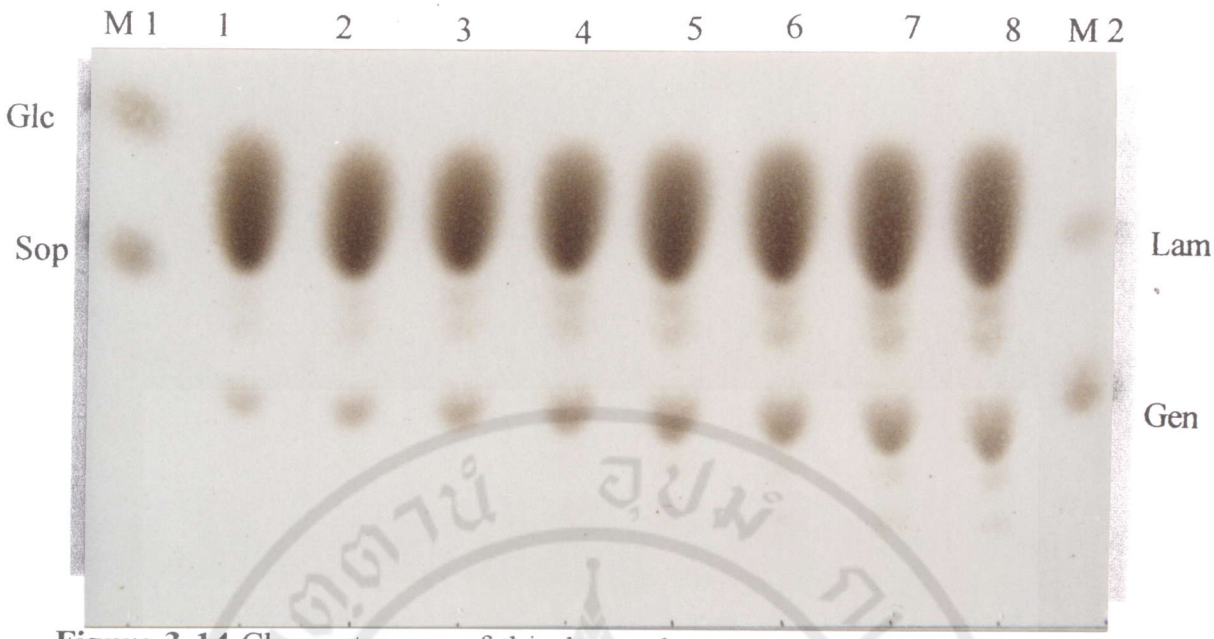

Figure 3-14 Chromatogram of thin-layer chromatography of oligosaccharide products from the synthesis by 0.2 U of immobilised enzyme with 50% (w/w) glucose as substrate in the total weight of 100 mg. Each sample was diluted 20 times and loaded for 2 μl.

Lane M 1 = markers: 4 μg glucose (Glc) + 4 μg sophorose (Sop)

1 = products at day 1 of synthesis

2 = products at day 2 of synthesis

3 = products at day 3 of synthesis

4 = products at day 4 of synthesis

5 = products at day 5 of synthesis

6 = products at day 6 of synthesis

7 = products at day 7 of synthesis

8 = products at day 8 of synthesis

M 2 = markers: 4 μg laminaribiose (Lam) + 4 μg gentiobiose (Gen)

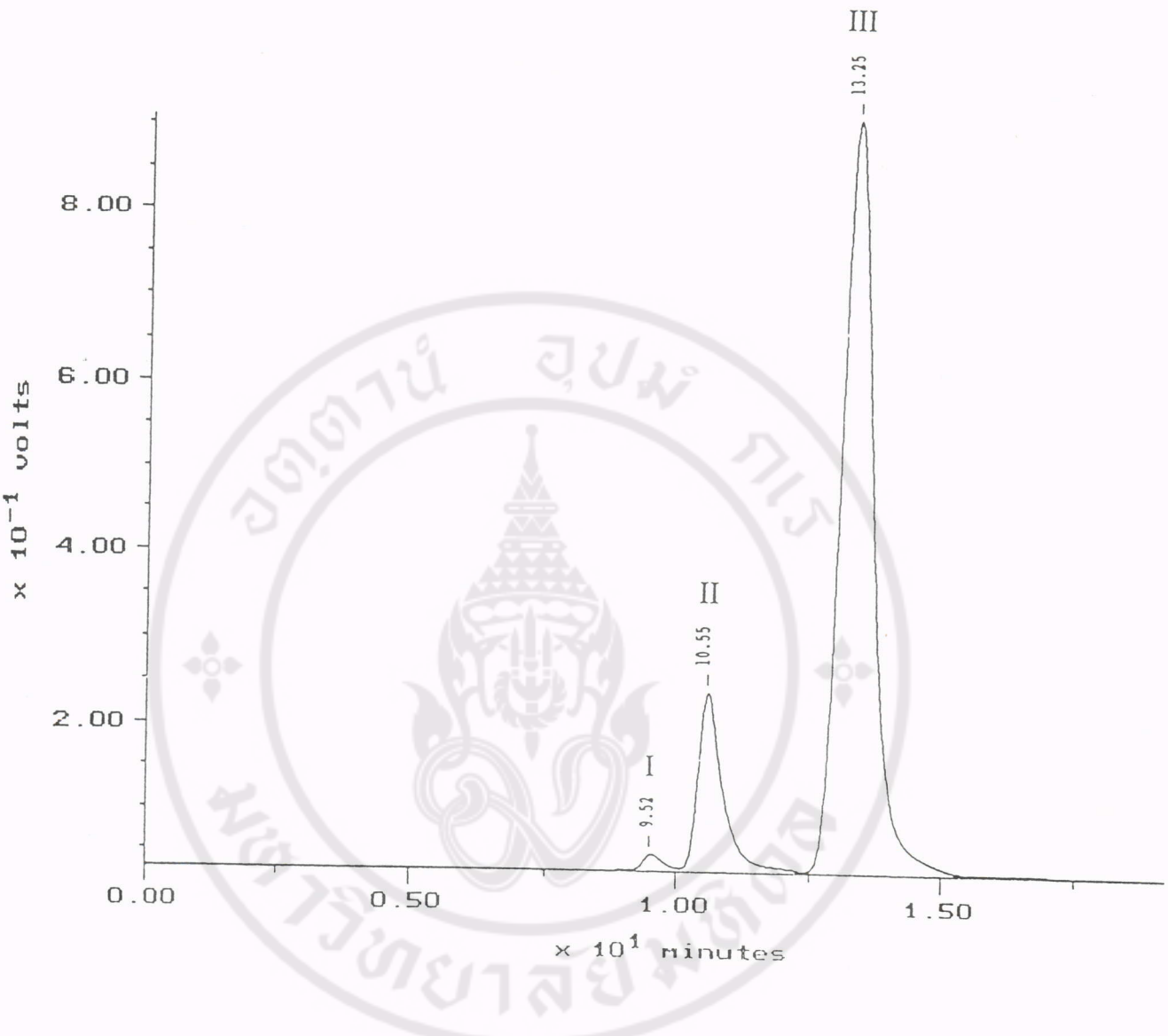

Figure 3-15 Representative h.p.l.c. chromatogram of time course of synthesis at day 6 by 0.2 U immobilised enzyme with 50% (w/w) glucose as substrate in the total weight of 100 mg. (I = trisaccharide, II = disaccharide, III = monosaccharide)

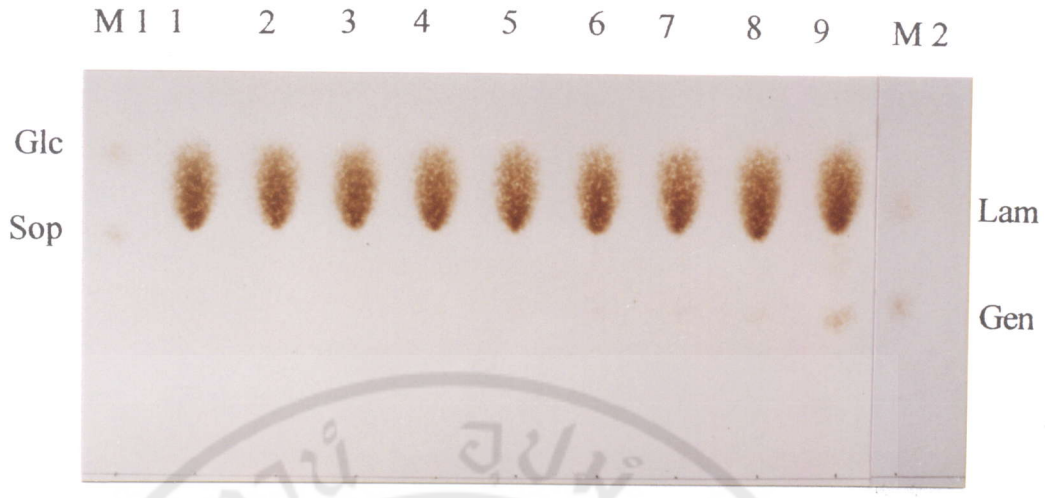

Figure 3-16 Thin-layer chromatogram of oligosaccharide products synthesised by 0.1 U crude free enzyme in the first 8 h of synthesis with 50% (w/w) glucose as substrate with the total weight of 100 mg. Each sample was diluted 20 times and loaded for 2 µl.

Lane M 1 = markers: 4 µg glucose (Glc) + 4 µg sophorose (sop)

1 = products at the zero hour of synthesis

2 = products at the first hour of synthesis

3 = products at the 2nd hour of synthesis

4 = products at the 3rd hour of synthesis

5 = products at the 4th hour of synthesis

6 = products at the 5th hour of synthesis

7 = products at the 6th hour of synthesis

8 = products at the 7th hour of synthesis

9 = products at the 8th hour of synthesis

M 2 = markers: 4 µg laminaribiose (Lam) + 4µg gentiobiose (Gen)

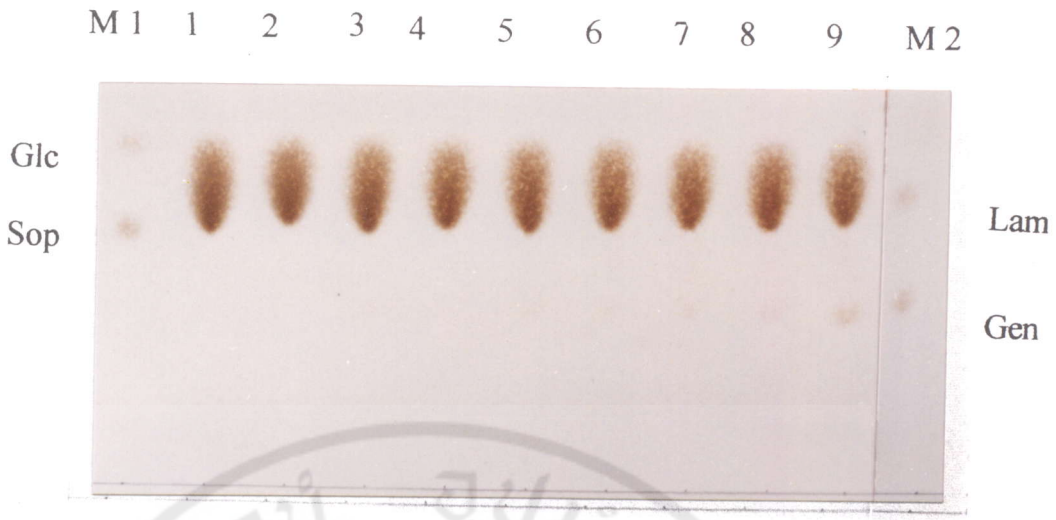

Figure 3-17 Thin-layer chromatogram of oligosaccharide products synthesised by 0.1 U immobilised enzyme in the first 8 h of synthesis with 50% (w/w) glucose as substrate in the total weight of 100 mg. Each sample was diluted 20 times and loaded for 2 µl.

Lane M 1 = markers: 4 µg glucose (Glc) + 4 µg sophorose (Sop)

1 = products at the zero hour of synthesis

2 = products at the first hour of synthesis

3 = products at the 2nd hour of synthesis

4 = products at the 3rd hour of synthesis

5 = products at the 4th hour of synthesis

6 = products at the 5th hour of synthesis

7 = products at the 6th hour of synthesis

8 = products at the 7th hour of synthesis

9 = products at the 8th hour of synthesis

M 2 = markers: 4 µg laminaribiose (Lam) + 4 µg gentiobiose (Gen)

3.5.2 Re-usability of enzyme immobilised on activated carbon in oligosaccharide synthesis

Immobilised enzyme of 0.2 U was used to synthesise oligosaccharide in the presence of 50% (w/w) glucose as substrate in a total reaction weight of 100 mg. The synthesis was performed for 5 days at 50°C and then immobilised enzyme was centrifuged to remove oligosaccharide products from the supernatant. Another 50% (w/w) glucose was added to the immobilised enzyme in the same volume as that taken, and incubation was performed for another 5 days. The reaction was centrifuged again to give immobilised enzyme and the supernatant containing products of the second usage of enzyme. Another volume of 50% (w/w) glucose was added to the immobilised enzyme, followed by incubation at 50°C for another 5 days. Centrifugation yielded the products of the third usage in the supernatant plus the immobilised enzyme bound to the activated carbon. The remaining activated carbon was then washed successively with distilled water (twice), 5% ethanol and 10% ethanol to remove bound sugars.

Free enzyme of 0.2 U was also incubated with 50% (w/w) glucose at 50°C for 15 days to compare with the products obtained by 3 uses of immobilised enzyme.

Figure 3-19 shows products from the 15th day of synthesis by free enzyme. To quantitate the amount of products obtained, peak I and II were analysed as higher oligosaccharide, peak III as disaccharide and peak IV as glucose substrate. The profile shows separation of disaccharides and higher oligosaccharides. Disaccharides were the major products synthesised. Figure 3-20 shows products from the 5th day of synthesis by immobilised enzyme in the first usage. Peak I was quantitated as trisaccharide, peak II as

disaccharide and peak I as glucose substrate. The results were similar to that obtained by free enzyme but shows no detectable oligosaccharide higher than trisaccharide formed. The major products obtained were also disaccharide in comparable amounts to free enzyme. All 3 fractions of products synthesised by immobilised enzyme are of the same pattern. Figure 3-20 is a representative h.p.l.c. chromatogram of products from the re-use of immobilised enzyme.

Table 3-7 shows the comparison between products from synthesis by free enzyme and the 3 fractions obtained with re-used immobilised enzyme. Analysis of products by h.p.l.c. shows no dramatic difference in amount of products between free enzyme and the 3 fractions obtained from re-used immobilised enzyme. The total disaccharide products from 3 combined fractions of immobilised enzyme were higher than with free enzyme, but the trisaccharide contents are comparable.

Figure 3-18 shows thin-layer chromatogram of products from synthesis by free enzyme compared with products from 3 fractions obtained by immobilised enzyme. There are no detectable difference of products found among free and immobilised enzyme with gentiobiose gentiobiose (glucose β1-6 glucose) as the major product.

After 15 days of synthesis by immobilised enzyme, the support containing enzyme was washed twice with distilled water and the adsorbed products were eluted by 5% ethanol followed by 10% ethanol. The results (Figure 3-21) show detectable amounts of disaccharide and higher oligosaccharide products eluted from the activated carbon. Figure 3-22 shows h.p.l.c. chromatogram of the products eluted by 5% ethanol, with profile from 10% ethanol elution being the same.

Table 3-7 Products from the synthesis at day 15 by 0.2 U crude enzyme compared with re-used products from the synthesis by 0.2 U crude immobilised enzyme.

Sample	Products	
	Disaccharide (mg)	Trisaccharide (mg)
Products from the synthesis by free enzyme	55.47	10.06
Products from the 1st use immobilised enzyme	43.31	4.31
Products from the 2nd use of immobilised enzyme	33.51	3.44
Products from the 3rd use of immobilised enzyme	35.94	4.15
Total products from the synthesis with immobilised enzyme	112.76	11.9

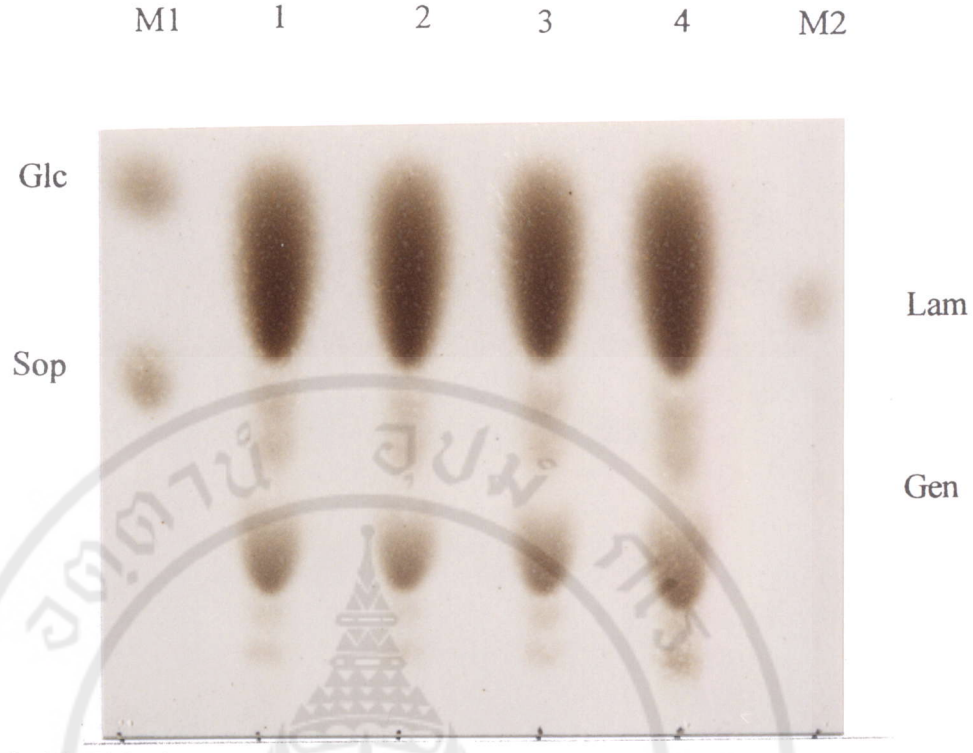

Figure 3-18 Thin-layer chromatogram of synthetic products by 0.2 U crude free enzyme compared with synthetic products by 3 fractions obtained from 0.2 U immobilised enzyme. Each sample was diluted 20 times and loaded for 2 µl.

Lane M1 = markers: 4 µg glucose (Glc) + 4 µg sophorose (Sop)

1 = synthetic products at day 15 by free enzyme

2 = synthetic products from 1st use of immobilised enzyme

3 = synthetic products from 2nd use of immobilised enzyme

4 = synthetic products from 3rd use of immobilised enzyme

M 2 = markers: 4 µg laminaribiose (lam) + 4µg gentiobiose (Gen)

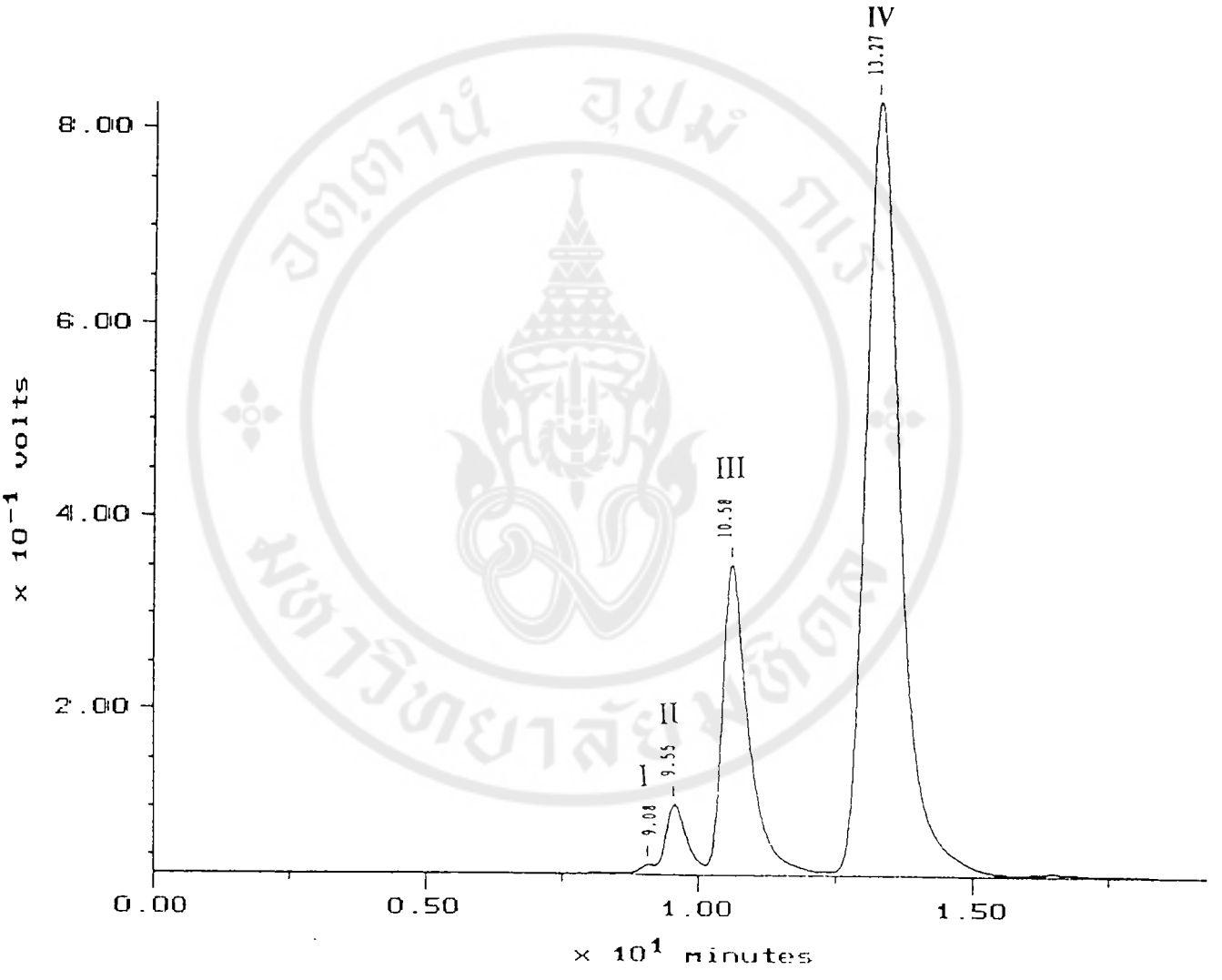

Figure 3-19 H.p.l.c chromatogram of synthetic products by 0.2 U free crude enzyme at day 15 of incubation at 50°C with 50% (w/w) glucose as substrate (I = higher oligosaccharide, II = trisaccharide, III =disaccharide, IV = monosaccharide)

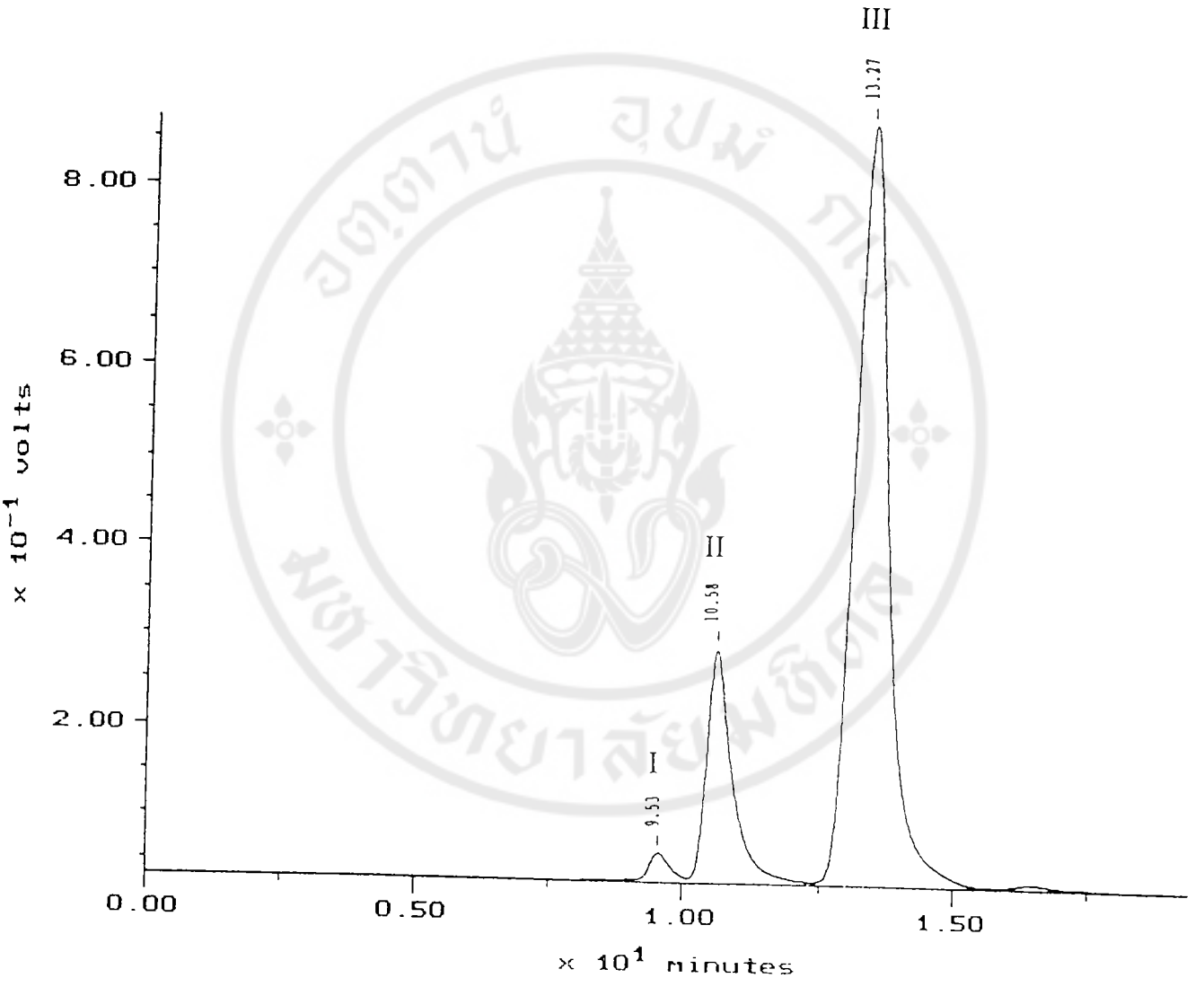

Figure 3-20 H p.l.c chromatogram of products from day 5 of synthesis by 0 2 U immobilised enzyme in the first usage (I = trisaccharide, II disaccharide, III = monosaccharide).

Figure 3-21 Thin-layer chromatogram of products of synthesis eluted from activated carbon after the reaction by 0.2 U immobilised enzyme.

Lane M1 = markers: 4μg glucose (Glc) + 4 μg sophorose (Sop)

1 = 5 μl products from elution by 5% ethanol

2 = 5 μl products from elution by 10% ethanol (2-fold diluted, loading volume: 2 μl)

M 2 = markers: 4μg laminaribiose (Lam) + 4 μg gentiobiose (Gen)

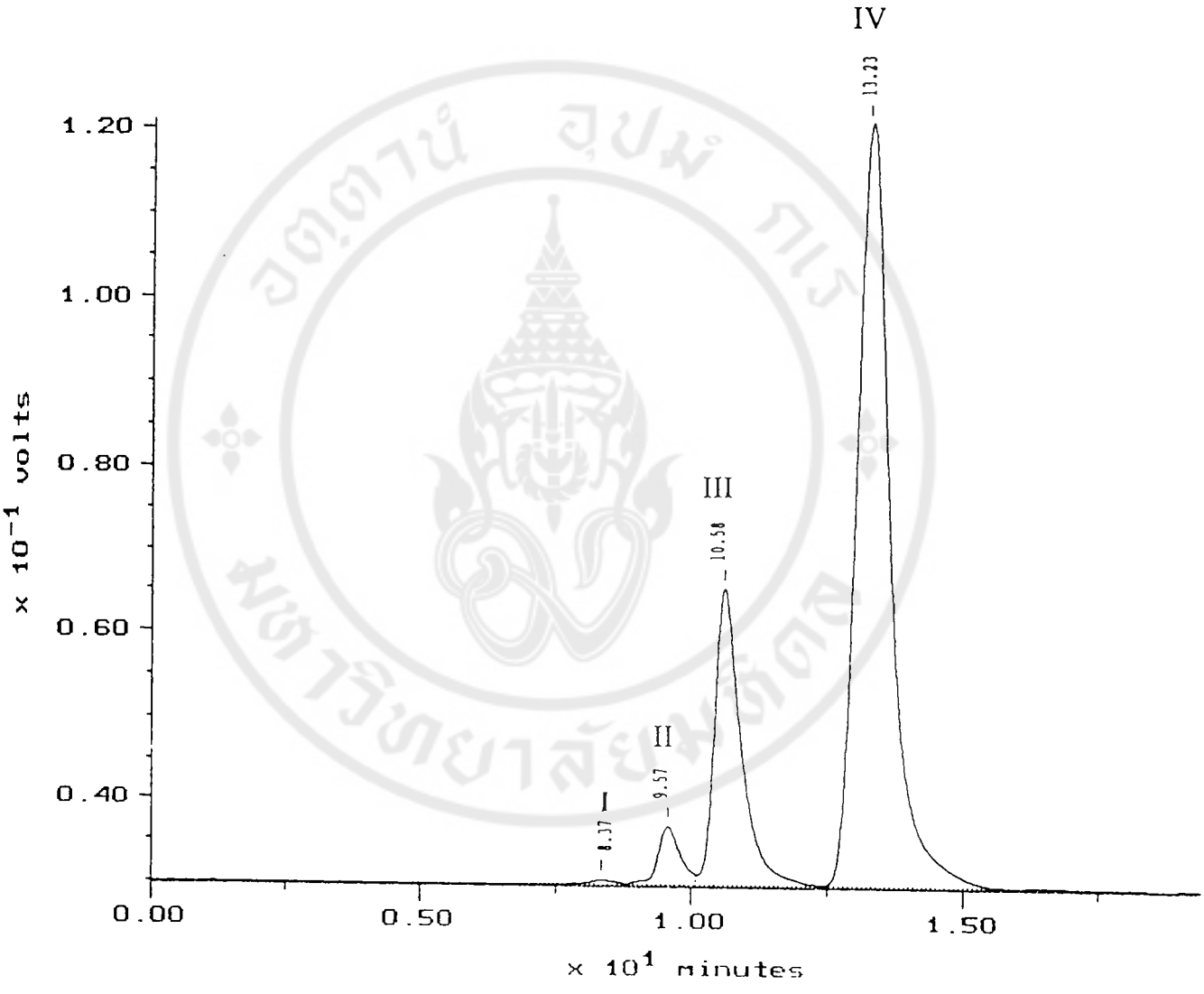

Figure 3-22 H.p.l.c. chromatogram of products of synthesis by 0.2 U immobilised enzyme obtained by eluting the activated carbon with 5% ethanol (I = trisaccharide, II = disaccharide, III = monosaccharide).

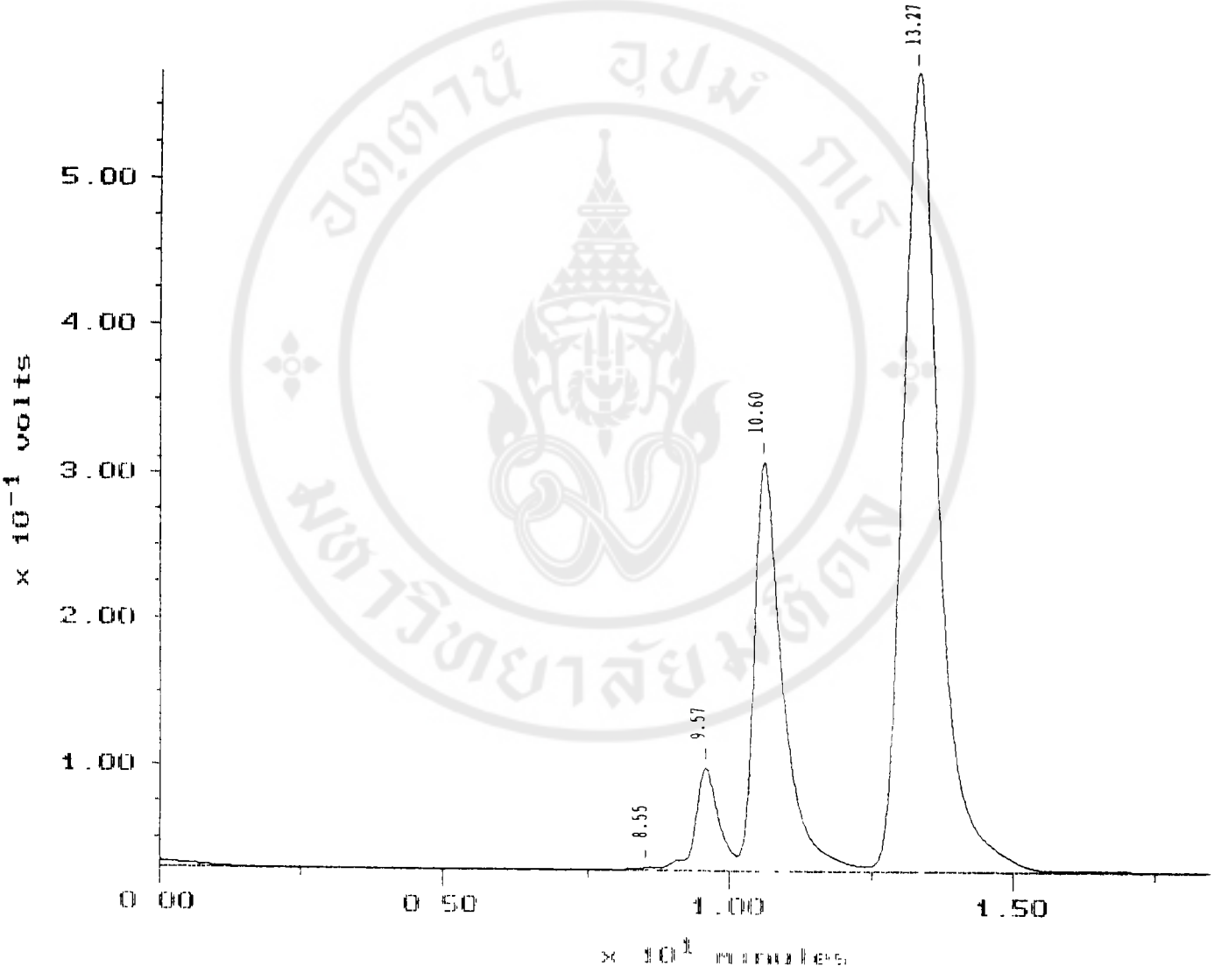

Figure 3-23 H.p l c chromatogram of products of synthesis by 0 2 U immobilised enzyme obtained by eluting the activated carbon with 10% ethanol (I = trisaccharide, II = disaccharide, III = monosaccharide).

3.5.3 Identification of oligosaccharide products on thin-layer chromatography

Since a large amount of glucose was present in the reaction mixture so disaccharide products from the synthesis, when detected by thin-layer chromatography, move more slowly and did not correspond to the loaded markers. Four disaccharide markers (glucose, sophorose, laminaribiose and gentiobiose) were loaded together with the sample to be detected with the results as shown in Figure 3-23. The results show that glucose appeared as the main product at the top of the plate. Laminaribiose has slower mobility than glucose and faster than sophorose and gentiobiose which is the slowest. The major product was therefore gentiobiose, with smaller amounts of sophorose and laminaribiose being formed.

Figure 3-24 Thin-layer chromatogram of product identification in thin-layer chromatography. Each sample was diluted 20 times and loaded for 2 µl.

Lane M 1 = 4 µg glucose

M 2 = 4 µg sophorose

1 = synthetic products

2 = synthetic products + 4 µg glucose

3 = synthetic products + 4 µg sophorose

4 = synthetic products + 4 µg laminaribiose

5 = synthetic products + 4 µg gentiobiose

M 3 = 4 µg laminaribiose

M 4 = 4 µg gentiobiose

CHAPTER IV
DISCUSSION

4.1 Preparation of crude β-glucosidase/β-fucosidase

Ammonium sulphate precipitation was the first step in purifying the enzyme and it was found to be an excellent method for removing a large amount of undesired proteins, while enzymes of interest were still quantitatively recovered in the 35-75% fraction.

4.2 Immobilisation of enzyme by activated carbon, celite, and cross-linkage with BSA by glutaraldehyde.

Maximum loading of crude enzyme immobilised on activated carbon and celite were 0.4 U/12.5 mg and 0.1 U/12.5 mg, with 50 and 53 percent immobilisation, respectively. While cross-linking 20 U of β-glucosidase with 5% BSA yielded the highest percent immobilisation of 13.5%. From these results, activated carbon seemed to be the best support to immobilise enzyme, since it could immobilise higher activity of enzyme for an equal amount of support compared to celite, yielded higher percent immobilisation, and used lower concentration of enzyme than cross-linking with BSA by glutaraldehyde. Moreover, immobilisation of enzyme on activated carbon also gave higher percent recovery yield than cross-linkage with BSA by glutaraldehyde.

By comparing the two adsorbents, the surface of activated carbon may be more active than celite since activated carbon was prepared by dehydration, carbonisation followed by activation by organic solvents. The steps of activated carbon preparation may make its surface become more readily available for any molecule to adsorb to than celite. While the components of celite are mainly silica and aluminum (49), its surface is less active than activated carbon since the physical force involved in the adsorption is mainly ionic binding.

When glutaraldehyde was added, cross-linking between the enzyme and BSA was observed. The added BSA was considered as the insoluble supporting matrix of the enzyme (39). By varying the amounts of BSA used in cross-linking with enzyme by 0.3% glutaraldehyde, 5% (w/v) BSA gave the highest percent immobilisation. Five percent (w/v) BSA and 0.3% glutaraldehyde may be the best ratio to make the enzyme insoluble. BSA was added into the reaction to act as a linker arm in order to increase the accessibility of substrate to enzyme.

The amount of activity of β-glucosidase from *Aspergillus phoenicis* immobilised on chitosan by glutaraldehyde varied from 1 to 10%, depending on the enzyme loading used (50). Less than 3% of the initial activity was found in the washings. Free enzyme treated with glutaraldehyde under the same conditions did not lose activity. When immobilisation was carried out in the presence of cellobiose, activity retention was somewhat greater.

The two methods of immobilising enzyme (adsorption and covalent bonding) did not improve the activities of the enzyme but the activities also decreased. This may be attributed to conformational changes in the enzyme structure or to steric hindrance, caused by the shielding effect of the matrix, that renders certain parts of the enzyme molecule less accessible to the

substrate, in the immediate vicinity of the enzyme molecules. It is well-established that the properties of the active sites of an enzyme molecule depend strongly on the 3-dimensional structure of the protein molecule. Thus, when an enzyme is adsorbed or covalently bound to a solid support, the interaction with the support may likely result in a modification of the enzyme conformation. Covalent bonds between the enzyme and the matrix or, in this case, between enzyme and BSA, or between enzyme molecules themselves, can stretch the whole molecule and thus alter the 3-dimensional structure of the active site. Fig 4-1 shows the effect of conformational changes and steric hindrances on enzyme behaviour. (46)

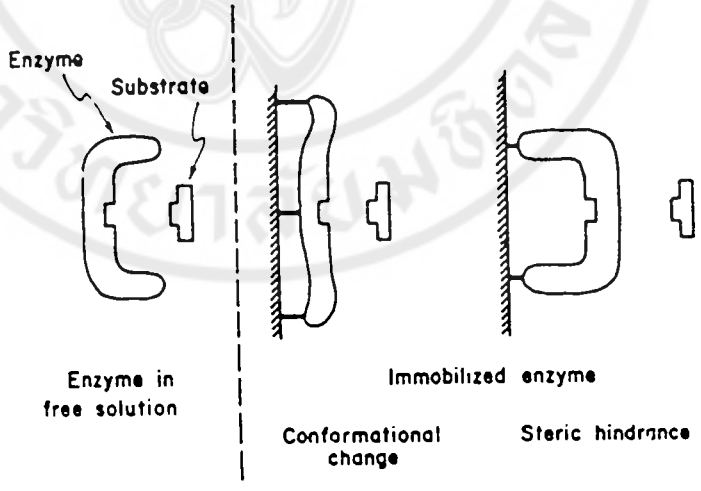

Figure 4-1 Schematic illustration of conformational changes and steric hindrance that may affect the behaviour of enzyme upon immobilisation (46).

4.3 Properties of crude enzyme compared with crude enzyme immobilised on activated carbon

4.3.1 Optimum pH of free and immobilised enzyme

pH optimum seems to be unchanged when enzyme is immobilised on activated carbon and celite when compared to free enzyme. This may be due to the microenvironment of immobilised enzyme itself, that does not differ from the macroenvironment or the bulk solution. The profile of enzyme immobilised on activated carbon is slightly broader than the free enzyme, suggesting that immobilised enzyme is less sensitive to pH change than the free enzyme which may be the result of immobilisation which restricts the conformation of enzyme to the support.

4.3.2 Temperature stability of free and immobilised enzyme.

Immobilised enzymes were less stable at 50°C than free enzyme, especially enzyme immobilised on activated carbon. This can be explained by the conformational changes upon immobilisation. Since the general principle of enzyme stabilisation consists of multipoint binding of the enzyme molecule to the support, this makes the conformation of the molecule becomes more rigid and more stable against unfolding and inactivation. But when multipoint binding is realised, it is only a small part of enzyme molecule surface that is bound to the support hence one should not expect that the whole of the molecule will become rigid (46). The addition of substrate or competitive

inhibitor may help stabilising the active site and heat stability may be improved.

4.3.3 Kinetics properties of free and immobilised enzymes

Kinetics studies showed that the K_m of free and immobilised β-glucosidase as determined by Michaelis-Menten and Lineweaver-Burk plots as being: 5.47 ± 0.4 mM for free enzyme and 4.57 ± 0.03 mM. K_m of β-fucosidase determined by the same way are 0.58 ± 0.06 mM for free enzyme and 0.94 ± 0.08 for immobilised enzyme.

Immobilisation of enzyme did not alter the affinity of enzyme to substrate as seen from the almost equal value of its K_ms. This may be due to the active site conformation was not affected much by immobilisation. A good shaking during the assay reaction may help reduce mass action effect so that products do not accumulate around the active site and made the substrate enable to access the enzyme.

β-Glucosidase from *Aspergillus* immobilised on chitosan also showed in change in K_m (49). K_m for cellobiose of free β-glucosidase appeared to be 0.8 mM cellobiose and of immobilised β-glucosidase was 3.7 mM. This may be explained by the rate of mass transfer of the substrate and products to and from immobilised enzymes, problems not found in free enzyme.

4.4 Oligosaccharide synthesis

4.4.1 Time course of oligosaccharide synthesis

For free enzyme, 0.1 U enzyme gave the slowest reaction rate, as seen from the slope in Figure 3-7, in the first few days of synthesis while 0.4 U enzyme gave the fastest. For disaccharide products, 0.1 U enzyme gave the lowest yield while 0.2 U enzyme gave the highest. For 0.4 U enzyme, its fastest reaction rate gave highest disaccharide products in the first few days so substrate was depleted more quickly. Free enzyme of 0.2 U was the most appropriate concentration since it gave a moderate rate of synthesis with highest yield of oligosaccharide at equilibrium.

For immobilised enzyme, as shown in Figure 3-18, all 3 concentrations of enzyme show gradual increase in oligosaccharide products with highest yield obtained from 0.4 U enzyme and the lowest from 0.1 U enzyme. From Figure 3-18, reaction with 0.4 U enzyme reached equilibrium in the later days of synthesis while the lower concentrations still continued to produce products. Immobilised enzyme of 0.2 U was also the most appropriate concentration due to its high yield per unit of enzyme. Concentration of 0.1 U also gave high yield per unit but its reaction rate was too slow.

By comparing between 0.4 U free and immobilised enzyme, free enzyme seems to reach equilibrium faster than immobilised enzyme, which may be also due to the unequal rate of glucose accessibility (diffusion) to the active site. However, total yield of products synthesised by 3 concentrations of both free and immobilised enzyme did not show a dramatic difference.

From time course of synthesis in the first 8 h for both free and immobilised enzyme, gentiobiose was the first and major product being synthesised. The results from thin-layer chromatography in Figure 3-14 and 3-15 show that the spot of gentiobiose can be readily seen at day 5 of synthesis for both free and immobilised enzymes. The bonding between glucose and glucose molecule in gentiobiose is β-1,6 which may be the bonding that can be formed easier than β-1,2 (sophorose) and β-1,3 (laminaribiose).

Enzyme concentration of 0.2 U was chosen for further experiment as it was the most appropriate concentration due to its moderate rate of synthesis, so equilibrium was not reached so soon.

Cross-linking between β-glucosidase from sweet almond and BSA by glutaraldehyde resulted in improved synthetic activity although the hydrolytic activity was decreased (43). It is possible that this immobilisation process creates a hydrophobic microenvironment at the active site that favours the synthetic activity.

4.4.2 Re-usability of enzyme immobilised on activated carbon for oligosaccharide synthesis

Immobilised enzyme of 0.2 U was used to synthesise oligosaccharide for 5 days and re-used twice with 5-day incubation period compared to 0.2 U free enzyme that was used to synthesis for a period of 15 days. The total products from 3 fractions of immobilised enzyme was compared to free enzyme. The results in Table 3-7 show that each fraction of products from the re-use of immobilised enzyme contained almost equal amount of disaccharide to that found with free enzyme but lower amount of trisaccharides. The total

disaccharide from 3 fractions of immobilised enzyme is higher than that from free enzyme but amount of trisaccharide is the same. This can be explained by the adsorption of oligosaccharide by activated carbon as shown in Figure 3-19. The elution profile of oligosaccharide from activated carbon by 5% and 10% ethanol shows a detectable quantity of disaccharide and other higher oligosaccharide eluted out.

CHAPTERV

SUMMARY

1. Crude β-glucosidase/β-fucosidase was prepared by 35-75% ammonium sulphate fractionation that can precipitate the enzyme in a quantitative manner.

2. Maximum loading of crude enzyme immobilised on activated carbon is 0.4 U/ 12.5 mg with percent immobilisation of 50% and on celite is 0.1 U/12.5 mg with percent immobilisation of 55-60%. Cross-linkage between 20 U β-glucosidase with 2.5% (w/v) BSA by 0.3% glutaraldehyde gave highest percent immobilisation of 2.5%.

3. pH optimum of free β-glucosidase/β-fucosidase were 5.5, 5.0-5.5 for crude β-glucosidase/β-fucosidase immobilised on activated carbon and 5.0 for crude β-glucosidase/β-fucosidase immobilised on celite.

4. Free enzyme is more stable at 50°C than enzyme immobilised on activated carbon and celite in which 5% activity was retained after 72 h of incubation. Enzyme immobilised on activated carbon lost almost all of its activities after 3 h of incubation while enzyme immobilised on celite retained 3% of its original activities after 48 h of incubation.

5. Kinetics studies show that K_m of β-glucosidase activity was 5.81 ± 0.19 mM for free enzyme and 4.57 ± 0.03 mM for crude enzyme immobilised on activated carbon. K_m of β-fucosidase was 0 73 ± 0.05 mM for free enzyme and 0.94 ± 0 08 mM for crude enzyme immobilised on activated carbon.

6. The suitable concentration of free and immobilised enzyme for oligosaccharide synthesis was 0.2 U. Concentration of 0.1 U gave too slow reaction and lower yield of products, while reaction with 0.4 U was too fast and gave lower yield of product per unit enzyme. Total products of synthesis

by 3 concentrations of both free and immobilised enzyme were almost equal as determined by h.p.l.c.

7. Gentiobiose was the first and major disaccharide products synthesised by free and immobilised enzyme.

8. Immobilised enzyme could be re-used for at least 2 times and gave no dramatic difference in the yield of products. Products obtained from synthesis by free enzyme for 15 days was similar to that obtained by immobilised enzyme. Total yield of disaccharide obtained from re-used immobilised enzyme was higher than that from free enzyme.

9. The synthesised oligosaccharides were partly adsorbed onto the activated carbon and could be eluted out by 5% and 10% ethanol.

BIBLIOGRAPHY

1. Esen A. β-Glucosidases: Overview. In: β-Glucosidases: Biochemistry and Molecular Biology. Edited by Esen A. ACS Symposium Series 533. American Chemistry Society. 1993: 1-14.

2. Conn EE. β-Glucosidase in Plants: Substrate Specificity. In: β-Glucosidase: Biochemistry and Molecular Biology. Edited by Esen A. ACS Symposium Series 533, Washington DC: American Chemical Society, 1993: 16.

3. Kuroki GW, Poulton JE. Arch Biochem Biophys. Comparison of Kinetics and Molecular Biology Properties of Two Forms of Amygdalin Hydrolase from Black Cherry (*Prunus serotina* Ehrh.) Seeds. 1986; 287: 433

4. Fan, TW-M, Conn EE. Isolation and Characterization of Two Cyanogenic β-Glucosidases from Flax Seeds. Arch Biochem Biophys. 1985; 243: 361.

5. Yeoh HH, Wee TC. Some Properties of β-Glucosidase from Some Tropical Plant Species. Phytochem. 1994; 35: 1391-1393.

6. Srisomsap S, Svasti J, Surarit R, Champattanachai V, Sawangareetrakul P, Boonpuan K, Subhasitanont P, Chokchaichamnankit D. Isolation and Characterization of an Enzyme with β-Glucosidase and β-Fucosidase activities from *Dalbergia cochinchinensis* Pierre. J Biochem. 1996;119: 585-590.

7. Zheng L, Poulton JE. Temporal and Spatial Expression of Amygdalin Hydrolase and (R)-Mandelonitrile Lyase in Black Cherry Seeds. Plant Physiol. 1995; 109, 31-39.

8. Campos N, Bako L, Brzobohty B, Feldwisch J, Zetti R, Bolana W, Palme K. Identification and Characterization of a Novel Phytohormone Conjugate Specific β-Glucosidase Activity from Maize. In: β-Glucosidase: Biochemistry and Molecular Biology. Edited by Esen A. ACS Symposium Series 533, Washington DC: American Chemical Society, 1994: 205-213.

9. Lecas M, Gunata ZY, Sapis JC, Bayonove CL. Purification and Partial Characterization of β-Glucosidase from Grape. Phytochem. 1991; 30: 451-454.

10. Glew RH, Gopalan V, Forsyth GW, VanderJagt DJ. The Mammalian Cytosolic Broad-Specificity β-Glucosidase. In: β-Glucosidases: Biochemistry and Molecular Biology. Edited by Esen A. ACS Symposium Series 533, Washington DC. 1993: 83.

11. Wright RM, Yablonsky MD, Shalita ZP, Goyal AK, Eveleigh DE. Cloning, Characterization and Nucleotide Sequence of a Gene Encoding *Microspora bispora* BgIB, a Thermostable β-Glucosidase Expressed in *Escherichia coli*. Appl Env Microb. 1922; 3455-3465.

12. Philippidis GP Smith TK, Wyman CE. Study of the Enzymatic Hydrolysis of Cellulose for Production of Fuel Ethanol by the Simultaneous Saccharification and Fermentation Process. Biotech Bioeng. 1993; 41: 846-853.

13. Sengupta S, Ghosh A, Sengupta S. Purification and Characterization of a β-Glucosidase (Cellobiase) from a Mushroom *Termitomyces clypeatus*. Biochimica et Biophysica Acta. 1991; 1076:215-220.

14. Watanabe T, Sato T, Yoshioka S, Koshijima T, Kuwahara M. Purification and Properties of *Aspergillus niger* β-Glucosidase. Eur J Biochem. 1992; 209: 651-659.

15. Chen H, Hayn M, Esterbauer H. Purification and Characterization of Two Extracellular β-Glucosidases from *Trichoderma reesei*. Biochimica et Biophysica Acta. 1992; 1121: 54-60.

16. Surarit R, Matsui H, Chiba S, Svasti J, Srisomsap C. Chemical Modification of β-D-Glucosidase/β-D-Fucosidase from *Dalbergia cochinchinensis* Pierre by Condural B Epoxide. Biosci Biotech Biochem. 1996; 60(8): 1265-1268.

17. Zeng YC, Li YT, Gu YZ, Zhang SZ. Purification and characterization of a Strictly Specific β-D-Fucosidase from *Aspergillus phoenisis*. Arch Biochem Biophy. 1992; 298 (1): 226-230.

18. Kempton JB, Withers SG. Mechanism of *Agrobacterium* β-Glucosidases: Kinetic Studies. Biochemistry. 1992; 31: 9961-9969.

19. De La Mata I, Estada P, Macarron R, Dominguez JM. Chemical Modification of β-Glucosidase from *Trichoderma reesei* QM 9414: pH Dependence of Kinetic Parameters. Biochem J. 1992; 283: 679-682.

20 Umezurike GM. The Mechanism of action of β-Glucosidase from *Botryodiplodia theobromae*. Biochem J. 1987; 241:455-462.

21. Dwek RA, Edge CJ, Harvey DJ, Wornmald MR. Analysis of Glycosyl-Associated Oligosaccharide. Ann Rev Biochem. 1993; 62: 65-100.

22. Kobata A. Use of Endo- and Exoglycosidases for Structural Studies of Glycoconjugates. Anal Biochem. 1979; 100: 1-14.

23. Pouson JC, Prieels JP, Glasgow LR, Hill RL. Sialyl- and Fucosyltransferases in the Biosynthesis of Aparaginyl-Linked Oligosaccharides in Glycoproteins. J Biol Chem. 1978; 253: 5617.

24. Tai T, Yamashita K, Ogata AM, Structural Studies of 2 Ovalbumin Glycopeptides in Relation to the Endo-β-N-Acetyl-Glucosaminidase Specificity. J Biol Chem. 1975; 250: 8569-8575.

25. Nilsson KG. Enzymatic Synthesis of Oilgosaccharides. TIBTECH 1988; 6: 256-264

26. Bucke C, Rastall RA. Synthesising Sugars by Enzyme in Reverse. Chem in Britain. 1990; July: 675-678.

27. Elbein AD. The Rate of N-Linked Oligosaccharides in Glycoprotein Function. TIBTECH. 1991; 9: 346-352.

28. Cote GL, Tao BY. Oligosaccharide Synthesis by Enzymatic Transglycosylation. Glycoconjugate J. 1990; 7: 145-162.

29. Ichikawa Y, Look GC, Wong CH. Enzyme-Catalysed Oligosaccharide Synthesis. Anal Biochem .1992; 202:215-238.

30. Surarit R, Svasti MRJ, Srisomsap C, Suginta W, Khunyoshying S, Nilwarangkoon S, Harnsakul P, Benjavongkulchai E, Screening of Glycohydrolase Enzymes in Thai Plant Seeds for Potential Use in Oligosaccharide Synthesis. J Sci Soc Thailand. 1995; 21: 293-303.

31. Chaplin MF, Bucke C. The Preparation and Kinetic of Immobilised Enzymes. In: Enzyme Technology. Edited by Chaplin MF and Bucke C. Cambridge University Press. 1990: 82.

32. Kierstan MPJ, Coughlan MP. Immobilization of Proteins by Noncovalent Procedures: Principles and Applications. In: Protein Immobilization: Fundamentals and Applications. Edited by Taylor RF. Marcel Dekker Inc. 1991: 13.

33. Baker SA, Kay I. In: Handbook of Enzyme Biotechnology. Edited by Wiseman A, Chichester. Ellis Horwood. 1975:89.

34. Marek PJ, Kierstan and Michael PC from Immobilization of Proteins by Noncovalent Procedures: Principles and Applications from Protein Immobilization edited by Richard F Taylor. Marcel Dekker, Inc. 1991; p 29.

35. Lee JM, Woodward J. Properties and Application of Immobilized β-D-Glucosidase Coentrapped with *Zymomonas mobilis* in Calcium Alginate. Biotech Bioeng. 1983; 25: 2441-2451.

36. Hagndal BH. An Enzyme Coimmobilized with a Microorganism: The Conversion of Cellobiose to Ethanol Using β-Glucosidase and *Saccharomyces cerevisiae* in Calcium alginate Gels. Biotech Bioeng. 1984; 26: 771-774.

37. Cabral JM, Kennedy JF. Covalent and Coordination Immobilization of Proteins. In Protein Immobilization: Fundamentals and Applications. Edited by Taylor RF. Marcel Dekker Inc. 1991: 73.

38. Goldstein G and Manecke G from The Chemistry of Enzyme Immobilization in Immobilized Enzyme Principles edited by Wingard LB, Katchalski-Katzir E and Goldstein L. Academic Press, N.Y. 1976.

39. Broun GB. Chemically Aggregated Enzymes from Methods in Enzymology edited by Mosbach K. Academic Press, N.Y.. 1976; 44: 263

40. Ravet C, Thomas D, Legoy MD. Gluco-Oligosaccharide Synthesis by Free and Immobilized β-Glucosidase. Biotech Bioeng. 1993; 42: 303-308.

41. Aguado J, Romero MD, Rodriguez L. Immobilization of β-Glucosidase from *Penicillium funiculosum* on Nylon Powder. Biotech Appl Biochem. 1993; 17: 49-55.

42. Broun G, Thomas D, Gellf G, Domurado D, Berjonneau AM, Guillon C. New Methods for Binding Molecules into a Water-Soluble Matrix: Properties after Insolubilization. 1973; 15: 359-375.

43. Baker JO, Oh KK, Grohmann K, Himmel ME. Thermal Stabilization of Fungal β-Glucosidase through Glutaraldehyde Crosslink. Biotech Letts. 1988; 10:325-330.

44. Tyagi R, Gupta MN. Noncovalent and Reversible Immobilization of Chemically Modified Amyloglucosidase and β-Glucosidase on DEAE-Cellulose. Process Biochemistry. 1994; 29: 443-448.

45. Horvath C. Pellicular Immobilized Enzymes. Biochim Biophys Acta. 1974; 358: 164-167.

46. Engasser JM, Horvath C. Diffusion and Kinetics with Immobilized Enzymes. In: Immobilized Enzyme Principles. Edited by Wingard LB, Katchalski KE, Goldstein L. Academic Press. NY. 1976; 1: 127

47. Evans CS. Properties of β-Glucosidase (Cellobiase) from Wood-Rotting Fungus, *Coviolus versicolor*. Appl Micro Biol. 1983; 22: 128-131.

48. Bradford MM. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing: Principles of Protein Dye Binding. Anal Biochem. 1976(1-2); 22: 248.

49. Celite: Manville's Series of Diatomic Bio-Catalyst Carrier. Celite R-535 from Manville (Fluka).

50. Bisset F and Sternberg D. Immobilization of Aspergillus phoenicis β-Glucosidase on Chitosan. App Env Microbiol. 1978; 35: 750-755.