การแยกและลักษณะสมบัติของไอโซฟอร์มของโซลูเบิลสตาร์ชซินเทสจากหัวมันสำปะหลัง *Manihot esculenta* CRANTZ Cultivar Kasetsart 50

นายธเนศ เปาอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISOLATION AND CHARACTERIZATION OF ISOFORMS OF SOLUBLE STARCH SYNTHASE FROM CASSAVA *Manihot esculenta* CRANTZ Cultivar Kasetsart 50 TUBERS

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Thesis Title	ISOLATION AND CHARACTERIZATION OF ISOFROMS OF SOLUBLE STARCH SYNTHASE FROM CASSAVA *Manihot esculenta* CRANTZ Cultivar Kasetsart 50 TUBERS
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มันสำปะหลังเป็นพืชเศรษฐกิจที่มีปริมาณการส่งออกสูง แป้งมันสำปะหลังสามารถนำไปใช้ประโยชน์ได้ในอุตสาหกรรมต่าง ๆ แป้งประกอบด้วยโมเลกุลของอะไมโลสและอะไมโลเพกติน เอนไซม์ที่เกี่ยวข้องกับการสังเคราะห์อะไมโลสและอะไมโลเพกตินในพืชมีหลายชนิด สตาร์ชซินเทส เป็นเอนไซม์ตัวหนึ่งที่เกี่ยวข้องกับการสังเคราะห์แป้ง สตาร์ชซินเทสแบ่งได้เป็น 2 ประเภท คือ granule-bound starch synthase (GBSS) และ soluble starch synthase (SSS) GBSS เกี่ยวข้องกับการสังเคราะห์อะไมโลส ส่วน SSS เกี่ยวข้องกับการสังเคราะห์อะไมโลเพคติน เอนไซม์สตาร์ชซินเทสทำหน้าที่สร้างพันธะ α-1,4 glucosidic linkage ในอะไมโลสและอะไมโลเพคติน ในการทดลองนี้ทำการศึกษา SSS ในหัวมันสำปะหลังสายพันธุ์เกษตรศาสตร์50 (KU50) โดยการสกัดแยกและทำให้บริสุทธิ์โดยการตกตะกอน 20-60% แอมโมเนียมซัลเฟตอิ่มตัว และวิธีคอลัมน์โครมาโทกราฟีโดยใช้คอลัมน์ phenyl sepharose และ Q-sepharose ตามลำดับ สามารถจำแนกสตาร์ชซินเทสได้ออกเป็น 3 ไอโซฟอร์ม คือ SSS1, SSS2 และ SSS3 ที่มีความบริสุทธิ์เพิ่มขึ้น 426, 513 และ 142 เท่าตามลำดับ เมื่อนำไปวิเคราะห์ด้วยอิเล็กโทรโฟเรซีสแบบเสียสภาพที่มีเอสดีเอส SSS1 พบแถบโปรตีน 2 แถบที่มีน้ำหนักโมเลกุล 92.4 และ 81.7 กิโลดาลตัน SSS2 พบแถบโปรตีน 3 แถบที่มีน้ำหนักโมเลกุล 112, 90.1 และ 81.7 กิโลดาลตัน SSS3 พบแถบโปรตีน 2 แถบที่มีน้ำหนักโมเลกุล 90.1 และ 79.8 กิโลดาลตัน สตาร์ชซินเทสที่เตรียมได้ทั้งสามไอโซฟอร์มสามารถเร่งปฏิกิริยาได้ดีที่ค่าความเป็นกรด-ด่างเท่ากับ 9.5, 7.0, 9.5 และอุณหภูมิ 37, 35, 37 องศาเซลเซียสตามลำดับ สำหรับตัวรับหน่วยของกลูโคส (primer) เราพบว่า SSS1, SSS2 และ SSS3 สามารถใช้ ตัวรับที่พอลิแซ็คคาไรด์ที่มีกิ่งและแป้งที่มีสัดส่วนที่มีอะไมโลเพกตินสูงได้ดีกว่าพอลิแซ็คคาไรด์สายตรง สตาร์ชซินเทสทั้งสามไอโซฟอร์มให้ค่า K_m ต่อ ADP-glucose เท่ากับ 0.09, 0.06, 0.08 mM และ rabbit liver glycogen เท่ากับ 1.46, 0.26, 0.13 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ การศึกษาผลของ thiol reagents ต่อสตาร์ชซินเทสพบว่า สตาร์ชซินเทสทั้งสามไอโซฟอร์มถูกยับยั้งโดยสารที่เปลี่ยนแปลงหมู่ –SH ซึ่งแสดงว่าหมู่ –SH มีความเกี่ยวข้องกับแอคติวิตีของ SSS

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....ธเนศ เปาอินทร์.....

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THANADE PAOIN : ISOLATION AND CHARACTERIZATION OF ISOFORMS OF SOLUBLE STARCH SYNTHASE FROM CASSAVA *Manihot esculenta* CRANTZ Cultivar Kasetsart 50 TUBERS. THESIS ADVISOR : ASSOC. PROF. TIPAPORN LIMPASANI, Ph.D., 99 pp.

Cassava starch is one of the major raw materials used in many industries. Starch has two major components, amylose and amylopectin, which were synthesized by starch synthase and other enzymes. Starch synthase exists in two forms, granule-bound and soluble starch synthase. They are enzymes that synthesize α(1,4)-glucosidic linkage in amylase and amylopectin. In this study, soluble starch synthase was extracted from cassava tubers and purified by precipitation at 20-60% saturated ammonium sulfate, followed by phenyl sepharose and Q-Sepharose column chromatographies. The purification step could isolate three isoform of soluble starch synthase that are SSS1, SSS2 and SSS3. These enzymes were purified up to 426, 513 and 142 fold, respectively. When analyzed the enzymes by SDS-polyacrylamide gel electrophoresis found that SSS1 showed two protein bands with molecular weight 92.4 and 81.7 kDa, SSS2 showed three protein bands with molecular weight 112, 90.1 and 79.8 kDa and SSS3 showed two protein bands with molecular weight 90.1 and 79.8 kDa. All isoforms of soluble starch synthase showed optimum pH and temperature at 9.5, 7.9, 9.5 and 37, 35, 37°C, respectively. SSS1, SSS2 and SSS3 used branched polysaccharides and starch with high amylopectin contents as primer better than linear polysaccharides. The apparent K_m values of SSS1, SSS2 and SSS3 for ADP-glucose were 0.09, 0.06 and 0.08 mM, respectively, whereas the K_m values of enzymes for rabbit liver glycogen were 1.46, 0.26 and 0.13 mg/ml, respectively. Moreover, all three isoforms could be inhibited by thiol modifying reagents, indicating the involvement of SH-group on cassava starch synthase activity.

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LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
ADP-glucose	Adenosine-5'-diphosphate glucose
°C	Degree of Celsius
BSA	bovine serum albumin
cm	centimeter
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethelenediaminetetraacetic acid
et al.	Et. Alii (latin), and others
GSH	Gluthaione reduced form
HCl	hydrochloric acid
KCl	potessium chloride
HPLC	High Performance Liquid Chromatography
kDa	Kilo Dalton
KU 50	Kasetsart 50
K_m	Michaelis constant
l	liter
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar

mol	mole
MW	molecular weight
NEM	*N*-Ethylmaleimide
nm	nanometer
PCMB	ρ-Chloromercuribenzoic acid
PMSF	Phenylmethylsulfonyl fluoride
rpm	revolution per minutes
SAX	strong anion exchanger
SDS	Sodium dodecyl sulfate
SSS	Soluble starch synthase
TEMED	N,N,N',N'-Tetramethylene ethylene diamine
V_{max}	maximal velocity
µg	microgram
µl	microliter

CHAPTER I

INTRODUCTION

1.1 Cassava

Cassava *(Manihot esculenta* Crantz) is the most important food crop which supplies around 500 million people in the world. Cassava is a native of Brazil and dispersed widely by the Portuguese in tropical and subtropical areas of Africa, Asia and the Caribbean during the 16^{th} and 17^{th} centuries. It became a staple food in many of these places because of its suitable characteristics. Cassava can grow and produce dependable yields in places where cereals and other crops can not grow or produce well. It can tolerate drought and can be grown on soils with low nutrient capacity, but also responds well to irrigation or higher rainfall conditions. Cassava has a high yield and high resistant to the damage from serious pests and diseases. Because cassava has no definite maturation point, it can be field-stored for several months or more and harvested when market, processing or other conditions are more favorable (Pluckneet *et al*, 2000).

Cassava is a dicotyledonous plant belonging to the botanical family Euphorbiaceae. All *Manihot* species, the normal cassava plants, have 36 chromosomes. Genetically, cassava is highly heterozygous. Both cross and self-pollination occur naturally. As a consequence of the very large variations found in cassava, both among the plants and within plants (as heterozygosity), the shrub, root shape and size, color and pigmentation of the petioles varied (Roger, 1963).

A B

C D

Figure 1.1 Cassava tree and its underground tuberous roots.

(A): Cassava tree

(B): Leaves

(C): Tuberous root

(D): Flower

The utilization of cassava as major food crops for a long time can cause health problems due to its deficiency of protein but cassava leaves can prevent this problem. Leaves are more nutritionally balanced than the roots and can be eaten as fresh vegetable, ground fresh and frozen in plastic bags, or dried and ground for sale in plastic bags (Pluckneet *et al*., 2000). Leaves, however, may be high in hydrocyanic acid which can cause goiter, neurological disorders, tropical ataxic neuropathy, respiratory poisoning and sometimes death. The HCN can be reduced to safe levels in most cases when the liquid is squeezed out after grinding and through evaporation during cooking (Osuntokun, 1973).

In addition to being used for human consumption, dried cassava is used as animal feed and cassava starch is used as raw material in many industries. The unmodified or native cassava starch is used for normal food products and for specialty markets, such as baby foods, non-allergenic products and food for hospitalized persons. Cassava starch can be modified to provide characteristics that are required for more specialized food and industrial products. Modified cassava starch can be used for the production of alcohol, starch for sizing paper and textiles, glues, MSG, sweeteners, pharmaceuticals, biodegradable products, butanol and acetone, manufacture of explosives and coagulation of rubber latex.

Thailand ranks third in the world's cassava production, and the top among cassava exporting countries (Table 1.1, 1.2 and Figure 1.2). The total area of cassava cultivation is 2.6 million acres, producing 17 million tons annually. Cassava is planted mainly in the Northeastern part of Thailand. In 2005, cassava exports in the form of dried pellets and starch was 4.044 million tons, accounting for 56% of world's cassava equivalent exports. (Figure 1.2) Main importing countries are the Netherlands, Portugal, Spain, China, Japan, South Africa, and Taiwan.

Table 1.1 World cassava production (FAO-GIEWS-Food Out look No.1 June 2006. http//www.fao.org)

	2004	2005	2006 Preliminary
		Thousand tonnes	
FLOUR AND STARCH			
Total	3,533	3,261	3,500
Japan	727	622	500
China	1,083	1,027	1,150
Of which Taiwan Province	604	502	550
Indonesia	229	349	350
Malaysia	193	229	200
Others	1,300	989	1,100
CHIP AND PELLETS			
Total	4,579	3,028	3,400
China	2,557	2,766	3,250
EU 25	186	1,246	150
Others	160	16	25

Table 1.2 Overview of cassava production and trade (Agriculture of Thailand "The heart of Thailand" 28 March 2007. http//www. moac.go.th)

	Unit	2003	2004	2005
World				
Production	Million tons	190.72	203.55	203.79
Trade (cassava equivalent)	Million tons	9.00	12.65	N/A
Thailand				
Planted area	Million acres.	1.03	1.08	1.04
Production	Million tons	19.72	21.44	16.94
Farm price	Baht per kg.	0.93	0.80	1.33
Domestic consumption	Million tons	4.41	4.16	3.70
Exports (cassava equivalent)	Million tons	5.37	7.02	4.98
Share of world trade	%	59.64%	55.46%	N/A

Figure 1.2 Share of world trade in cassava equivalent exports. (Agriculture of Thailand "The heart of Thailand" 28 March 2007. http//www. moac.go.th)

1.2 Starch

Starch, the storage carbohydrate of most higher and lower plants, occurs as large complex insoluble granule located both in leaf chloroplasts (transient starch) and in the amyloplasts of the plant storage tissue cells (storage starch). It contains at least two types of polysaccharides, amylose and amylopectin. Starch contains varied ratio of amylose and amylopectin in different plants, resulting in different properties of plant starch. (Table 1.3)

Table 1.3 Percent of amylose and amylopectin in reserve plant starch (Young, 1984 and Nilmanee, 2000)

Starch	Amylose (%)	Amylopectin (%)
Rice *	13.6	86.4
Waxy rice **	0	100
Corn *	23.1	76.9
Wheat **	28	72
Barley **	22	78
Waxy barley **	0	100
Oat **	27	73
Maize **	28	72
Waxy maize **	0.8	99.2
Tapioca **	16.7	83.3
Potato **	20	80
Sweet potato **	17.8	82.2
Smooth pea **	35	65
Wrinkled pea **	66	34

* data from Nilmanee, 2000

** data from Young, 1984

Amylose is a homopolymer of glucose units which are linked by α (1→4) glucosidic bond with a very few α (1→6) branches (about 1 branch per 1,000 glucan residues). Amylose can be separated from starch granule by dissolving the starch granule in water and removing the amylose as an insoluble complex with a polar organic solvent. The relatively smaller amylose polymers are soluble in warm water, and will crystallize from the solution if the temperature is lowered (John, 1992).

Amylopectin is chemically similar to glycogen in that both are α (1→4)-linked, α (1→6)-branched glucose homopolymers, although the major difference between them is the organization of the latter into large, insoluble, semi-crystalline granules. The branched nature of amylopectin occurs about every 20 glucose units (5% of the links). The chains make up the amylopectin molecule vary in length from 12-60 glucoside units, with an average chain length of around 20 units (John, 1992). The basic structure of the granule is dictated by the packing of amylopectin molecules in organized arrays. Amylopectin consists of chains of α-1,4 linked glucose units, branched by α-1,6 linkage. There is general agreement that the granules are radically arranged with their non-reducing ends pointing toward the surface, and are organized into alternating crystalline and amorphous lamellae with a periodicity of 9 nm (Jenkins *et al*., 1993). The lamellae are believed to reflect the arrangement of chains into clusters. Within clusters, where the branches are concentrated, the chains are likely to be in an amorphous structure, while in the region where the chains are predominantly linear, with fewer branch points, chains associate to form double helices that pack together in ordered arrays to give the crystalline lamellae (Smith *et al*., 1997) (Figure 1.3). Three categories of chains have been distinguished within the amylopectin structure. A-chains, short amylose chains

substituted only at the reducing end where they are joined to the rest of the molecule by a single 1,6 bond. B-chains substituted at the reducing end and at one or more C-6-OH group by A-chains or by other B-chains. C-chain, a single, substituted at one or more C-6-OH groups, but unsubstituted at the reducing end (Figure 1.4). Each amylopectin contains one C-chain, and thus one reducing group (John, 1992). The major characterize of amylose and amylopectin are summarized in Table 1.4.

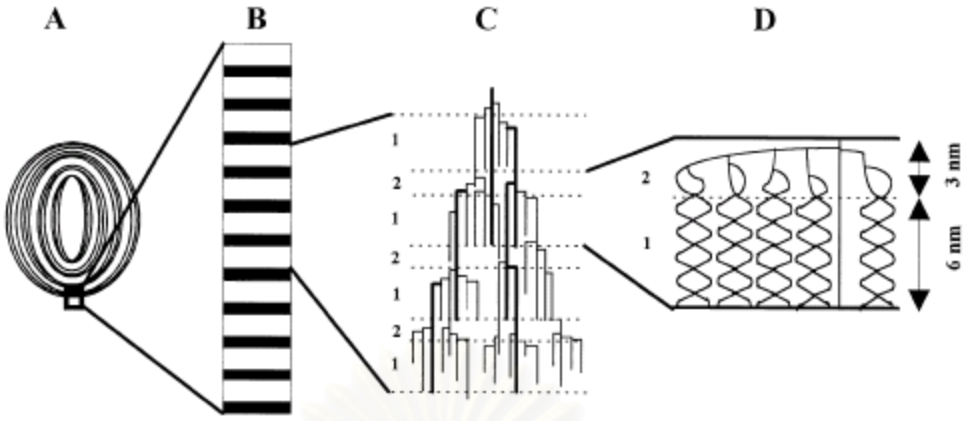


Figure 1.3 Starch granule organizations (Ball *et al*., 1996)

A: A schematic view of a 1.5 μm thick starch granule with its succession of amorphous and crystalline growth ring

B: A section of a crystalline growth ring of the granule is related to the molecular organization of amylopectin. Each shaded and plain section represents an amorphous and a crystalline lamella respectively. Thus the crystalline growth ring enlarged in this panel contains a regular succession of 11 amorphous and crystalline lamellae. This would amount to a 0.1μm thick growth ring.

C: This panel enlarges a succession of 7 lamellae and relates them to the primary structure of a portion of an amylopectin molecule. Each line represents an α-1,4 linked glucan chain. The chains are hooked together by α-1,6 branches. The dotted line delimits the sections appearing chain clusters and that the glucans are pointing towards the granule's surface.

D: This panel relates a part of primary structure depicted in (C) to the secondary structure of a single cluster displaying the double helical structures. The 6 nm size of the crystalline portion corresponds to a length of 18 glucose residues.

Table 1.4 Summarizes the major characterize of amylose and amylopectin (Banks and Muir, 1980)

Property	Amylose	Amylopectin
Molecular configuration	Essentially linear	Highly branched
Average molecular weight	Ca. 10^6	Ca. 10^8
X-ray diffraction	Crystalline	Amorphous
Action of (3-amylase and Z-enzyme	Complete hydrolysis	Residual dextrins of high molecular weight
Complex formation	Readily forms complexes with iodine and polar substances	Very limited complex formation
Solubility in aqueous solution	Unstable, tends to retrograde	Stable

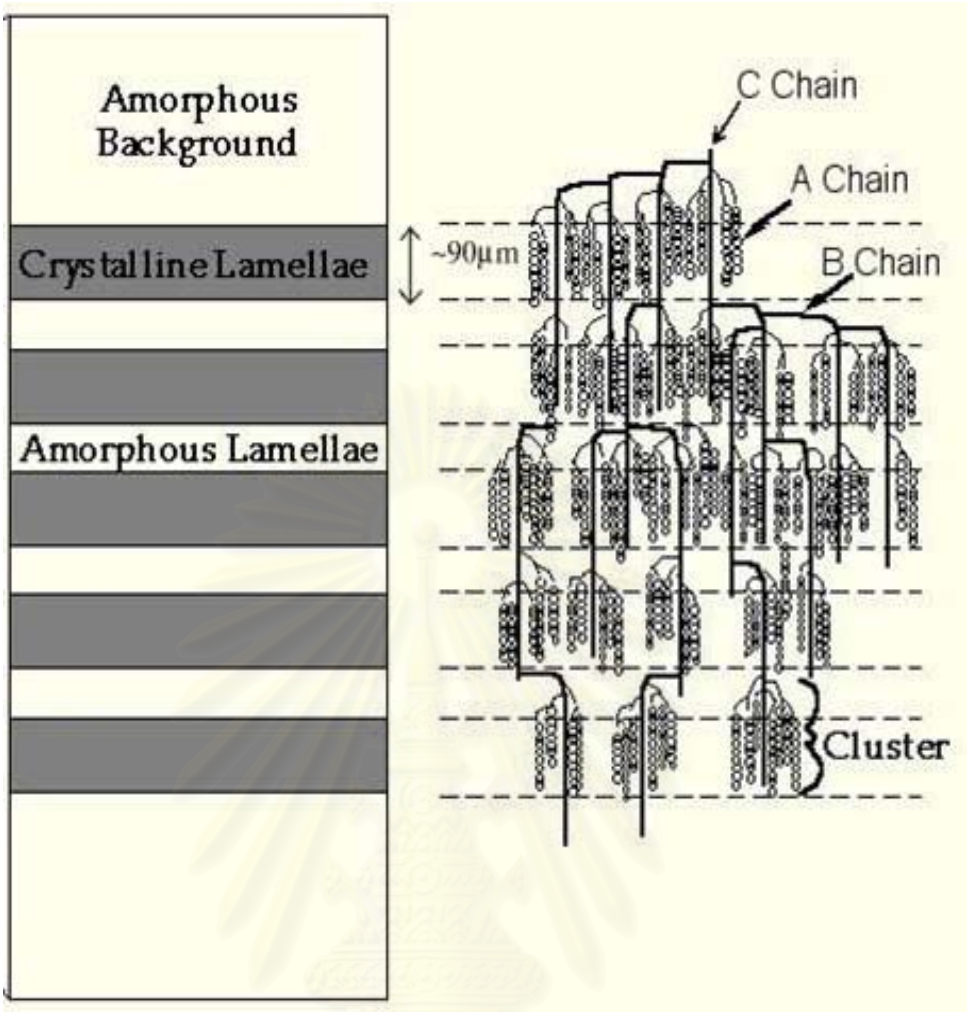


Figure 1.4 The branch structure of amylopectin (John, 1992)

1.3. Starch biosynthesis

There are many enzymes involved in starch biosynthesis. ADP-glucose pyrophosphorylase (AGPase) (EC 2.7.7.27) is responsible in all plant organs for the synthesis of ADP-glucose, the substrate for the synthesis of starch polymers. AGPase catalyzes the reaction converting Glucose- l-phosphate and ATP to ADP-glucose and this reaction is the rate-limiting step of starch biosynthesis and control starch quantity. Starch synthase (SS) (EC 2.4.1.21), starch branching enzyme (SBE) (EC 2.4.1.18), debranching enzyme (DBE) (EC 2.4.1.25) and disproportionating enzyme (D-enzyme) (EC 2.4.1.25) play roles in starch quality, physical and biochemical properties (Myers *et al*., 2000). Starch biosynthesis depends on amylose and amylopectin biosynthesis.

1.3.1 Amylose biosynthesis

Amylose molecules appear to exist as single helix within the starch granules, interspersed with amylopectin in amorphous regions. Amylose synthesis in storage organs is a specific function of the granule-bound starch synthase I (GBSSI) an isoform of starch synthase which catalyzes the transferring of glucosyl units from ADP-glucose to non-reducing ends of growing polysaccharides via new α(1→4) linkages. The glucose acceptor or primer for GBSSI activity is the short glucans and malto-oligosaccharides (Denyer et al, 1999). Amylose synthesis occurs within the matrix of the starch granule (Tatge et al, 1999). The space available in the matrix, created by the synthesis of amylopectin, may be an important factor determining the amylose content of storage starch.

1.3.2 Amylopectin biosynthesis

It is widely accepted that amylopectin is elaborated at the surface of the starch granule by starch synthases (SSs) and starch branching enzymes (SBEs) in the soluble fraction of the amyloplast. Soluble starch synthase elongates very short chains at the peripheral of the granule. Initially these chains are of insufficient length to act as substrates of SBE, which acts preferentially upon chains in double helical conformation, and they remain unbranched. When they reach an appropriate length for branching to occur, branches are created through the action of SBE by catalyzing the cleavage of α(1→4) linkages and transfer of the released reducing end to a C6 hydroxyl, creating a new α(1→6) linkage. The other two enzymes which are, potentially, involved in amylopectin biosynthesis are debranching enzyme (DBE) and disproportionating enzyme (D-enzyme) (Myers et al, 2000). DBE catalyzes the hydrolysis of α(1→6) linkages and D-enzyme catalyzes the transfer a segment of one linear chain to another (Figure 1.5). Amylopectin do not accumulate to normal level when both enzymes are missing. The branch linkage hydrolysis is required for net amylopectin production. DBE removes the outer chains from the unorganized glucan created by SBE and SS. This will prevent phytoglycogen synthesis and leave out the tightly spaced branched that will generate the next amorphous lamellae (Figure 1.6). Amylopectin content is significantly decreased by the mutation of DBEs and the abnormal phytoglycogen accumulates. Branch frequency in phytoglycogen is approximately 10%, about twice that in amylopectin. Phytoglycogen does not exhibit the higher order structures of amylopectin, presumably because the chain length distribution is weighted toward shorter linear segments, and long B-chains with multiple branches are lacking (Myers et al, 2000). D-enzyme may play a direct role in amylopectin

formation, or it could be involved in indirect recycling of glucosyl unit from water-soluble polysaccharide or pre-amylopectin back into amylopectin biosynthesis. Thus, D-enzyme should be considered in addition to SS and SBE as a potential direct determinant of chain length distribution. However, it is not clear how D-enzyme acts on amylopectin biosynthesis (Myers *et al*., 2000).

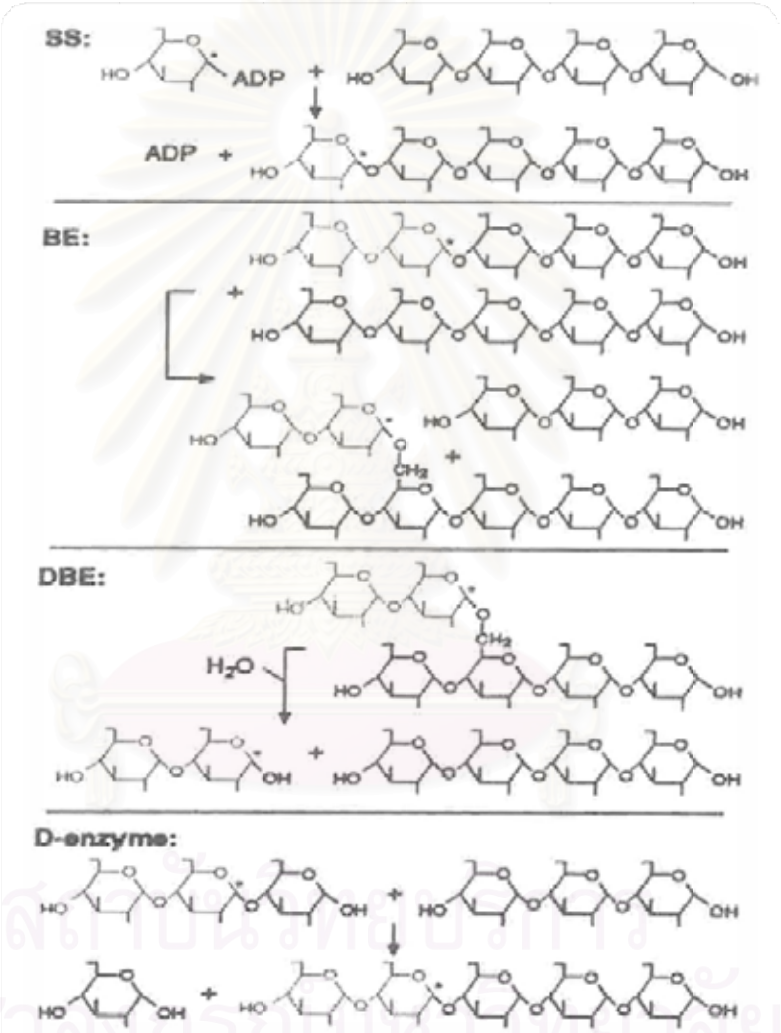


Figure 1.5 Reaction of enzyme involve in amylopectin synthesis (Myers *et al*., 2000)

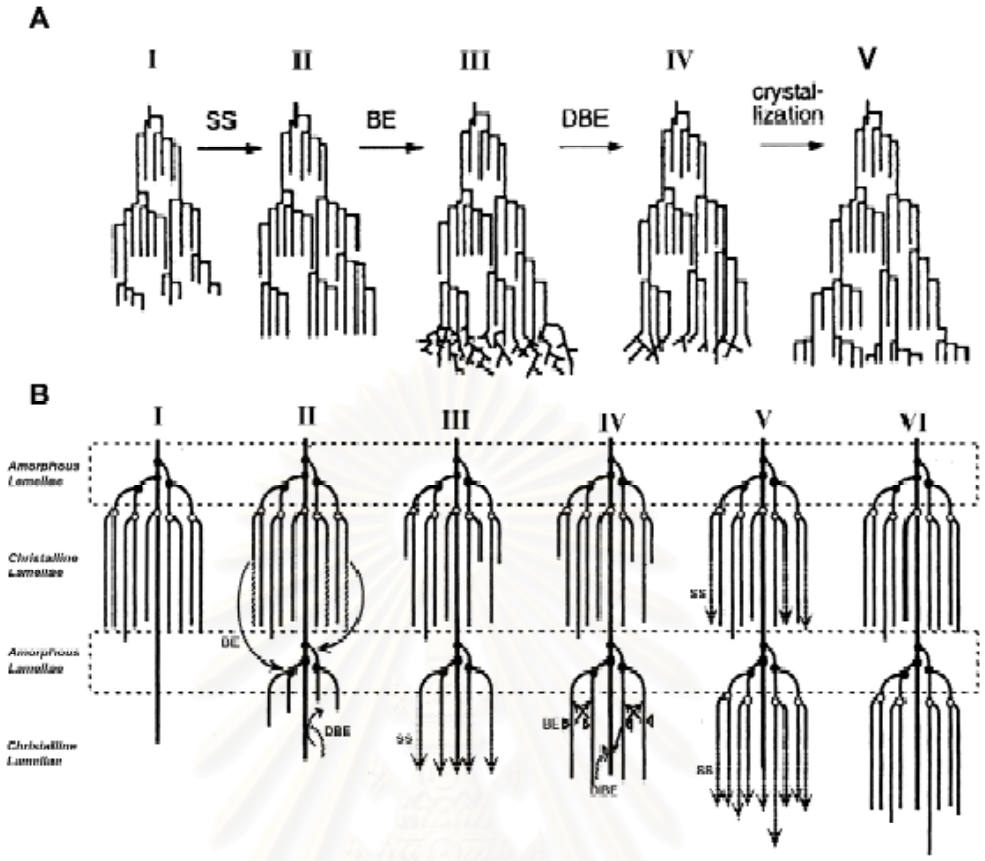


Figure 1.6 Amylopectin biosynthesis model (Boren, 2005)

A: Glucan trimming model: I→II Branch elogation, II→III New branches form, III→IV Excess branches are removed, IV→V After removal of excess branches newly formed parallel branches can crystallize. (After Ball et al. (1996))

B: two step branching and improper branch clearing model: II Branches are formed by moving strands from the previous layer. III Newly formed branches elongate IV Intra cluster branching to facilitate double helix formation V Elongation of all branches VI Double helix formation and crystallisation. (After Nakamura (2002))

1.4 Starch synthase

Starch synthase (EC 2.4.1.21) was first observed by Leloir, De Fekete and Cardini, 1961. It catalyzes the transfer of glucosyl units from nucleotide-glucose donor to non-reducing ends of growing polysaccharides, such as amylose, amylopectin and glycogen, via new α(1→4) linkages. This reaction was first described using UDP-glucose as the nucleotide glucose donor. Subsequently, Recondo and Leloir (1961) showed that ADP-glucose was a better substrate both in terms of V_{max} and affinity (Recondo and Leloir, 1961). Starch synthases are defined in two types, granule-bound and soluble starch synthase.

1.4.1 Granule-bound starch synthase

Granule-bound starch synthase (GBSS), which was the form bound to starch granule, was studied in *waxy* plants in which starch granule contains only amylopectin but no amylose. The missing of granule-bound starch synthase activity was detected. It appeared that the major granule-bound starch synthase, referred to GBSSI, was responsible for the production of amylose. The GBSSI, also known as waxy gene product which was absent in waxy plants, has been identified as a 58 or 60 kDa protein in rnaize, 60 kDa protein in rice, 68 kDa protein in amaranth, 60 kDa protein in pea and 60 kDa protein in potato.

GBSSI synthesized amylose within the matrix of the starch granule. Amylose in the transgenic potatoes, which contained reducing level of GBSSI, was wholly or largely confined to a central region of the granule (Tatge *et al*., 1999). Consequently, this core region stained blue with iodine whereas the peripheral zone stained red. By making extensive measurements of the relative sizes of granules and their blue staining core in tubers over a range of developments, the blue core increased in size as the granule grows. The extent of the increase in size

of the blue core was greater in potatoes with higher levels of GBSSI. These data showed that amylose synthesis occurred within the matrix of the granule and were consistent with the idea that the space available in the matrix might be an important determinant of the amylose content of storage starch (Tatge *et al*., 1999).

GBSSI used ADP-glucose and malto-oligosaccharide as substrates while amylopectin acted as an effector increasing the rate at which GBSSI, elongated maltooligosaccharides. The affinity of GBSS I for amylopectin as an effector was greater than its affinity for amylopectin as a substrate. These results suggested that specific interaction with amylopectin in the matrix of starch granule was a unique property of GBSSI and is critical in determining the nature of its products. The interactions between GBSSI and amylopectin within matrix of the granule might be essential to allow elongation of malto-oligosaccharide (Leloir *et al*., 1961).

Novel GBSS was discovered in waxy wheat, in which amylose was observed in pericarp, embryo and aleurone layer. The novel GBSS was different from GBSSI by molecular weight, isoelectric point and N-terminal amino acids. Its molecular weight was 56 kDa and the N-terminal was 40-50% similar to GBSSI and cross-react with GBSSI antisera of maize and potato. Therefore, the 56 kDa protein was suggested to be in GBSSI class and it was a waxy protein isoform. Its expression was controlled by development stage and may be tissue specific (Nakamura *et al*., 1988; Fujita and Taira, 1998)

Most of specific properties of GBSSI are determined by a C-terminal region that includes the KTGGL 'look-alike' motif and the tail (C-terminal extension). The tail is specific to GBSSI proteins. The KTGGL 'look-

alike' motif is also specific to GBSSI because it resembles the N-terminal KTGGL motif identified as a site of ADP/ADP-glucose binding in bacterial glycogen synthase by labeling with pyridoxal phosphate derivative. The structure similarities between the N-terminal KTGGL and C-terminal KTGGL 'look-alike' motif therefore suggest that both are involved in binding ADP/ADP-glucose (Edwards *et al*., 1999).

In cassava, the size of waxy protein (GBSSI),was predicted to be about 58.61 kDa (530 amino acids) from cDNA and exhibits high amino acid sequence identity with potato GBSS (74%) and also with GBSS from other plant species (60-72%) (Salehuzzaman *et al*., 1993). Cassava GBSSI was most prominent in tubers while GBSSII was leaf-specific and produced different kinds of amylose. GBSSI might be committed to the production of amylose for storage while GBSSII produced a kind of amylose, which was easier to form, and breakdown. Cassava GBSSII showed only 30% homology to GBSSI. The major difference being the N-terminal region comprising the transit peptide as well as extra 193 amino acids on GBSSII. However, within the C-terminal region of GBSSII, there were regions of up to 40 amino acids, which contained as much as 70% identity with corresponding regions on the cassava GBSSI amino acid sequences. Comparison of the predicted amino acid sequence of cassava GBSSII with GBSSII sequence of potato and pea revealed high homology over C-terminal (70% identity). The KTGGL 'look-alike' motif thought to be responsible for ADP=glucose binding was also located in this region (Munyikwa *et al*., 1997).

1.4.2 Soluble starch synthase

Soluble starch synthases (SSSs) locate in soluble phase (stroma) of the plastid. It is the enzymes concerning in amylopectin synthesis. Anion exchange column chromatography showed the isoforms of SSS in different plants: 4 isoforms in spinach leaf (Ozbun *et al*., 1972), 2 isoforms in maize kernel (Ozbun, Sawker and Preiss, 1971), 3 isoforms in potato tuber (Shwker, Ozbun and Preiss, 1972), 1 isoform in grape leaf, 2 isoforms in maize leaf (Hawker and Downton, 1974), 2 isoforms in rice grain (Pisigan and Rosario, 1976), 3 isoforms in sorghum seed (Boyer, 1985), and 1 isoform in castor bean endosperm (Goldner and Beevers, 1989). Some isoform(s) of starch synthase can catalyze the synthesis of α-(1→4) linkage without glucose acceptor, called primer, such as glycogen, amylopectin and amylose. Some soluble starch synthase isoform(s) were, associated with the surface of starch granule referred as starch synthase which was not classified as granule-bound or soluble starch synthase, such as in potato (Larsson *et al*., 1996; Marshall *et al*., 1996), maize (Foster *et al*., 1996), and pea (Edwards *et al*., 1996).

Maize SSSs have been studied for about 30 years, The 2 isoforms of SSSs were discovered in maize kernel (Ozbun *et al*., 1971). SSSI used glycogen as primer better than amylopectin and it had unprimed activity. Its optimum temperature was 42°C and optimum pH was about 8.0 (Cao *et al*., 2000). SSSI showed the molecular weight about 76 kDa and was 90% associated with starch granule (Foster *et al*., 1996). Its amino acid sequence was compared with that deduced from the cDNA of *E. coli* glycogen synthase. The N-terminal extension was observed about 93 amino acids. Its N-terminal extension was suggested to regulate the primer binding because the N-terminal truncation decreased the enzyme affinity for amylopectin but did not have the effect on enzyme activity (Imparl-Radosevich *et al*.,

1998). Moreover, the antiserum against SSSI eliminated approximately 60% of the total SSS activity (Cao *et al*., 1999). This result indicated that SSSI was the major SSS of maize kernels.

There were some different properties between maize SSSII and SSSI unlike SSSI, SSSII used amylopectin as primer better than glycogen and did not have unprimed activity (Ozbun *et al*., 1971). It's optimum temperature was 37°C and optimum pH was about 8.5 (Cao *et al*., 2000). The study in maize kernels homozygous for the recessive *dull* allele *(du)* found lower SSS activity (Boyer and Preiss, 1981). The *dul-* mutation defined a gene with an important function in starch synthesis enzymes, especially for SSSII and SBEIIa. The *Dul* gene was predicted coding for SSSII because its similarity of predicted amino acid sequence to potato SSIII and glycogen synthase (Gao *et al*., 1998). The other information to confirmed *Dul* gene coding for SSSII was the *Dul* antisera eliminated 20-30% of total SSS activity from the kernel extract. Otherwise, the C-terminal of *Dul* gene product contained the conserved sequence for SS and glycogen synthase (Cao *et al*., 1999). The *dul* antisera detected a soluble endosperm protein more than 200kDa that was lacking in *du-* mutants. It was related the 188 KDa protein predicted by cDNA of *Dul* gene (Gao *et al*., 1998).

The major isoform of SS in the soluble fraction from potato tuber, referred to as SSIII, contributed to 70-80% of total activity in soluble fraction. SSII, which was both granule-bound and present in soluble form in the stroma of amyloplast, accounted for only about 10-15% of the total soluble SS activity in potato tuber (Edwards *et al*., 1995). The molecular weight of SSIII was determined to be about 140 kDa distinct from GBSSI and SSII. An antibody that strongly

recognized SSII only very weakly recognized SSIII and its predicted amino acid sequence differed substantially from those of the other two isoforms. Its amino acid sequence is about 30% and 50% similar to GBSSI and SSII, respectively. The reduction in activity of SSII alone had relatively little effect on the morphology of the granule, but affecting in amylopectin structure and starch properties. The reduction of SSIII alone caused serious disruption of granule morphology. It contributed the effect in amylopectin structure and starch properties (Edwards *et al.,* 1999). The reduction of both isoforms had no measurable effect on the starch content, in term of amylose and amylopectin ratio of the tuber, either during growth or maturity. They caused change in starch granule morphology, the branch lengths of amylopectin, and the gelatinization behavior. This result indicated that different isoforms of starch synthase made distinct contributions to the synthesis of amylopectin, and that they acted in a synergistic manner, rather than independently, during amylopectin synthesis (Edwards *et al.,* 1999).

1.5 Some important cassava cultivars developed in Thailand

In Thailand, a cassava-breeding program was started with the release of Rayong1 in 1975. Rayong1 was developed from a selection of local cultivars, and was the first variety bred as a source for industrial raw material. Since then, there have been many cassava varieties being produced with different physical and chemical properties (Table 1.5). Cassava breeding program in Thailand aims to improve starch yield and adaptability to a wider range of growing conditions. Starch yield is the function of starch content and root dry matter yield. There has been no systematic institutional breeding of cassava for improved cooking quality in Thailand. From the many varieties developed, there are only a few that are widely adopted (Table 1.6). At

present, the Rayong Field Crops Research Center (RAY-FCR), Department of Agricultural, Ministry of Agriculture is still continuously running breeding program.

Table 1.5 Cassavas varieties in Thailand (Yaiyen, 2003)

	Rayong 1	**Rayong 2**	**Rayong 3**	**Rayong 5**	**Rayong 60**	**Rayong 90**	**KU 50**	**Sriraja 1**	**5 minute**
Stem color	Metallic green	Pale brown	Pale brown	Greenish brown	Pale brown	Orangish brown	Metallic green	Metallic green	Greenish brown
Petiole color	Purple	Greenish purple	Pale green	Pale purple	Greenish brown	Pale green	purple	Purple green	Light green
Heights (cm.)	200-300	180-220	130-180	170-220	170-250	160-200	200-300	231	250-350
Number of branches	Little	Medium	High	Little	Medium	High	Little	Little	Little
Tuber's color	Pale brown	Pale brown	Pale brown	Pale brown	Pale brown	Dark brown	Brown	Yellow-white	Dark-brown
Production (tree/rai)	3.22	3	2.73	4.02	3.52	3.65	3.67	-	2-3
Starch (%)	18.3-24	18.3-24	23-28	22.3	18.5	23.7	23.3	21.9	14

Table 1.6 Comparison of cassava varieties in Thailand (Yaiyen, 2003)

Varieties	Advantage	Disadvantage
Rayong 1	High yield, good plant type	Low content starch
Rayong 2	Good taste	Low quantitative starch content, tuber up to environment
Rayong 3	High root dry matter	Short shrub and high branch, difficult to take care
Rayong 5	High yield, well adapted to environment	High disease in plant
Rayong 60	Early harvest, high yield	Tuber has color, low starch content
Rayong 90	High root dry matter, high yield	Short age
KU 50	High root dry matter, high yield, good plant type	Difficult to take care if low environment
Sriraja 1	High root dry matter, good plant type	Tuber has color, low content starch
Five minute	Low cyanic acid, Good taste	Low yield

1.6 Objectives

Cassava starch has been used as raw material in many industries. Different starch quality will offer wider applications of starch. The study of starch biosynthetic enzymes in cassava mill proud important information and tools for improvement of cassava starch quality and quantity and consequently, added value to cassava starch. There is little report on soluble starch synthase in cassava.

The objectives of this thesis are:

1. To purify and characterize starch synthase isoforms from cassava tubers.

2. To study kinetic parameters of the purified cassava starch synthase isoforms.

3. To study the parameters affecting cassava starch synthase activity.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant materials

Cassava tubers cultivar Rayong 1and Kasetsart 50 (KU50) from Rayong Field Crops Center at Rayong province, Thailand.

2.2 Chemicals

Chemical	Company
Acrylamide	Merck, Germany
ADP-glucose	Sigma, USA
Aquasorb	Fluka, Switzerland
Ammonium dihidrogen phosphate	May&Baker, England
di-Ammonium hydrogen phosphate	May&Baker, England
Ammonium persulfate	Merck, Germany
Ammonium sulphate	Carlo, Germany
Amylopectin, corn	Sigma, USA
Amylose, corn	Sigma, USA
Bicine (*N*,*N*-bis[2-hydroxyethyl]glycine)	Sigma, USA
Bromophenol Blue	BDH, England
Cassava starch	Thaiwa, Thailand
p-Chloromercuribenzoic acid (PCMB)	Sigma, USA
Corn starch	Maizena, Thailand
Coomassie Blue G-250	Sigma, USA
Coomassie Blue R-250	Sigma, USA
Dithiothreitol (DTT)	Sigma, USA
Ethelenediaminetetraacetic acid (EDTA)	Fluka, Switzerland
N-Ethylmaleimide (NEM)	Aldrich, USA

Chemical	Company
Glucose	Sigma, USA
Glutathione, Reduced form	Sigma, USA
Glycine	Sigma, USA
Glycogen, Oyster	Sigma, USA
Glycogen, Rabbit liver	Sigma, USA
lodoacetic acid (IAA)	Aldrich, USA
Maltose	Sigma, USA
Maltotriose	Sigma, USA
Maltotetraose	Sigma, USA
Maltopentaose	Sigma, USA
Maltohexaose	Sigma, USA
Maltoheptaose	Sigma, USA
β-Mercaptoethanol	Sigma, USA
N,*N*-methyl-bis-acrylamide	Merck, Germany
MOPS (3-[*(N*-Morpholino)propanesulfonic acid]	Sigma, USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, USA
Phenyl Sepharose High Performance	Pharmacia, Sweden
Potato starch	Sigma, USA
Q-Sepharose	Pharmacia, Sweden
Rice starch	Thaiwa, Thailand
Servalyt pH 3-10	Serva, USA
Sephadex G-200	Pharrnacia, Sweden
Sodium laulyl sulphate (SDS)	BDH, England
Standard low molecular weight marker proteins	Ptiarmacia, USA
N,*N*,*N'*,*N'*-Tetramethylene ethylene diamine (TEMED)	BDII, England
Trichloroacetic acid	Fluka, Switzerland
Tris (hydroxymethly) aminomethane	Fluka, Switzerland

The other common chemicals were reagent grade from BDH, Carlo-Erba and Fluka.

2.3 Equipments

Equipment/Model	Company
Centrifuge/ J2-21	Beckman, USA
Centrifuge/ 1000 series	Labquip, England
Conductivity meter/ CDM83	Radiometer, Denmark
Electrophoresis Unit/ Mighty Small Il	Hoefer Pharmacia Biotech, USA
Fraction collector/ Redi Frac	Pharmacia LKB, Sweden
HPLC/ LC 1050 series	Hewlett-Packard, USA
Lyophilyzer/ Flexi-Dry µP	FTS Systems, USA
Peristaltic pump/ P-1	Pharmacia Biotech, Sweden
Electrophoresis power supply/ EPS300	Pharmacia Biotech, Sweden
Gyrotary water bath shaker/ G76D	New Brunswick Scientific, USA
Spectrophotometer/ DU650	Beckman, USA

2.4 Purification of starch synthase from cassava tubers

2.4.1 Preparation of cassava crude enzyme

Cassava tubers were peeled and the cortex was removed. The parenchyma was chopped and homogenized in a blender to collect crude extract and adjust with ice-cold 50 mM Tris-acetate pH 8.5 with 10mM EDTA, 2mM DTT final concentration (Ozbun, Hawker and Preiss, 1971). The buffer was added with 1 mM PMSF and 20% glycerol to prevent protease activity and stabilize enzyme. The homogenate was filtered and centrifuged at 12,000 rpm for 60 minutes at 4°C to remove starch and lipid. The supernatant was collected as crude enzyme and kept at 4°C for further works.

2.4.2 Ammonium sulfate precipitation

Solid ammonium sulfate was slowly added to cassava crude enzyme to reach 20% saturation. Afterward, the supernatant was collected by centrifugation at 12,000 rpm for 60 minutes at 4°C. Solid ammonium sulfate was further added to the supernatant to attend 60% saturation. The pellet was collected by centrifugation at 12,000 rpm for 60 minutes at 4°C and dissolved in 50 mM Tris-acetate pH 8.5 containing 1 M ammonium sulfate, 10 mM EDTA and 2 mM DTT (Ozbun, Hawker and Preiss, 1971).

2.4.3 Phenyl Sepharose column chromatography

Phenyl Sepharose high performance column (2 x 10cm.) was washed with 2 column volumes of sodium hydroxide 0.5 N followed with 500 ml distilled water. The column was then equilibrated with elution buffer (50 mM Tris-acetate pH 8.5, 10 mM EDTA and 2 mM DTT) containing 1 M ammonium sulfate. The sample from ammonium sulfate precipitation step was centrifuged to remove undissolved particles and loaded onto the column and eluted with 15 column volumes of elution buffer at flow rate of 1 ml/min controlled by peristaltic pump. The enzyme was step-wise eluted with 15 column volumes of 0.5 M, 0.2 M, 0.1 M, and 0 M ammonium sulfate in elution buffer, respectively. Fractions of 4 m1 were collected using fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm using spectrophotometer (DU series 650, Beckman, USA). The enzyme activity was detected by the method described in section 2.5. The fractions with enzyme activity were pooled in dialysis bag (molecular weight cut off 12kDa) and concentrated using aquasorb to reduce volume for further purification step.

2.4.4 Q-Sepharose column chromatography

Hitrap Q-Sepharose column (1.5 x 2.5cm.) was washed with 2 column volumes of 0.5 N sodium hydroxide followed with 50 ml of distilled water. The column was then equilibrated with 100 m1 of elution buffer. The fraction from phenyl Sepharose column chromatography was loaded onto the Q-Sepharose column and washed with 10 column volumes of equilibration buffer to remove unbound proteins. The enzyme was then eluted with 10 column volumes of a linear gradient of 0 - 0.5 M potassium chloride in elution buffer at flow rate of 1 ml/min. Fractions of 2 ml were collected using fraction collector. The elution profile was monitored for protein and enzyme activity as previously described in section 2.4.3. The fraction with SS activity was pooled. The pooled fractions were dialyzed in elution buffer. The dialyzed enzyme was concentrated by aquasorb and collected for next purification step and characterization.

2.5 Determination of starch synthase activity

2.5.1 HPLC assay

Starch synthase activity was determined by HPLC using the developed method of Hawker (Hawker et al. 1974) and Viola (Viola et al., 1999). ADP-glucose was used as a nucleotide sugar donor for this reaction.

$$\text{ADP-glucose} + \text{Primer}(\text{glucose}_n) \xrightarrow{\text{starch synthase}} \text{primer}(\text{glucose}_{n+1}) + \text{ADP}$$

In the reaction, when 1 nmole of glucose units was incorporated into primer, 1 nmole of ADP was released and represented the amount of incorporated glucose units. The assay mixture contained 100 mM Bicine-KOH pH 8.5, 25 mM potassium acetate, 10 mM glutathione (reduced form), 5 mM EDTA, 1 mM ADP-glucose, 20 mg/ml glycogen and enzyme in a final volume of 200 µl. After incubation at 37°C for 15 minutes, the reaction was stopped by heating in boiling water for 5 minutes and centrifuged at 12,000 rpm for 10 minutes. The supernatant was collected and assayed for amount of ADP released by using HPLC system (Hewlett-Packard LC 1050 series with UV detector, USA.). Aliquot (20µl) of supernatant was injected onto Sphereclone-SAX (5µ, 250 x 4.6mm: Phenomenex, USA.). Mobile phases were (A) 10mM ammonium phosphate, pH 3.0 and (B) 450mM ammonium phosphate, pH 4.5. Flow rate was l ml per minute. The column was equilibrated with 100% A and 0% B before sample injection. After injection, the gradient of mobile phase was established as followed: isocratic 0% B for 1 minute, linear increases to 100% B for 30 minutes, linear decreases to 0% B for 1 minute and isocratic 0% B for 5 minutes. The detector was set at 254 nm. Under the conditions, ADP-glucose was eluted at 16.2 minutes and ADP was eluted at 18.7 minutes. Unit activity of starch synthase was defined as 1 nmole of glucose incorporated per minute at 37°C. Specific activity was defined as unit activity per mg protein. The calculation of activity and specific activity was described in Appendix A.

2.5.2 Radioactive assay

Starch synthase activity was assayed essentially as described by Pollock and Preiss (C. Pollock, J. Preiss, 1980). Assay mixture (200 µl) contained 100 mM Bicine-NaOH pH 8.5, 5 mM EDTA, 0.25 M Potassium acetate, 1mM

Gluthathione, 1mg rabbit liver glycogen, 0.7 mM ADP-[^{14}C]Glc (500 cpm/nmol), 0.25 mM ADP-glucose and enzyme in a final volume of 200 µl. After incubation at 37°C for 15 minutes, the reaction was stopped by heating in boiling water for 5 minutes. The stopped reaction was incubated with 500 µl of 70% methanol for 5 minutes and centrifuged at 12,000 rpm for 5 minutes. Then, the pellet was washed 2 times by 500 µl of 70% methanol and centrifuged at 12,000 rpm for 5 minutes. After that, the pellet was dissolved with 20 µl distilled water and 1.5 ml scintillation fluid was added. The amount of ^{14}C-glucose was counted by scintillation counter (LS 6500 Mmulti-Purpose Scintillation Counter, Beckman Counter USA) for 10 minutes.

2.6 Determination of protein concentration

Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard (see Appendix B). The 100 µ1 of sample was mixed with l ml of Coomassie blue reagent and left for 5 minutes before measured absorbance at 595 nm. The Coomassie blue reagent was the mixture of 100 mg Coomassie blue G250, 50 ml ethanol, 100 ml 85% phosphoric acid, and 850 m1 distilled water toward total of 1 liter.

2.7 Polyacrylamide gel electrophoresis (PAGE)

2.7.1 Non-denaturing glycogen-PAGE

Non-denaturing polyacrylamide gel was prepared as described in Appendix C, with addition of 0.3% (w/v) glycogen in the gel solution on slap gel (10x8x1.5cm.) 0f 6% (w/v) separating gel and 4% (w/v) stacking gel. Cold Tris-glycine buffer pH 8.3 was used as electrode buffer. To preserved activity 2 mM

DTT was added to electrode buffer. The electrophoresis was performed at constant current of 20 mA. At the end of the run, the gel was incubated in incubating buffer containing 5 mM ADP-glucose and 10 mg/ml of rabbit liver glycogen at 30° C for 3 hours (Appendix C). The gel was rinsed with distilled water and stained with fresh I_2/KI solution (5 g KI, 0.5 g I_2 in 500 ml distilled water) (Martha, Myers, Jason, et all, 2004).

2.7.2 SDS-PAGE

The denaturing gel electrophoresis was carried out with 0.1% (w/v) SDS in 10% (w/v) separating gel and 4.0% (w/v) stacking gel and Tris-glycine buffer pH 8.3 containing 0.15 (w/v) SDS was used as electrode buffer (Appendix C). Sample to be analyzed were treated with sample buffer and boiled for 5 minutes before application to the gel. The electrophoresis was preformed at constant current of 20 mA per slab at room temperature on a electrophoresis unit from cathode towards anode and stained for protein with Coomassien blue G250 (Appendix C).

2.8 Characterization of starch synthase

2.8.1 Effect of pH on starch synthase activity

The Q-Sepharose purified starch synthase was used to study the effect of pHs on its activity. The enzyme was assayed as described in section 2.5 at various pHs. The enzyme was incubated with substrate solution prepared in universal buffer as described in Appendix E. After the reaction was stopped by heating in boiling water for 5 minutes, ADP was detected and measured as described in section 2.5.1. The enzyme activity unit was calculated as described in Appendix A.

2.8.2 Effect of temperature on starch synthase activity

The Q-Sepharose purified starch synthase was used to study the effect of temperature on its activity. The enzyme was assayed by incubation at 20, 25, 28, 30, 35, 37, 45, 50, 55 and 60°C for 30 minutes. After the reaction was stopped by heated in boiling water for 5 minutes, the amount of ADP was detected and measured as described in section 2.5.1. The enzyme activity was calculated as described in Appendix A.

2.8.3 Temperature stability of starch synthase

The temperature stability of starch synthase was studied. The Q-Sepharose purified enzyme was incubated at 25, 30, 35, 40, 45, 50, 55 and 60°C for 30 minutes. Afterward, the activity of incubated enzyme was assayed at 37°C for 30 minutes. The reaction was stopped by heating in the boiling water for 5 minutes. The ADP was detected and measured as described in section 2.5. The enzyme was incubated at the highest temperature, which the enzyme activity still remained, at 0, 3, 6, 12, 18, 24, 30 and 36 hours and collected to assay as described previously. After the reaction was stopped by heated in boiling water 5 minutes, ADP was measured as described in section 2.5.1. The enzyme activity unit was calculated as described in Appendix A.

2.8.4 Effect of primer on starch synthase activity

The effect of various primers on the activity of starch synthase was studied. The reaction mixture was prepared as described in section 2.5.1. The rabbit liver glycogen was replaced by 10 mg/ml of either oyster glycogen, amylopectin, amylose, cassava starch, rice starch, potato starch, corn starch. 20 mM of maltopentaose or maltohexaose. The reaction was stopped by heated in boiling

water after incubated at 37 °C for 30 minutes. ADP was measured as described in section 2.5.1. The enzyme activity unit was calculated as described in Appendix A.

2.8.5 Effect of sulfhydryl group reagents on starch synthase activity

The effect of the sulfhydryl group reagents on starch synthase activity was studied. Different concentrations (2 mM, 5 mM, and 10 mM) of DTT, NEM, IAA, and PCMB were added to different reaction mixtures. The reactions were stopped by heated in boiling water after incubated at 37°C for 15 minutes. ADP was measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

2.8.6 Kinetic constant for ADP-glucose

The purified starch synthase was used to study for kinetic constant for ADP-glucose, which is the substrate of this enzyme. The reaction mixture consisted of various concentrations of ADP-glucose, 0.03125, 0.0625, 0.125, 0.25, 0.5, 0.625, 1.0, 1.25, 2.0, 2.5, 4.0, and 5.0mM with fixed excess amount of rabbit liver glycogen. The reaction was incubated at 37°C for 30 minutes, stopped by heated in boiling water and the ADP was detected and measured as described in section 2.5.1. The enzyme activity unit was calculated as described in Appendix A.

2.8.7 Kinetic constant for rabbit liver glycogen

The kinetic constant for primer of starch synthase was determined. The rabbit liver glycogen, which showed the highest activity, was used as the primer for this reaction. The reaction mixture consisted of various concentrations of rabbit liver glycogen, 0.625, 1.0, 1.25, 2.0, 2.5, 4.0, 5.0, 8.0, 10.0, and 20.0 mg/ml with fixed concentration of excess ADP-glucose. The reaction was stopped by heated in boiling water after incubation at 37°C for 30 minutes. The ADP was

detected and measured as described in section 2.5.1. The enzyme activity unit was calculated as described in Appendix A.

CHAPTER III

RESULTS

3.1 Developmental pattern of starch synthase in cassava tubers

Soluble starch synthase (SSS) activity was monitored in tubers of two cassava cultivars Rayong 1 and Kasetsart 50 (KU 50) at 3, 6, 9 and 12 months, using assay method described in section 2.5.1. The SSS activity increased gradually from 3 – 12 months crude extract in Rayong 1 whereas in KU 50 SSS increased to a maximum level at 9 months. At 9 months, SSS in KU 50 were about 5 times higher than Rayong 1, but the level of SSS in Rayong 1 still increased at 12 months while SSS in KU 50 dropped. Therefore, 9 month old tubers of KU 50 was used as source of SSS for purification and characterization study because of the much higher level of the enzyme and the shortly harvest time of the cultivar when SSS attained maximum level.

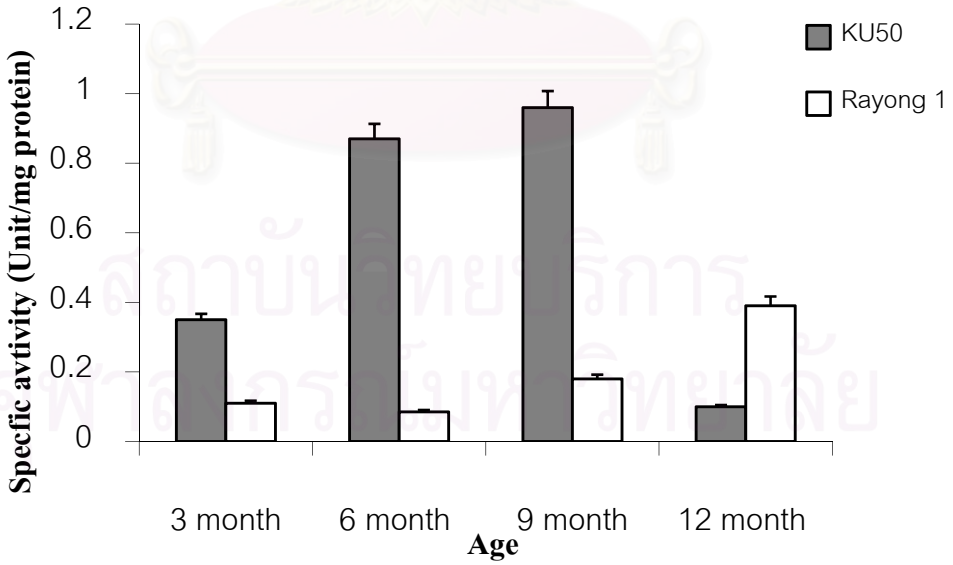


Figure 3.1 Comparison of soluble starch synthase activity in tubers of cassava cultivar Rayong 1 and Kasetsart 50 at different ages

3.2 Purification of starch synthase from cassava tuber cultivar Kasetsart 50

3.2.1 Preparation of crude enzyme

Crude SSS from tubers of 9 month old KU 50 was prepared from parenchyma tissue as described in section 2.4.1. The crude SSS fraction contained 2,276 mg proteins and 2,967 units of SSS activity in total volume of 400 m1 from starting material of 5 kg tubers.

3.2.2 Ammonium sulfate precipitation

Crude SSS was purified by ammonium sulfate precipitation as described in section 2.4.2. SSS activity was monitored by the radioactive method (section 2.5.2). Preliminary experiment to determine the suitable ammonium sulfate concentration for precipitation of starch synthase was performed by step-wise increase of ammonium sulfate at 10 % increment showed starch synthase activity in the 20-30, 30-40, 40-50, and 50-60% with highest activity in 30-40%. To harvest most of SSS, protein fractionation was performed in the range of 20-60% saturated ammonium sulfate precipitation. The pellet was dissolved in 50 mM Tris-acetate buffer pH 8.5 containing 1M ammonium sulfate, 10 mM EDTA, 2 mM DTT and stored for the next step of purification. The protein remained was 766.92 mg with starch synthase activity recovered at 2,400 units, about 80.8 % recovery from crude enzyme.

3.2.3 Phenyl Sepharose column chromatography

Cassava starch synthase from 20-60% saturated ammonium sulfate precipitation was dissolved in 50 mM Tris-acetate pH 8.5 containing 1 M ammonium sulfate, 10 mM EDTA and 2 mM DTT and centrifuged. The supernatant was loaded onto Phenyl sepharose column chromatography as described in section 2.4.3. The chromatographic profile was shown in Figure 3.2.

The unbound proteins were eluted from Phenyl Sepharose with elution buffer containing 1 M ammonium sulfate until SSS activity decreased to base line. The other proteins, which were bound to the column, were similarly eluted by step-wise method with elution buffer containing 0.5, 0.2, and 0.1 M ammonium sulfate. Starch synthase activity was monitored by radioactive method as described in section 2.5.2. Starch synthase activity was found in peaks eluted with 1M ammonium sulfate (unbound protein and named SSS1), One activity peaks was eluted at 0.5 M ammonium sulfate and named SSS2 and the third activity peak was eluted by elution buffer without salt and named SSS3 (Figure 3.2). Each activity peak was pooled and concentrated by aquasorb. The protein and SSS activity obtained in each peak was 21.42, 16.50, 15.12 mg protein with 238.39, 150.02 and 227.33 activity units for SSS1, SSS2 and SSS3 respectively. SSS1, SSS2 and SSS3 were purified by 9, 7, 12 folds respectively.

3.2.4 Q-Sepharose column chromatography

The pooled SSS1, SSS2 and SSS3 fraction from Phenyl sepharose was separately loaded onto Q-Sepharose column chromatography as described in section 2.4.4.

3.2.4.1 Soluble starch synthase1 (SSS1)

The unbound proteins were eluted from Q-Sepharose column with elution buffer. Starch synthase bound to Q-Sepharose column was eluted with linear gradient from 0 M - 0.5 M potassium chloride. The enzyme was eluted at 0.16 M potassium chloride (Figure 3.3). The activity fractions were collected and dialyzed against elution buffer. The dialyzed enzyme contained 1.12 mg protein with 619.54 activity units. The specific activity of the enzyme was 553.16 nmol of glucose incorporated/min/mg protein. From this step, the enzyme was purified to 426

folds.

3.2.4.2 Soluble starch synthase2 (SSS2)

The unbound proteins were eluted from Q-Sepharose column with elution buffer. Starch synthase bound to Q-Sepharose column was eluted with linear gradient from 0 M - 0.5 M potassium chloride. The enzyme was eluted at 0.18 M potassium chloride (Figure 3.4). The activity fractions were collected and dialyzed against elution buffer. The dialyzed enzyme contained 0.22 mg protein with 146.78 activity units. The specific activity of the enzyme was 667.14 nmol of glucose incorporated/min/mg protein. From this step, the enzyme was purified to 513 folds.

3.2.4.3 Soluble starch synthase3 (SSS3)

The unbound proteins were eluted from Q-Sepharose column with elution buffer. Starch synthase bound to Q-Sepharose column was eluted with linear gradient from 0 M - 0.5 M potassium chloride. The enzyme was eluted at 0.14 M potassium chloride (Figure 3.5). The activity fractions were collected and dialyzed against elution buffer. The dialyzed enzyme contained 0.86 mg protein with 158.73 activity units. The specific activity of the enzyme was 184.57 nmol of glucose incorporated/min/mg protein. From this step, the enzyme was purified to 142 folds.

3.2.5 Summary of cassava soluble starch synthase purification

Cassava soluble starch synthase was extracted and purified by ammonium sulfate precipitation and column chromatography as described previously. The summary of this enzyme is shown in table 3.1

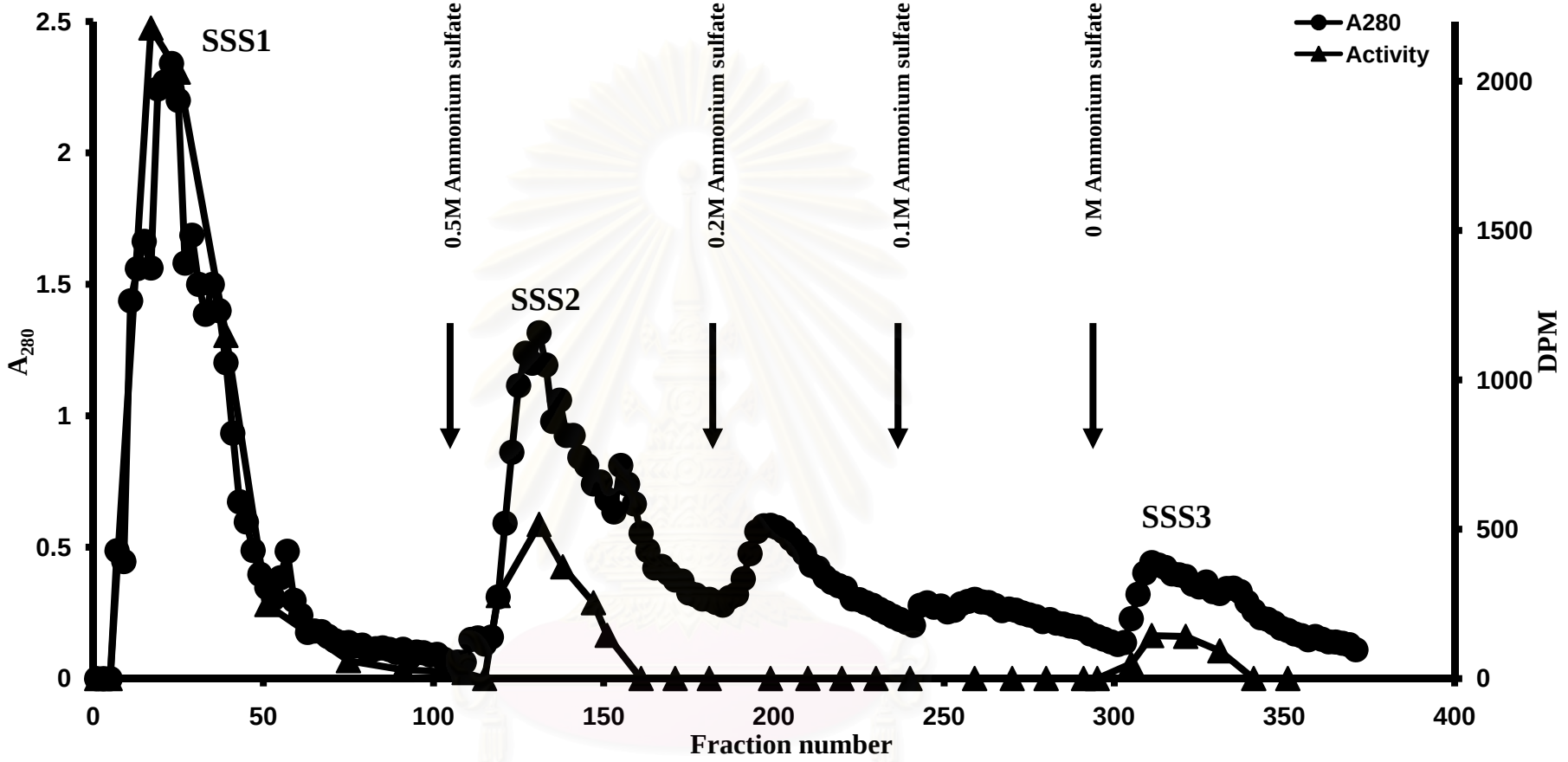


Figure 3.2 Chromatographic profile of 20–60% saturated ammonium sulfate precipitated fraction from cassava parenchyma on Phenyl Sepharose High Performance

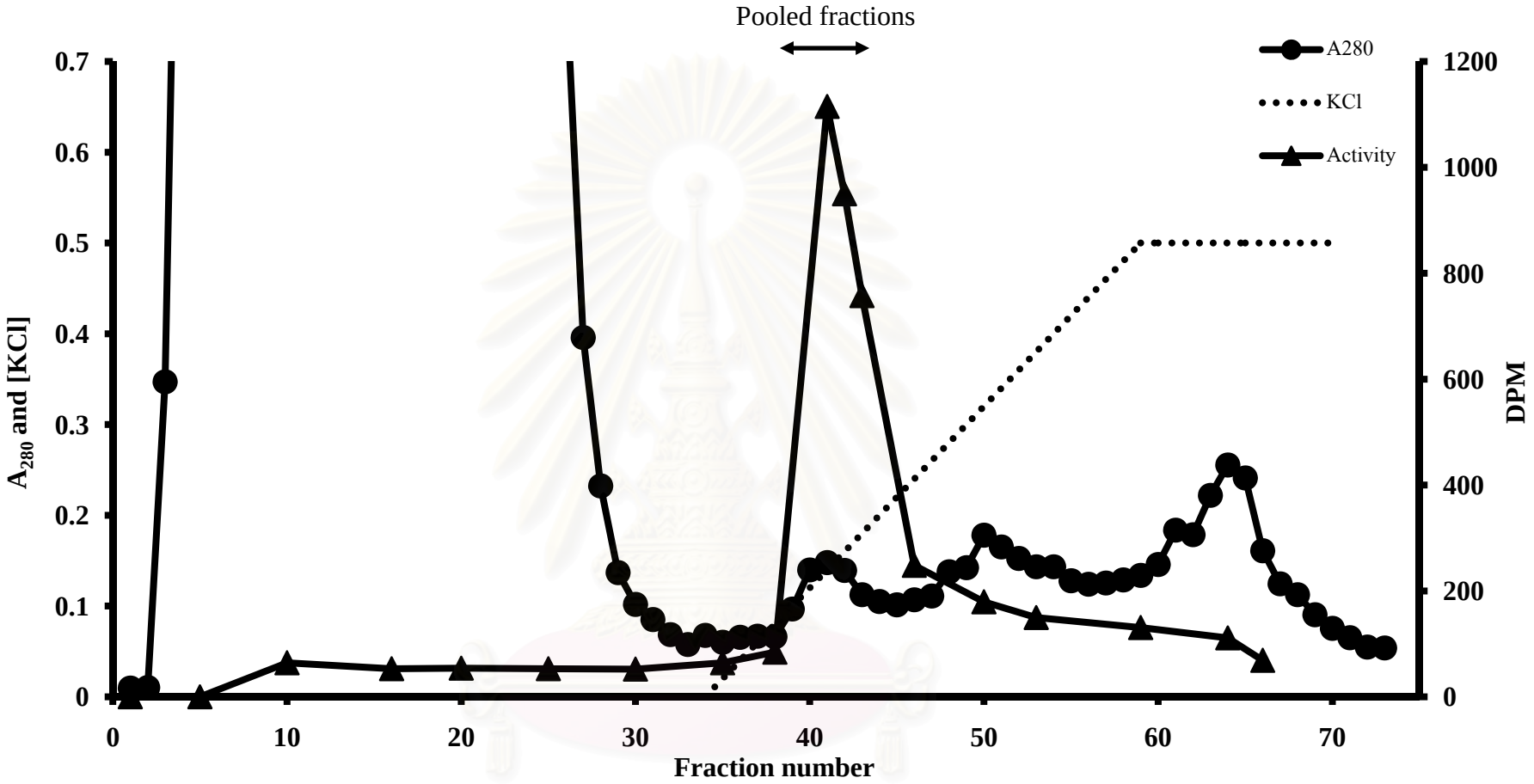


Figure 3.3 Chromatographic profile of SSS1 from Phenyl Sepharose column on Q-Sepharose column

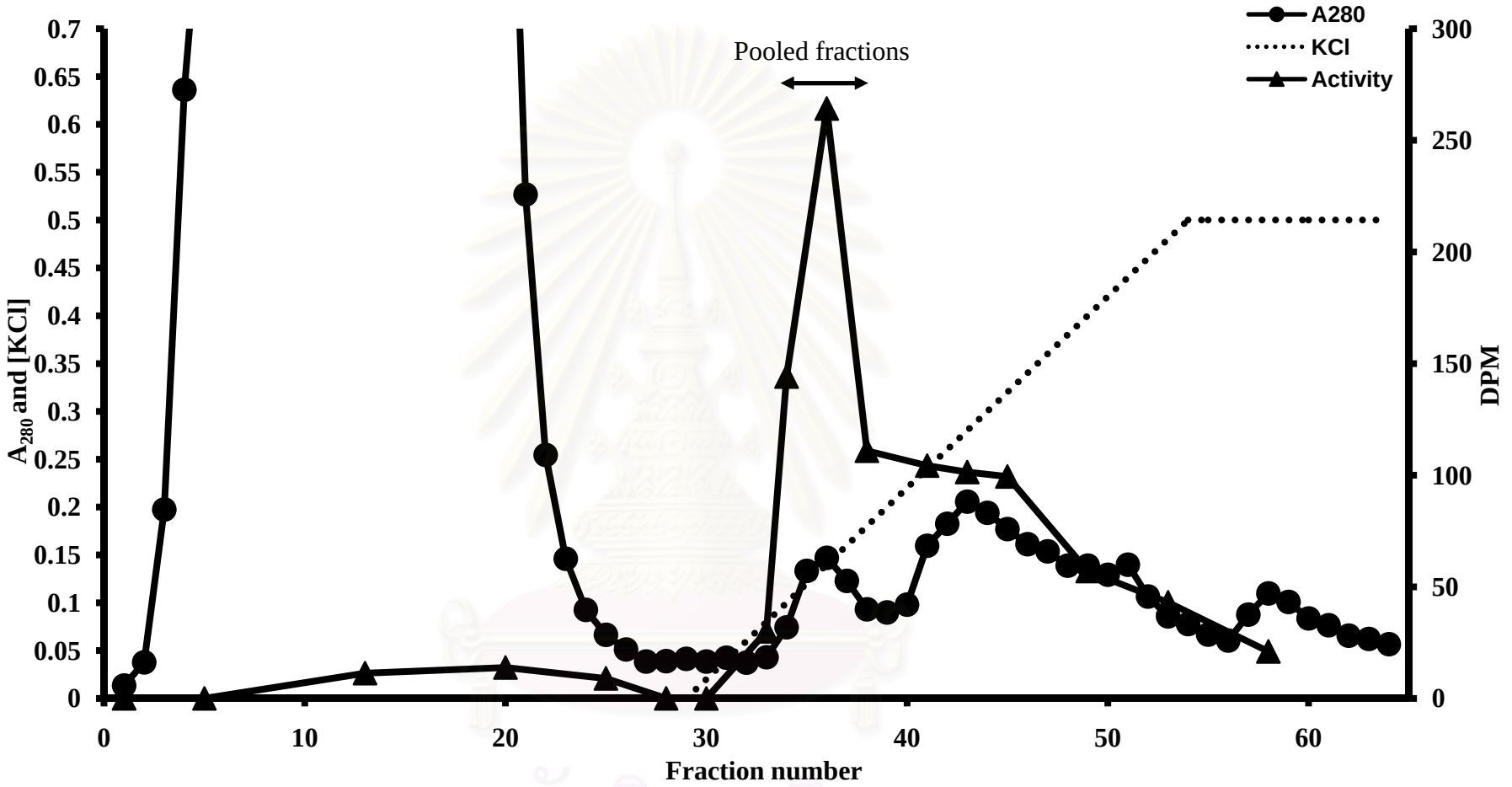


Figure 3.4 Chromatographic profile of SSS2 from Phenyl Sepharose column on Q-Sepharose column

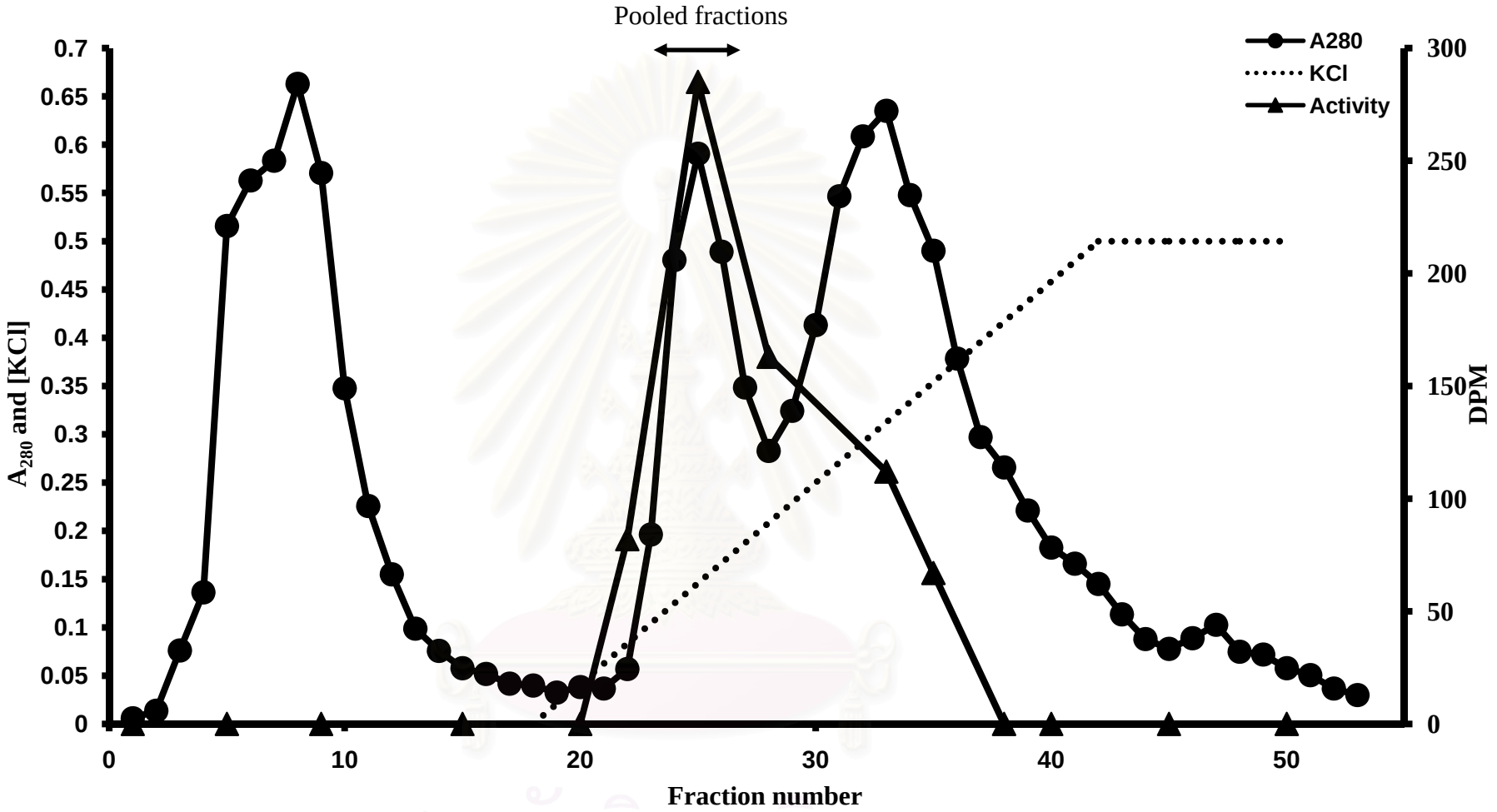


Figure 3.5 Chromatographic profile of SSS3 from Phenyl Sepharose column on Q-Sepharose column

Table 3.1 Purification table of cassava soluble starch synthase

Fraction	Total protein (mg)	Total Activity (unit)	Specific activity (unit/mg)	Purification fold	Recovery (%)
Crude	2276.00	2967.03	1.30	1	100
20-60% $(NH_4)_2SO_4$	766.92	2400.67	3.13	2	80.9
Phenyl Sepharose					
- SSS1	21.42	238.39	11.13	9	8
- SSS2	16.50	150.02	9.09	7	5
- SSS3	15.12	227.33	15.04	12	7
Q-Sepharose FF					
- SSS1	1.12	616.54	553.16	426	20
- SSS2	0.22	146.78	667.14	513	4
- SSS3	0.86	158.73	184.57	142	5

Activity unit : The nmol of glucose incorporated in 1 minute at 37 °C

3.3 Characterization of cassava soluble starch synthase

The three cassava soluble starch synthase isoforms purified from Q-Sepharose column chromatography were used for the characterization studies.

3.3.1 Determination of protein pattern on polyacrylamide gel electrophoresis

3.3.1.1 Non-denaturing glycogen-PAGE

The enzyme from each step of purification was analyzed on Non-denaturing glycogen-PAGE and stain activity as described in section 2.7.1. The activity band of SSS from cassava was clearly visible as brown color in crude enzyme, especially in highly concentrate enzyme. Staining of the less concentrated crude SSS and the three isoforms appeared as smear of brown color with more intense bands in the crude fraction.

3.3.1.2 SDS-polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed for purity and protein pattern by SDS-PAGE (Figure 3.7) as described in section 2.7.2.

SSS1 was showed 1 protein band with the molecular weight in denaturing state of 81.7 kDa. SSS2 showed 3 bands with the molecular weight of 112, 90.2 and 81.7 kDa. SSS3 was showed 2 bands with the molecular weight of 90.2 and 79.8 kDa.

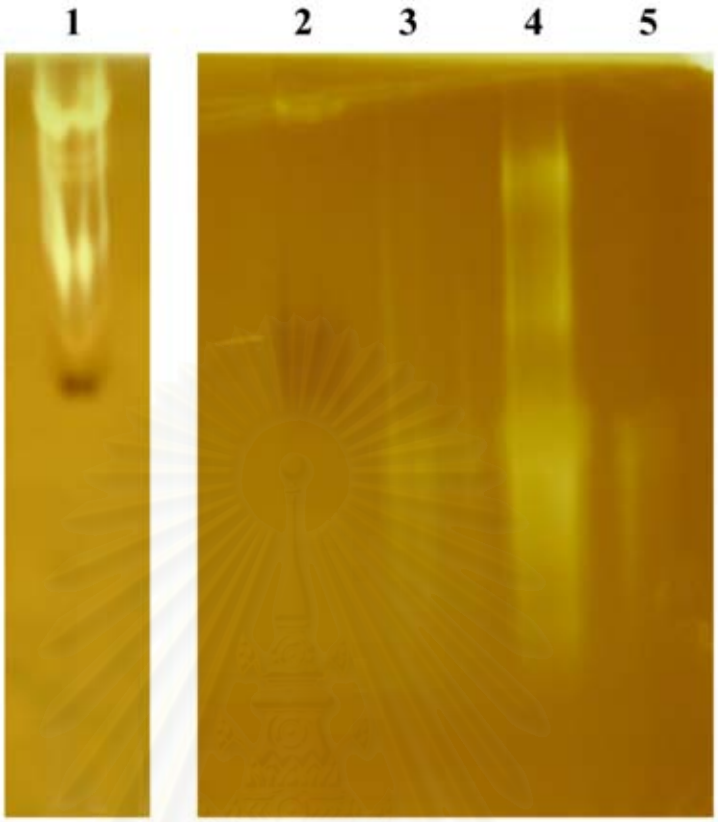


Figure 3.6 Non-denaturing glycogen-PAGE of SSS from different purification steps

Lane 1 : highly concentration crude SSS (≈150 µg protein)

Lane 2 : Crude SSS (30 µg protein)

Lane 3 : SSS1 from Q-sepharose column (15 µg protein)

Lane 4 : SSS2 from Q-sepharose column (15 µg protein)

Lane 5 : SSS3 from Q-sepharose column (15 µg protein)

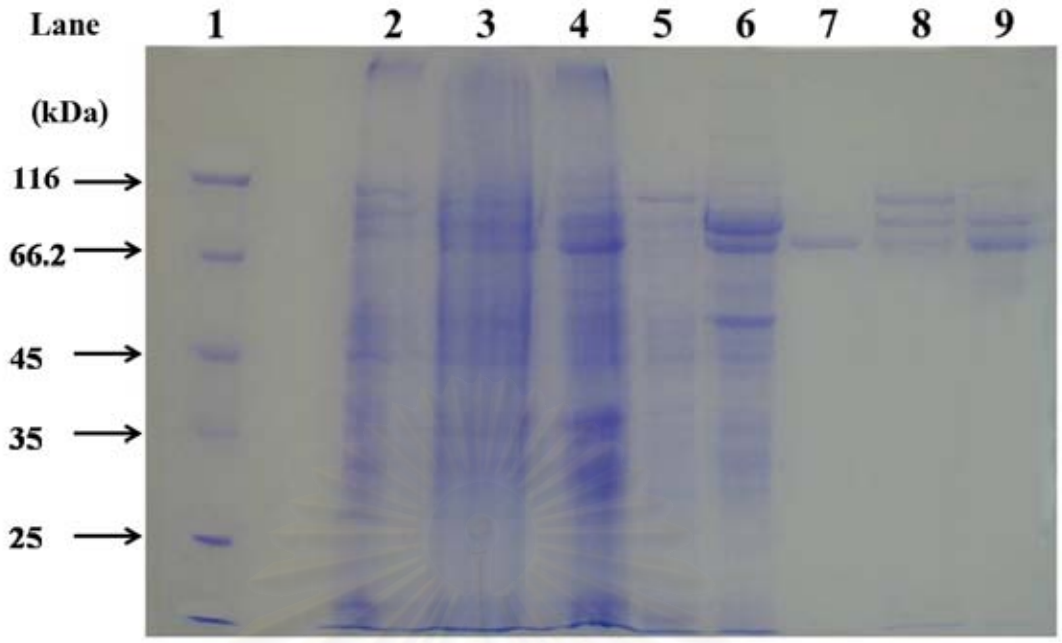


Figure 3.7 SDS-PAGE analysis of soluble starch synthase from cassava tubers in each step of purification on 10% polyacrylamide gel

Lane 1 : Protein molecular weight marker
[β-galactosidase (116kDa), Bovine serum albumin (66.2kDa), Ovalbumin (45kDa), Lactate dehydrogenase (35kDa), REase Bsp98I (25kDa), β-galactoglobulin (18.4kDa) and Lysozyme (14.4kDa)]

Lane 2 : Crude enzyme (30 µg protein)

Lane 3 : 20 – 60% ammonium sulfate precipitation (30 µg protein)

Lane 4 : SSS1 from phenyl sepharose column chromatography (20 µg protein)

Lane 5 : SSS2 from phenyl sepharose column chromatography (20 µg protein)

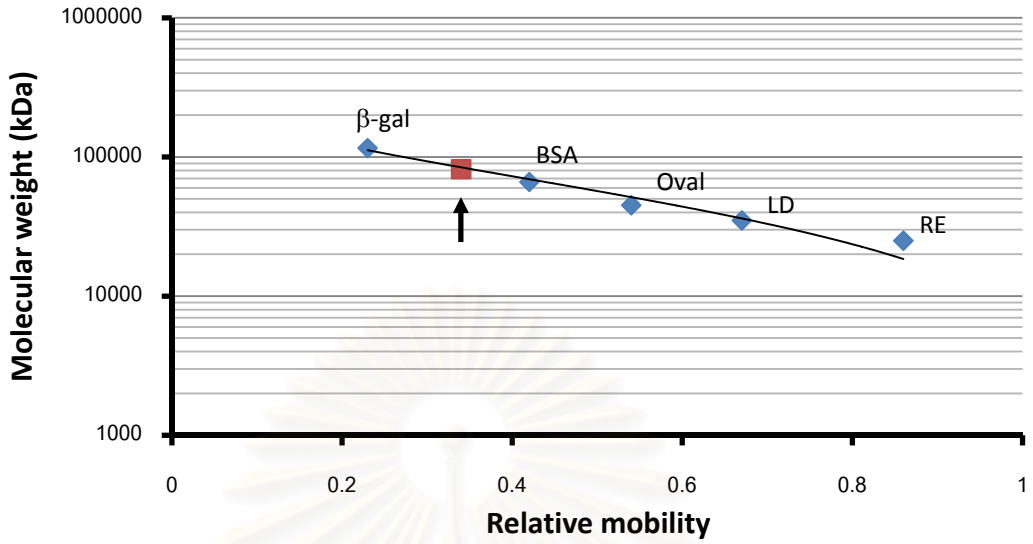
Lane 6 : SSS3 from phenyl sepharose column chromatography (20 µg protein)

Lane 7 : SSS1 from Q-sepharose column chromatography (15 µg protein)

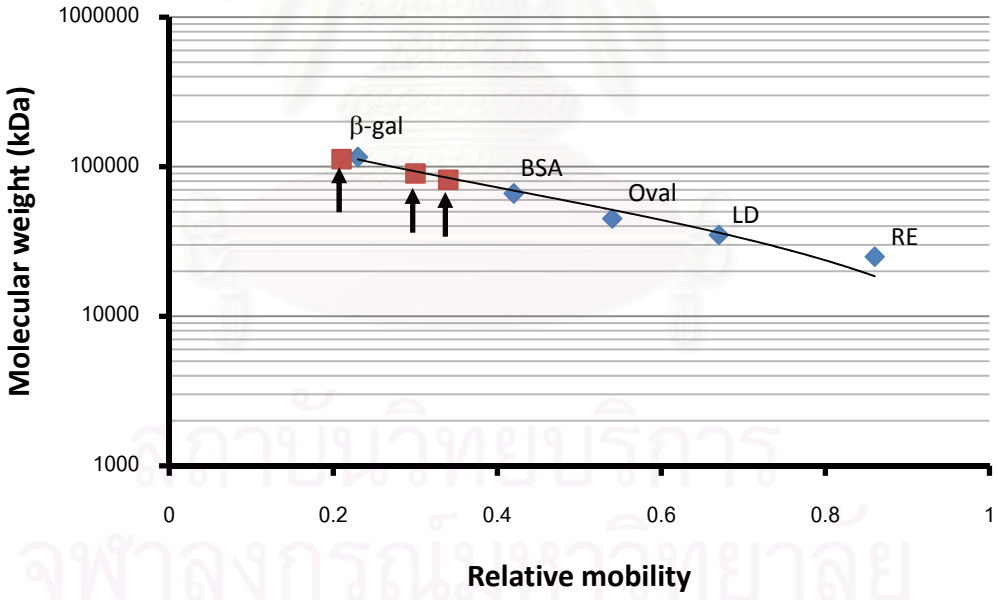
Lane 8 : SSS2 from Q-sepharose column chromatography (15 µg protein)

Lane 9 : SSS3 from Q-sepharose column chromatography (15 µg protein)

(A)


1000000
100000
10000
1000
Molecular weight (kDa)
β-gal
BSA
Oval
LD
RE
0
0.2
0.4
0.6
0.8
1
Relative mobility

(B)


1000000
100000
10000
1000
Molecular weight (kDa)
β-gal
BSA
Oval
LD
RE
0
0.2
0.4
0.6
0.8
1
Relative mobility

(C)

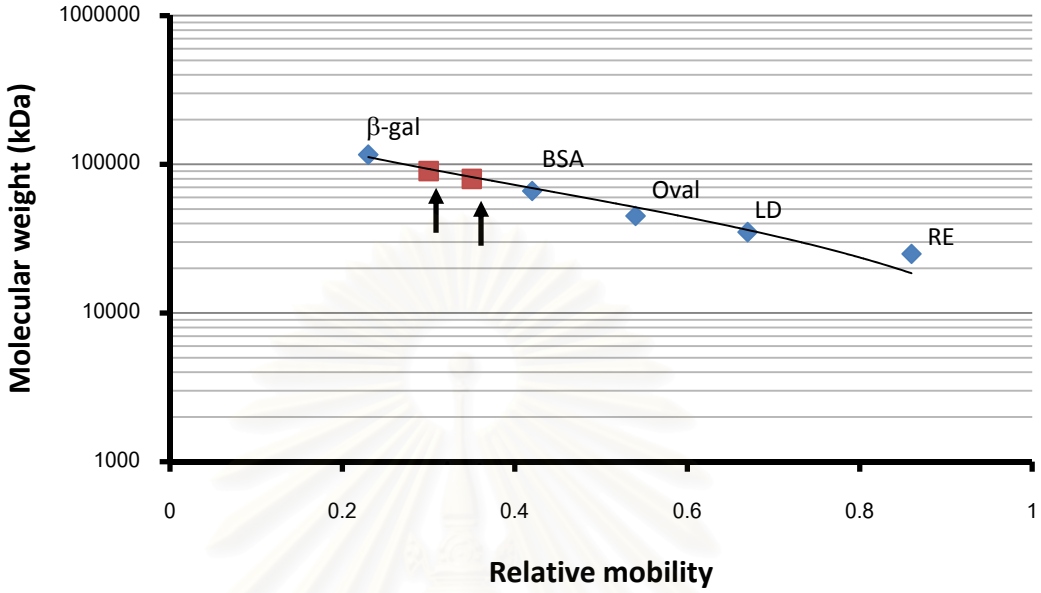


Figure 3.8 Calibration curve for molecular weight on SDS-PAGE of SSS1 (A), SSS2 (B) and SSS3 (C)

β-gal	= β-galactosidase	MW = 116 kDa
BSA	= Bovine serum albumin	MW = 66.2 kDa
Oval	= Ovalbumin	MW = 45 kDa
LD	= Lactate dehydrogenase	MW = 35 kDa
RE	= REase Bsp98I	MW = 25 kDa

3.3.2 Optimum pH of cassava soluble starch synthase activity

Cassava soluble starch synthases were assayed at various pH's as described in section 2.8.1. The results were shown in Figure 3.9. Soluble starch synthase activity was expressed as % relative activity with the highest activity referred as 100%.

SSS1 showed highest activity or optimum pH at 9.5 and this was defined as 100% activity. At pH 7.0 - 9.0, SSS1 activity was in the range 70 – 80% Below pH 7.0, SSS1 activity rapidly dropped to near zero at pH 4.0. At pH 11.0. SSS1 activity was retained at 80%.

SSS2 showed highest activity or optimum pH at 7.0 and this was defined as 100% activity. At pH 7.0-9.0, SSS2 activity was over 80% and gradually dropped to 60% at pH 11.0. Below pH 7.0, SSS2 activity gradually dropped to about 30% at pH 4.0.

SSS3 showed highest activity or optimum pH at 9.5 and this was defined as 100% activity. The activity profile of SSS3 was almost similar to SSS1 except SSS3 activity at pH lower than 7.0 dropped slowly and activity was still retained at around 20% at pH 4.0.

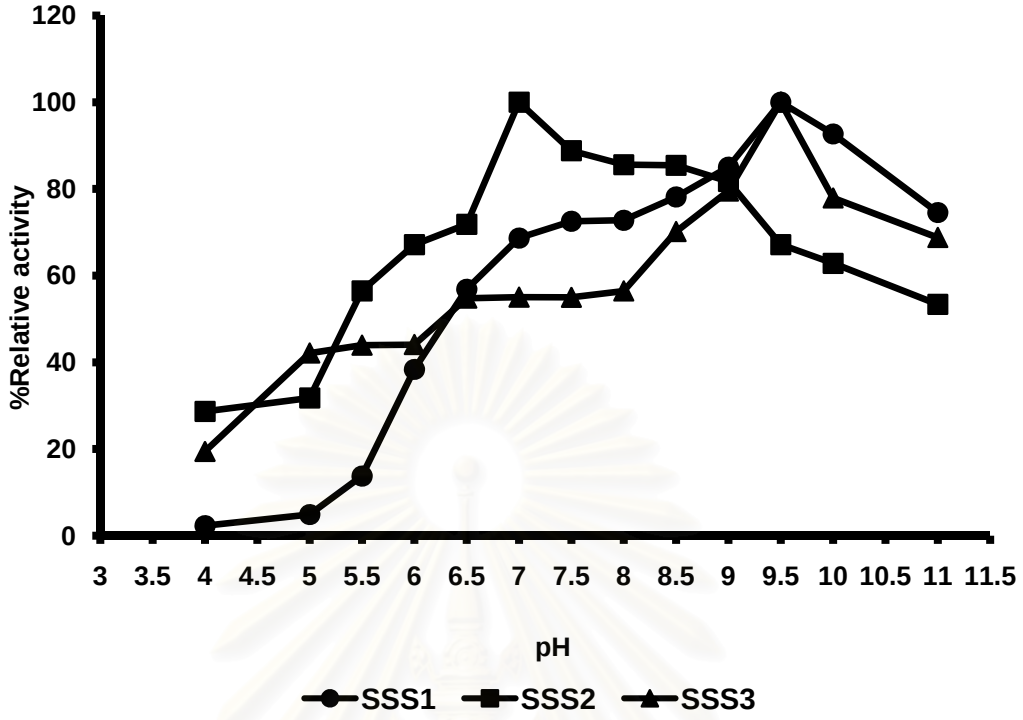


Figure 3.9 Effect of pH on cassava soluble starch synthase activity

3.3.3 Optimum temperature of cassava soluble starch synthase

Cassava soluble starch synthase was assayed at various temperatures as described in section 2.8.1. The results were shown in Figure 3.10. Soluble starch synthase activity was expressed as % relative activity with the highest activity referred as 100%.

SSS1 and SSS3 showed highest activity at 37 ◦C and was defined as 100% activity. SSS3 showed higher activity at temperatures above and below 37 ◦C. At 20 ◦C SSS2 and SSS3, retained 50% activity while SSS1 retained only 25% activity. At temperature range 45 – 60 ◦C SSS3 retained 25% of it activity but SSS1 and SSS2 rapidly lost then activities to zero. SSS2 showed the highest activity at 35 ◦C which was defined as 100% activity. Between 25 ◦C and 40 ◦C, SSS2 retained it activity above 60%.

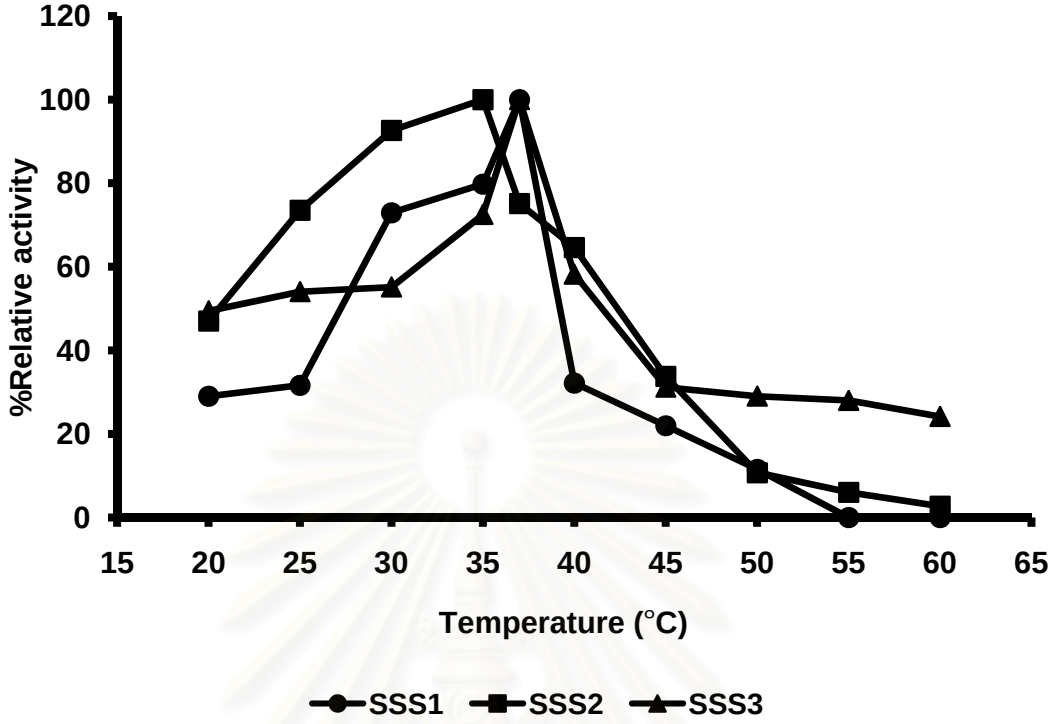


Figure 3.10 Effect of temperatures on cassava soluble starch synthase activity

3.3.4 Temperature stability of cassava soluble starch synthase

Cassava soluble starch synthase was pre-incubated at various temperatures for 30 minutes before assayed as described in section 2.8.3. The result was shown in Figure 3.11. The soluble starch synthase activity of non pre-incubated enzyme was defined as 100% activity. Its activity was significantly reduced when the temperature was over 37 °C.

The enzyme was further incubation at 37 °C and checked for its activity every 6 hours. The result was shown in Figure 3.12. The starch synthase activity at start was defined as 100% activity. After 3 hours the enzymes activity started to decline. SSS3 was more stable than SSS2 and SSS1. After being left at 37 °C for 36 hours, SSS3 and SSS2 retained 40% and 30% activity respectively, while activity SSS1 dropped to almost zero.

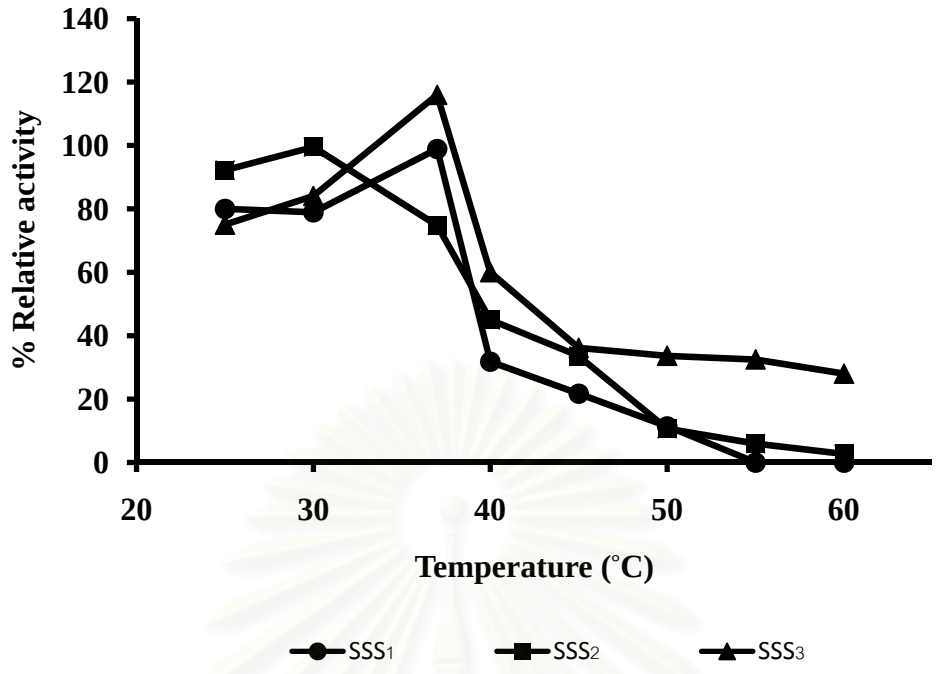


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Figure 3.11 Temperature stability of cassava starch synthase

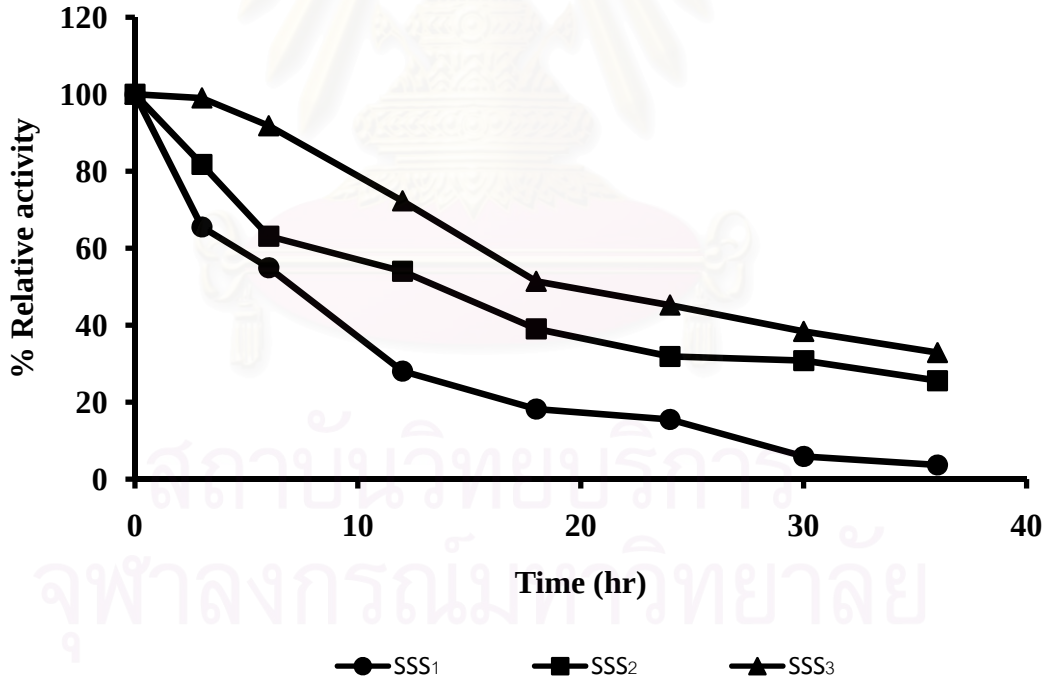

Figure 3.12 SSS stability at 37 ˚C

3.3.5 Effect of primer on cassava soluble starch synthase activity

Cassava starch synthase was assayed using various primers as described in section 2.8.4. The primers can be defined into 3 groups. First, the homopolysaccharides of glucose, rabbit liver glycogen (RLG), oyster glycogen (OG) and amylopectin (AP). The second group is starch from different sources: cassava, rice, potato, and corn, and the last group were un-branched oligosaccharides potato amylose (AM), maltohexose and maltopentaose. The effects of primers were shown in Table 3.2 and Figure 3.13. The starch synthase activity when rabbit liver glycogen was used as primer which was highest was defined as 100% activity.

For branched homopolysaccharides of glucose SSS1, SSS2 and SSS3 can use glycogens better than amylopectin with SSS3 showed lowest activity towards oyster glycogen(OG). For primer in the starch group, SSS1 showed higher activity towards, cassava starch than other starches SSS2 and SSS3 showed the highest activity with rice starch. All three enzymes showed less activity towards amylose and malto-oligosaccharides.

Table 3.2 Effect of primer on cassava soluble starch synthase activity

Primer	%Relative activity		
	SSS1	SSS2	SSS3
Rabbit liver glycogen*	100	100	100
Oyster glycogen *	115	86	70
Potato amylopectin *	94	97	102
Cassava starch *	91	92	65
Rice starch *	78	108	95
Potato starch*	63	61	73
Corn starch *	68	91	90
Potato amylose *	18	46	37
Maltopentaose**	6	29	28
Maltohexaose**	12	40	47

* Concentration 20 mg/ml **Concentration 20mM

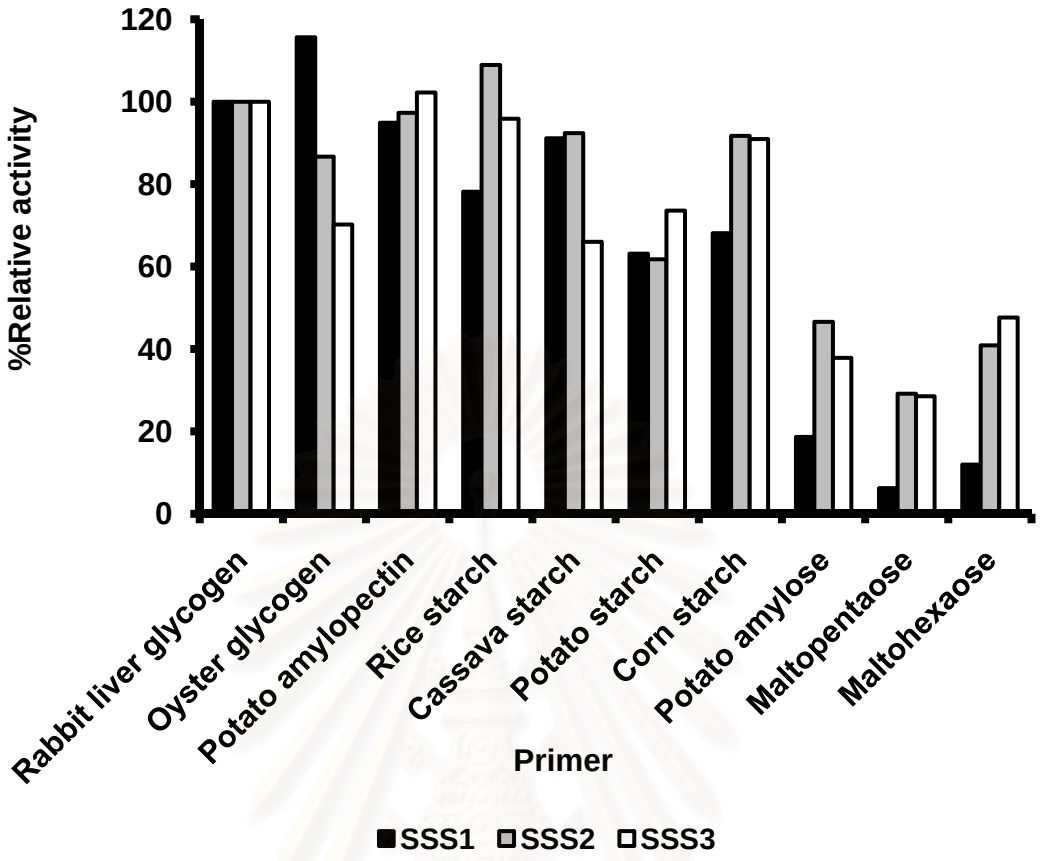

Figure 3.13 Comparison of the effect of primers on cassava soluble starch synthase activity

3.3.6 Effect of sulfhydryl reagent on cassava soluble starch synthase activity

Cassava soluble starch synthase was assayed with added sulfhydryl reagent to determine the effect of SH-group on its activity as described in section 2.8.5. The result was shown in Figure 3.14 Control was the reaction mixture without adding sulfhydryl reagent. DTT showed significant activation effect on SSS1 and SSS2 but no effect on SSS3. Other reagents showed inhibitory effects on all isoforms at all concentration.

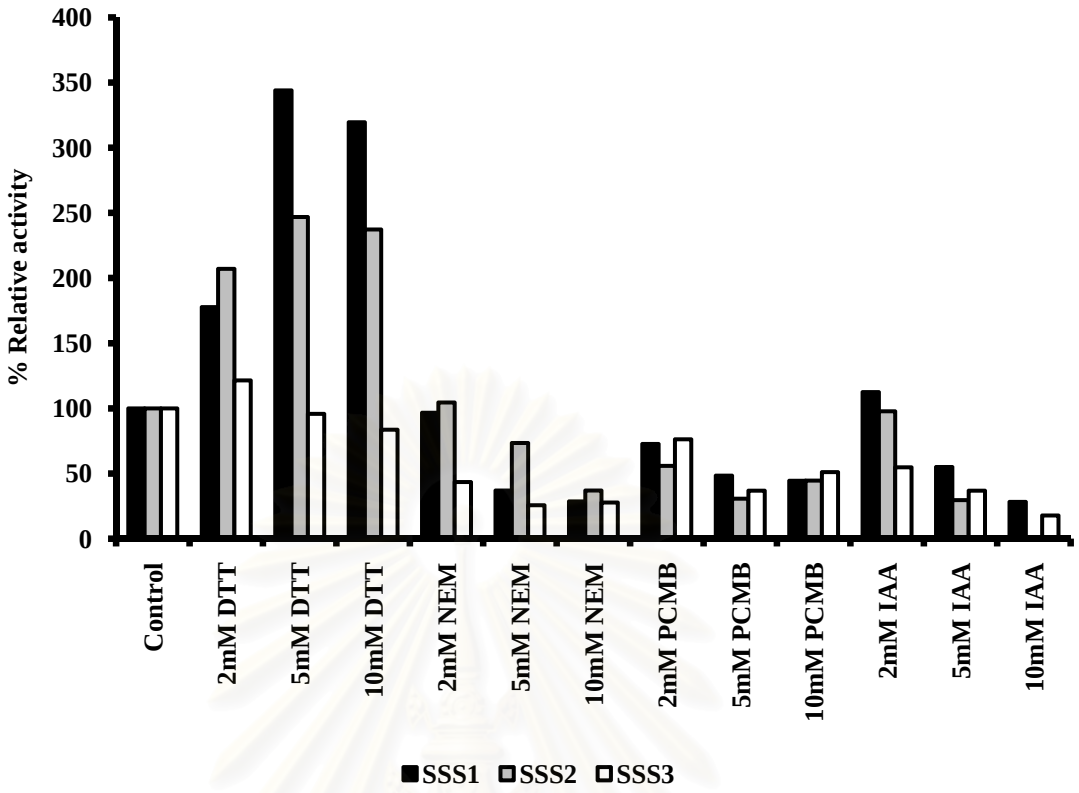

Figure 3.14 Effect of sulfhydryl reagents on soluble starch synthase activity

3.3.7 Kinetic constant for ADP-glucose

Cassava starch synthase was assayed at 37 °C for 15 minutes as described in section 2.5.1. The saturated curve of ADP-glucose were shown in Figure 3.15, 3.19 and 3.23 and Lineweaver-Burk plot were shown in Figure 3.16, 3.20 and 3.24 for SSS1, SSS2 and SSS3 respectively. The K_m and V_{max} values were summarized in Table 3.3.

3.3.8 Kinetic constant for rabbit liver glycogen

All three cassava starch synthases were assayed at 37 °C for 15 minutes as described in section 2.5.1. The rabbit liver glycogen was used as primer because it showed highest activity as primer for 3 starch synthase isoforms as shown in section 3.3.4. The saturated curves of rabbit liver glycogen were shown in Figures 3.17, 3.21 and 3.25 and Lineweaver-Burk plot were shown in Figures 3.18, 3.22 and 3.26 for SSS1, SSS2 and SSS3 respectively. The K_m and V_{max} were summarized in Table 3.3.

Table 3.3 Kinetic constant for ADP-glucose and rabbit liver glycogen

Enzyme	ADP-glucose				Rabbit glycogen			
	K_m (mM)		V_{max}*		K_m (mg/ml)		V_{max}*	
	S	L	S	L	S	L	S	L
SSS1	0.08	0.09	13.80	14.50	1.00	1.46	10.3	13.15
SSS2	0.05	0.06	8.00	8.40	0.41	0.26	12.33	12.50
SSS3	0.09	0.08	8.80	8.54	0.25	0.13	14.40	14.28

* = nmol of glucose incorporated/min

S = Determination by saturation curve

L = Determination by Lineweaver-Burk plot

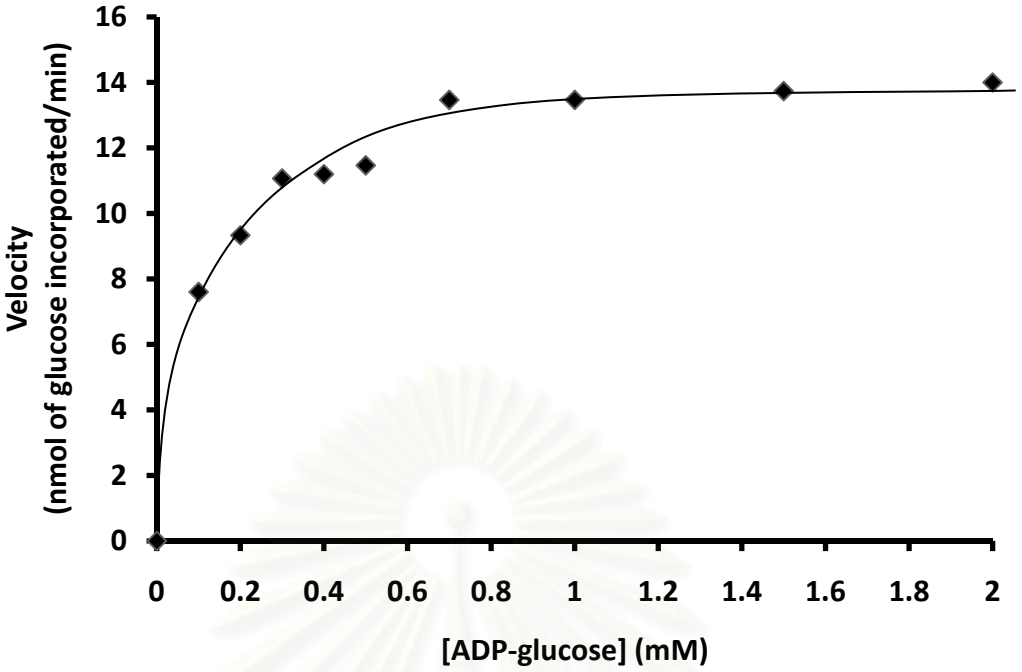

Figure 3.15 Saturation curve of ADP-glucose for cassava starch synthase1 activity (SSS1)

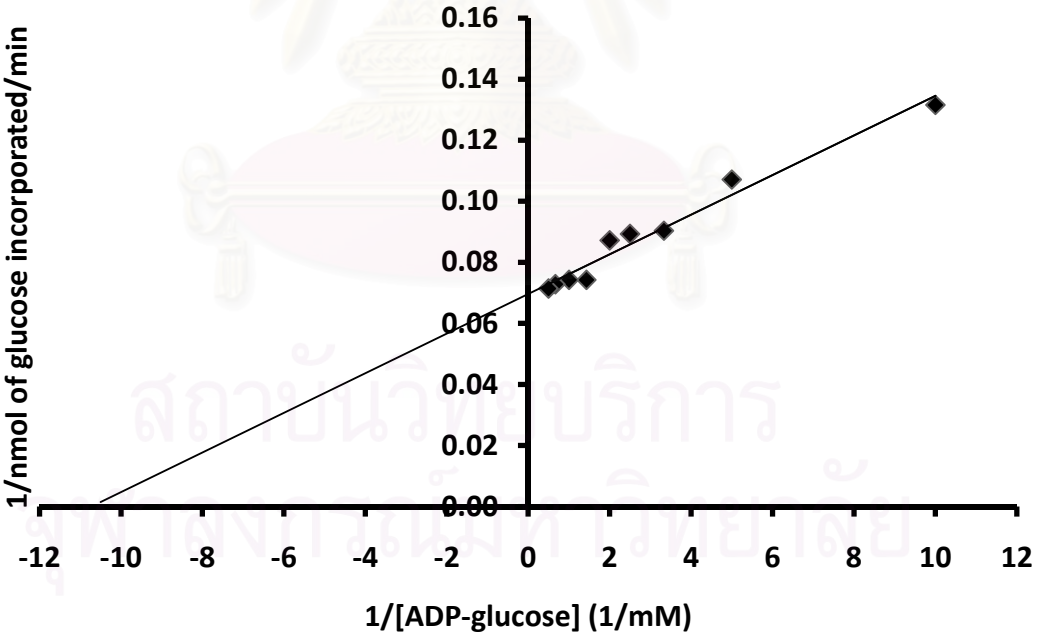

Figure 3.16 Lineweaver-Burk of ADP-glucose for cassava soluble starch synthase1 (SSS1)

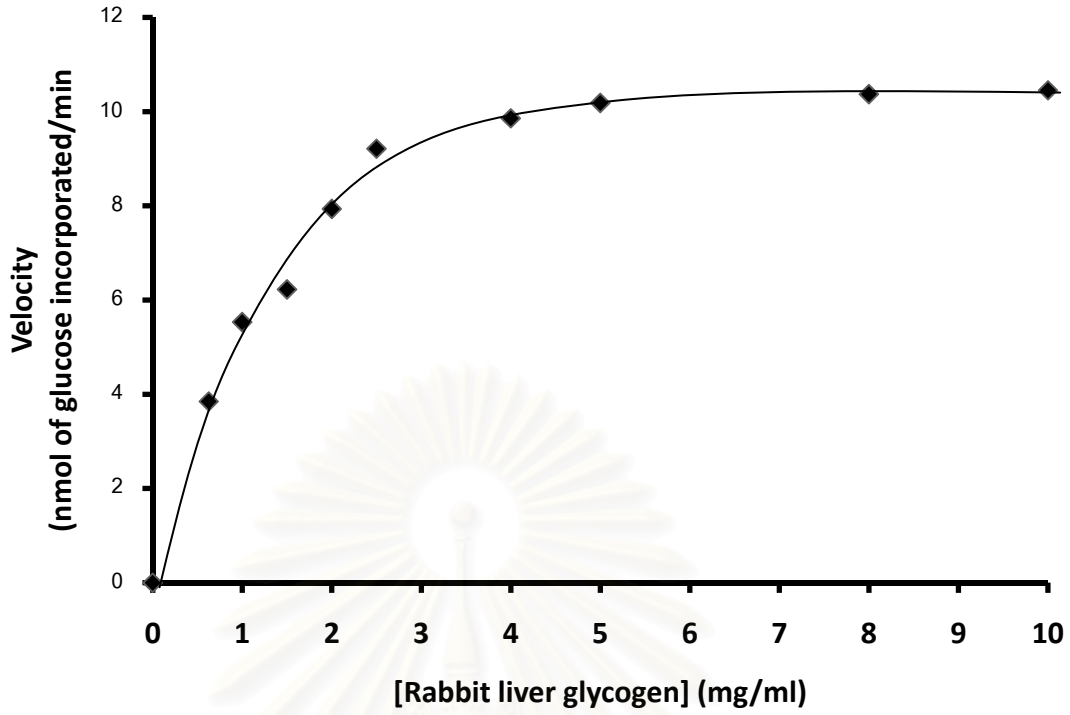

Figure 3.17 Saturation curve of rabbit liver glycogen for cassava starch synthase1 activity (SSS1)

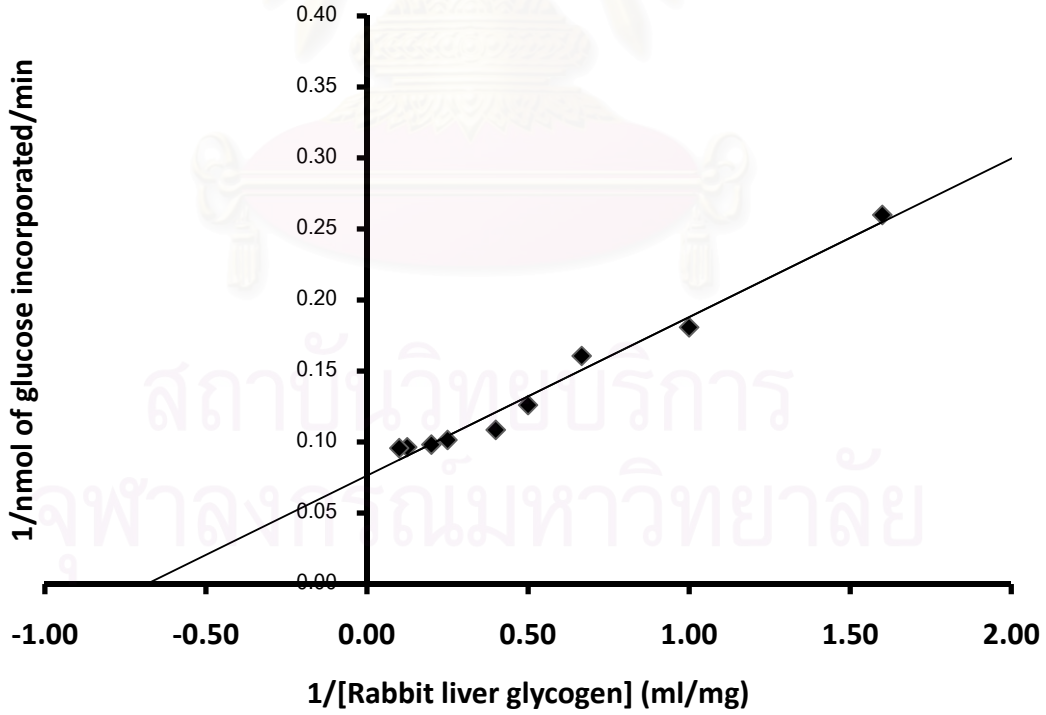

Figure 3.18 Lineweaver-Burk of rabbit liver glycogen for cassava soluble starch synthase1 (SSS1)

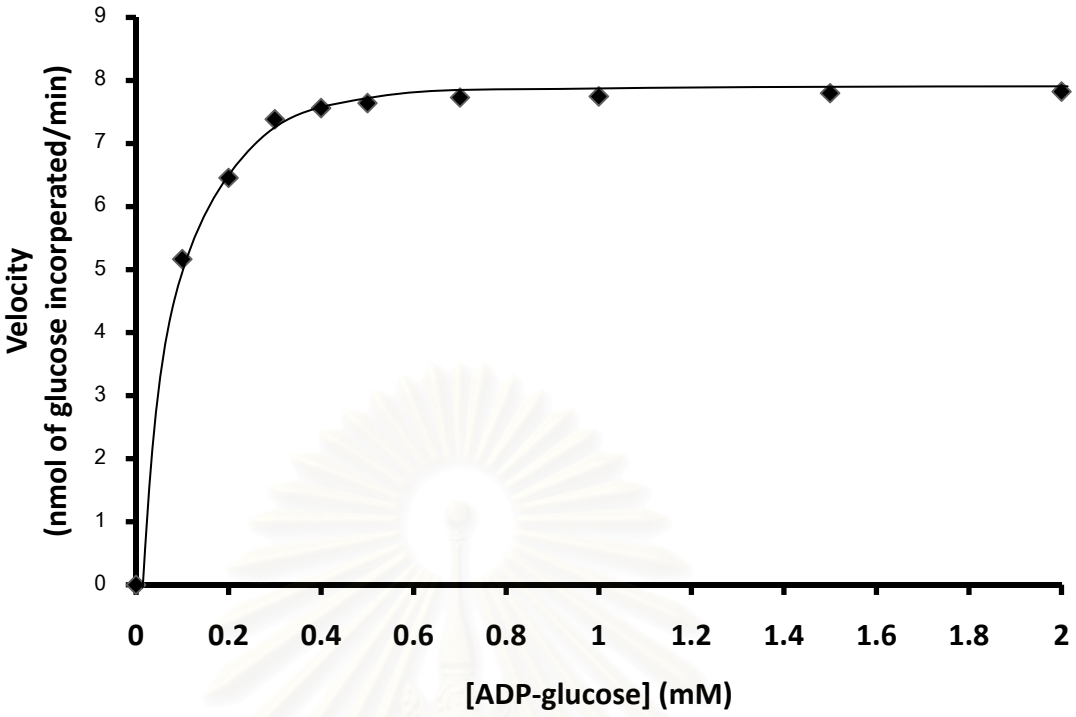

Figure 3.19 Saturation curve of ADP-glucose for cassava starch synthase2 activity (SSS2)

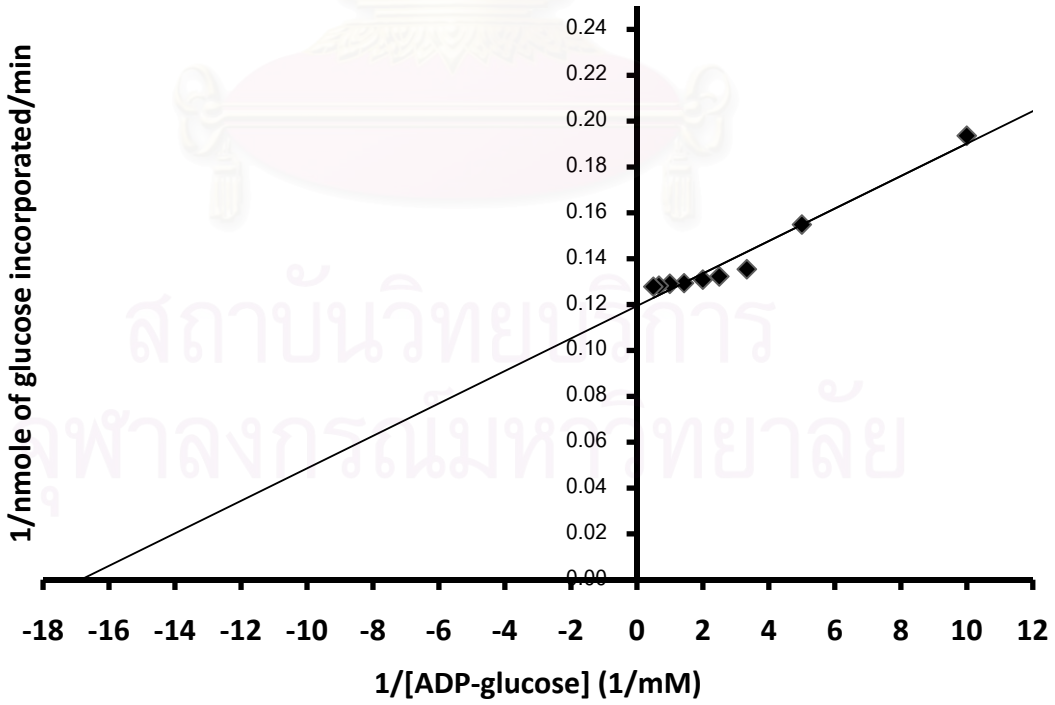

Figure 3.20 Lineweaver-Burk of ADP-glucose for cassava soluble starch synthase2 (SSS2)

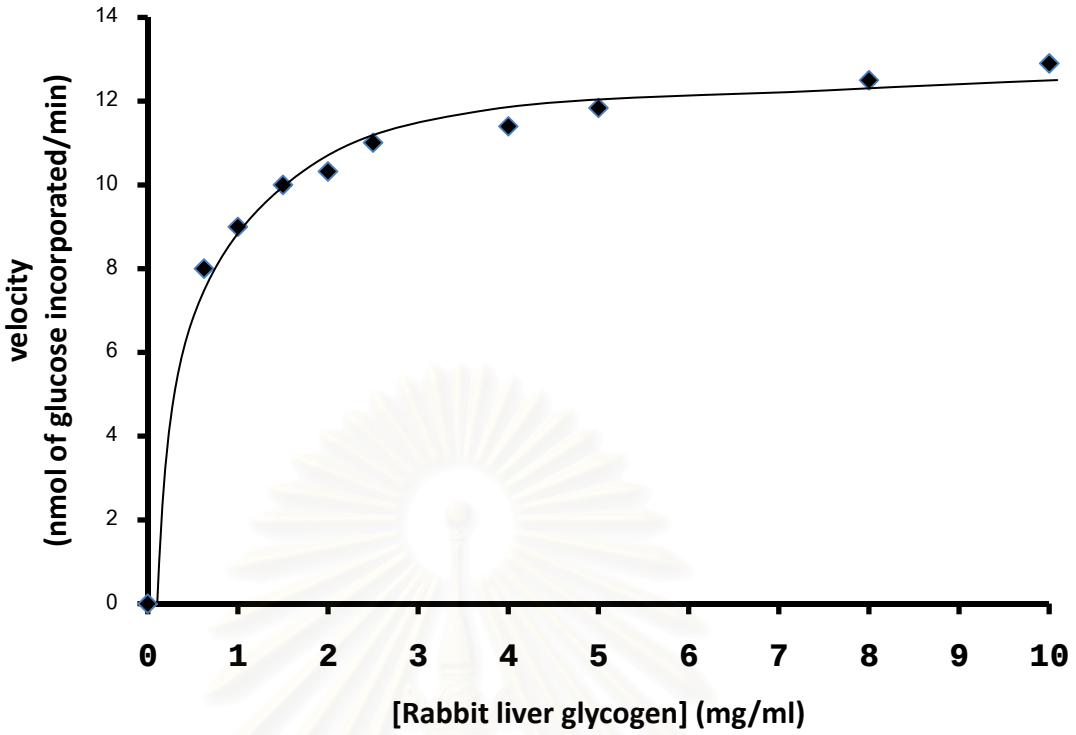

Figure 3.21 Saturation curve of rabbit liver glycogen for cassava starch synthase2 activity (SSS2)

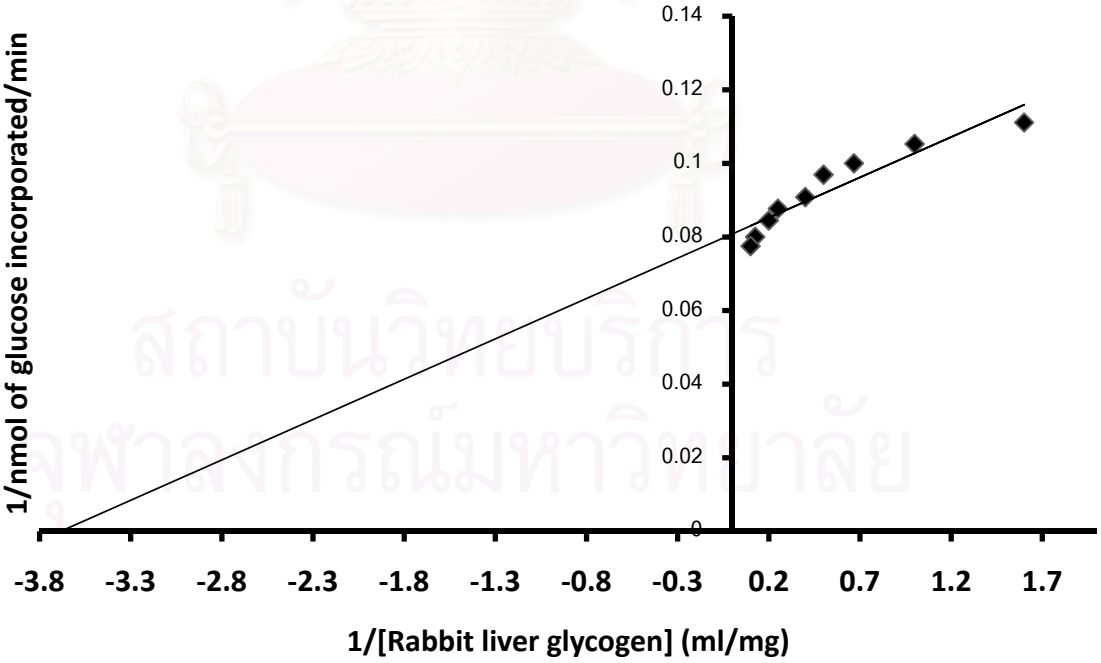

Figure 3.22 Lineweaver-Burk of rabbit liver glycogen for cassava soluble starch synthase2 (SSS2)

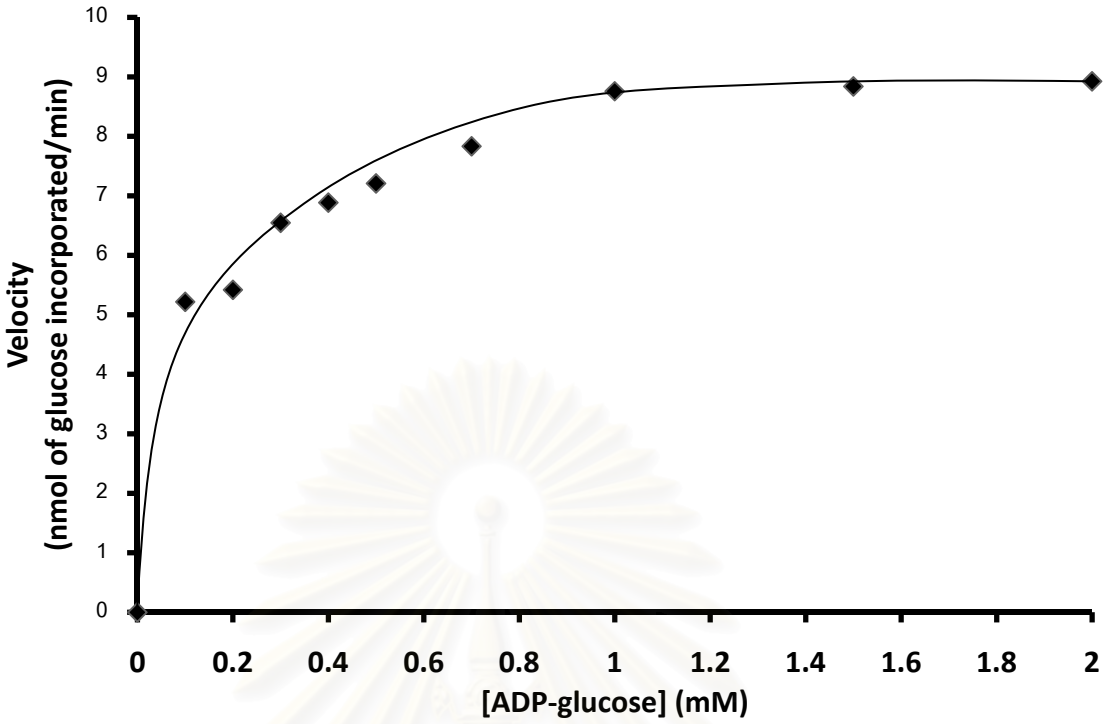

Figure 3.23 Saturation curve of ADP-glucose for cassava starch synthase3 activity (SSS3)

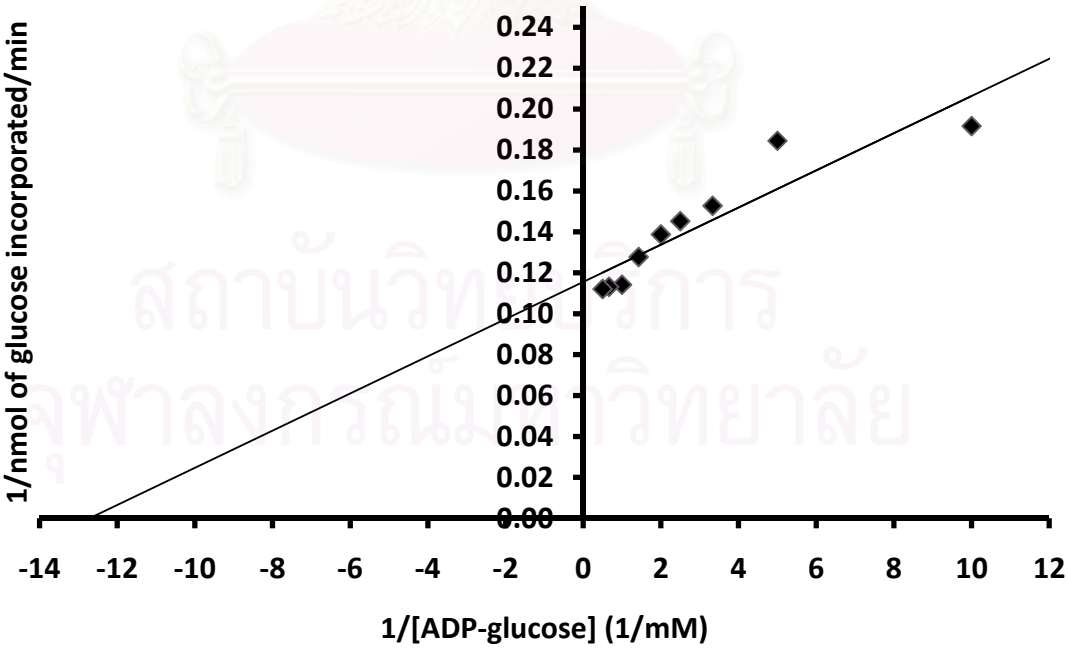

Figure 3.24 Lineweaver-Burk of ADP-glucose for cassava soluble starch synthase3 (SSS3)

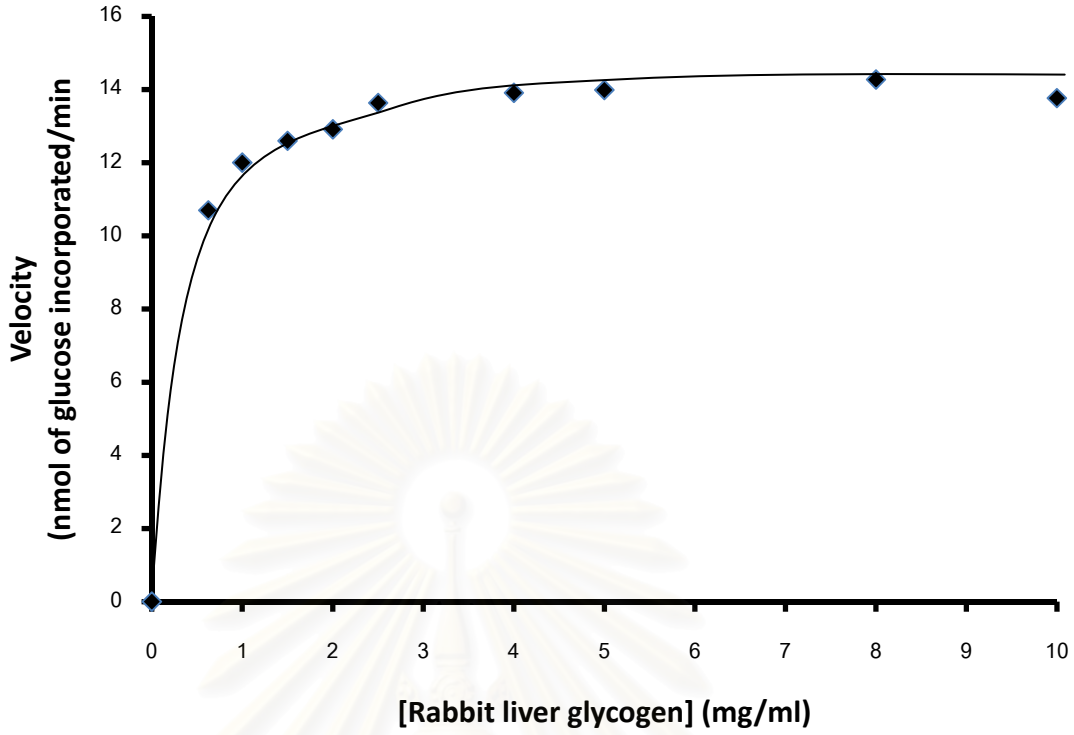

Figure 3.25 Saturation curve of rabbit liver glycogen for cassava starch synthase3 activity (SSS3)

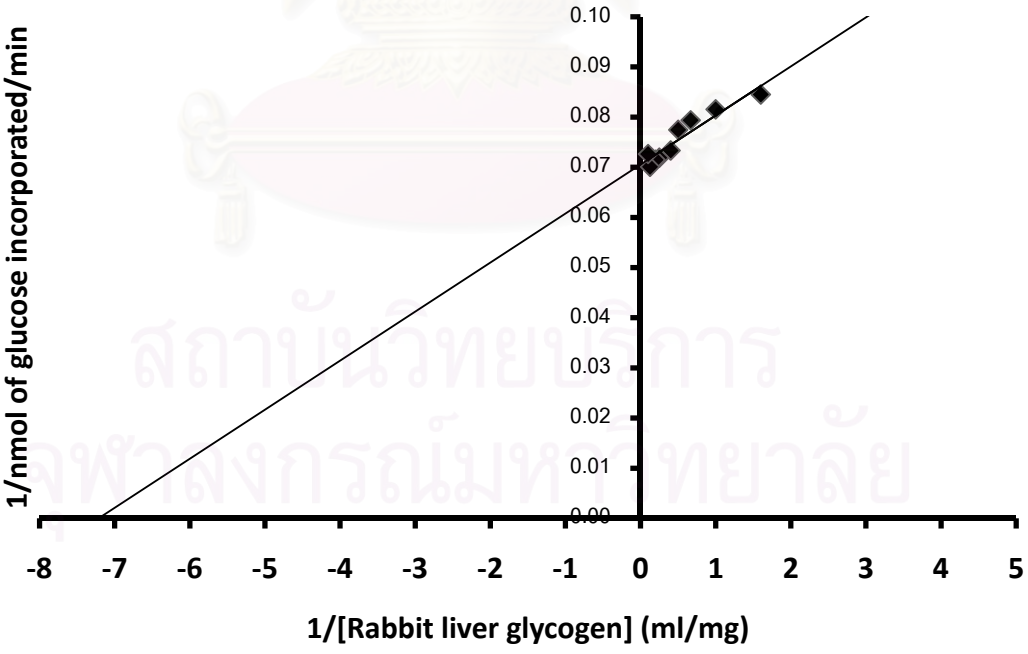

Figure 3.26 Lineweaver-Burk of rabbit liver glycogen for cassava soluble starch synthase3 (SSS3)

CHAPTER IV

DISCUSSION

Cassava is one of the most economically important crops which are produced in large scale and most of it is exported from Thailand as raw materials. The problems of cassava trade in Thailand are the falling price and the decrease of demand of cassava products in EC market, the most important venue for cassava product traded internationally and the main cassava import market. To solve this problem and increase the value, cassava should be more traded in the form of starch and other product because it can be used in many industries. However, cassava starch contains some undesirable quality for certain industries. The study of cassava starch biosynthesis is the important key to improve starch quality. This thesis concentrated on the understanding of soluble starch synthase in cassava tuber which is one of the enzymes involved in amylopectin biosynthesis and starch quality, with emphasis on the cultivar developed in Thailand for cassava market.

4.1 Assay method for starch synthase

So far, there are 2 methods reported for the assay of starch synthase, radioactive and non-radioactive assay. In 1980, Pollock and Preiss presented the assay method incubating the enzyme with radioactive ADP[U-^{14}C]-glucose in bicine buffer pH 8.5 including glutathione (reduced form), EDTA, potassium acetate, and glycogen the ^{14}C-glucose moiety of ADPG was transferred onto the glycogen molecule which was precipitated with 70% methanol (section 2.5.2). Since then, this method was widely used and accepted for assaying starch synthase activity. In

1999, Viola *et al.* presented the alternative method based on quantitative non-radioactive assay of starch synthase using HPLC. From stoichiometry of the reaction catalyzed by starch synthase (section 2.5.1) with ADP-glucose and a primer as substrates, the amount of ADP produced in the reaction was equivalent to the amount of glucose units incorporated to the primer. The Spherisorb SAX column used in the system can separate ADP from other compounds, especially ADP-glucose, and the amount of ADP can be measured by UV detector at wavelength 254nm. The advantages of HPLC method over radioactive method are its simplicity, low cost, safety, and no release of the radioactive waste to environment. In addition, the problem of using radioactive method on starch synthase activity is contamination of amylolytic enzyme activity in the crude enzyme extract and early steps of purification. Amylolytic enzyme can hydrolyze α-1,4 glucosidic linkage produced by starch synthase, therefore, will result in hydrolysis of the incorporated ^{14}C-glucose in the product. This resulted in lower amount of calculated ^{14}C-glucose incorporation obtained leading to inconsistency and the error in a calculation of starch synthase activity. The determination of ADP produced by HPLC method directly measured the glucose donor and was not affected if there was amylase contamination. This method is also more sensitive with a lower limit of 0.7 - l.0 nmol of ADP (Viola et al., 1999). The HPLC method was employed throughout the characterization work which required more accurate data. Experiment was also performed to ensure that there was no hydrolytic activity of ADP-glucose itself by autolysis and heat. ADP was not detected in the blank (no enzyme) or control (enzyme heated before reaction). Incubation of ADP-glucose and enzyme without primer also showed negligible trace of ADP-glucose hydrolysis. However, this method is time consuming and not practical for experiments with

large number of samples such as fractions from column chromatography. Therefore, radioactive method was used in monitory SSS activity in the purification process.

4.2 Developmental pattern of SSS in cassava tubers of cultivars Rayong 1 and Kasetsart 50

Rayong 1 and Kasetsart 50 were two of many cultivars of cassava developed in Thailand, a cassava-breeding program started with the release of Rayong 1 in 1975. Rayong 1 was developed from a selection of local cultivars, and was the first variety bred as source for industrial raw material. Kasetsart 50 (KU50) was cross breeding between Rayong 1 and Rayong 90 by Kasetsart University KU50 gives high tuber yield and high starch content. (Table 1.5)

The SSS activity expressed as specific activity was much higher in cultivar Kasetsart 50 up to 9 months but dropped drastically at 12 month. This may reflect the characteristics of this cultivar that it tubers contain high starch content compared to Rayong 1. Similar patterns were observed for starch branching enzyme (Yaiyen, 2003) and debranching enzyme (unpublished data). Maximun storage starch content of Kasetsart 50 tubers may be attained around 9 months or later, but before 12 months. The decrease in SSS at 12 months may reflect the control of starch biosynthesis activity to a lower level, just enough to keep storage starch content at a constant level. Monitoring the developmental patterns of starch, amylose and amylopectin may be useful in explaining the observation. The different age of tubers with maximum SSS activity should be due to the different growth profile of each cultivar.

4.3 Purification of starch synthase from cassava tubers

In maize and potato, the cDNA of starch synthase was cloned *in E. coli.* to produce starch synthase which was purified to homogeneity and characterized (Edwards et al., 1995; Marshall et al., 1996; Imparl-Radosevich et al., 1998; Edwards et al., 1999 and Cao et al., 1999). There were only a few reports on the attempt to purify soluble starch synthases by conventional method from plant tissues (Boyer and Preiss, 1981; Cao, James and Myers, 2000). They were unable to purify starch synthase to homogeneity using Q-sepharose and Sephacryl S-200 column chromatography. In our work, we tried to purify starch synthase from cassava tubers by column chromatographies and were able to obtain SSS1, SSS2 and SSS3 purification folds up to 426, 513 and 142 folds.

In primary experiment, SSS was located in parenchynna of cassava tubers which was used to prepare crude SSS by the modified method of Ozbun et al. (1971). The purification procedures used were ammonium sulfate precipitation followed by 2 kinds of column chromatography: Phenyl Sepharose, and Q-Sepharose. Primary experiment showed that SSS were found in precipitation from 20-60% ammonium sulfate. At this step, more than half of the proteins were removed but about 20% of the enzyme activity was lost. The loss of significant portion of the starch synthase activity may be caused by the removal of some factors important for stabilizing the enzyme activity. Moreover, crude fraction may contain the ADP-glucose hydrolytic enzyme which can hydrolyze ADP-glucose and over producing ADP resulting in high amount of ADP detected. The calculation of starch synthase activity in crude preparation, therefore, was higher than it should be. This may lead to the apparent great activity loss in the first step. An experiment by-passing ammonium sulfate precipitation by direct application of the crude enzyme to

DEAE-cellulose column was also performed. It was found that there was great loss of starch synthase as well with less amount of proteins removed. Furthermore, samples obtained from DEAE-cellulose column needed to be desalted and concentrated for the next step of' purification which led to further loss of enzyme activity. In addition, the column can not clearly separate SSS isoforms. Therefore, it was decided that ammonium sulfate precipitation followed by phenyl sepharose column as more appropriate in the first two steps to avoid desalting and concentration of samples. Although activity loss was unavoidable, its was less than using DEAE-cellulose column. The sample from ammonium sulfate precipitation can be directly apply to phenyl sepharose column without desalting since the hydrophobic nature of the column required elution started from high concentration of ammonium sulfate followed by decreasing concentrations of ammonium sulfate, either stepwise or gradient. Phenyl sepharose column separated SSS activity clearly into 3 isoforms, SSS1, SSS2 and SSS3.

Q-Sepharose contributed significantly in the purification procedures. Q-Sepharose removed more contaminating proteins, resulting in purification of SSS1, SSS2 and SSS3 up to 426, 514 and 142 folds. However, the yield obtained was rather low due to rapid loss of enzyme activity. Each isoforms were separately purified by Q-Sepharose column and were eluted at quite close salt concentration. Q-Sepharose is an ion-exchange column, same as DEAE cellulose. This correlated well with the observation that DEAE cellulose con not separate the isoforms from crude SSS. The 3 isoforms may contain amount of charges in the same rank and therefore, can not be separated by ion exchange column. Since SSS lost activity rapidly, the purified isoforms were kept in 20% (v/v) glycerol which helped in conserving the activity for longer period for characterization.

4.4. Characterization of cassava starch synthase

4.4.1 Non-denaturing glycogen PAGE

Activity stain of soluble starch was performed with glycogen added in the slab gel preparation. Staining was performed on the basis that SSS extended the polysaccharide branches of glycogen with glucose from ADP-glucose to a suitable length that iodine can bind and formed the color complex. Figure 3.6 showed that highly concentrated crude SSS appeared as intense brown band. On the other band, the less concentrated crude enzyme and SSS1, SSS2 and SSS3 purified from Q-sepharose appeared as smear of brown color. The more concentrated SSS in lane 1 may contain the amount of enzyme which can form enough products to form color complex with iodine to the detectable level by this method. On the other hand, less concentrated samples in lanes 2, 3, 4, and 5 can not form enough products to the detectable level. The light brown smear in lanes 2, 3 and 4 may be caused by some hydrolytic activity on the branches of glycogen to produce linear polysaccharide which can form some iodine complex.

4.4.2 Molecular weight determination

The crude SSS preparation and its purified isoforms were separated on 10% SDS-PAGE. The protein bands separated on SDS-PAGE showed that there were overlapping protein bands in the Q-sepharose fractions of all three isoforms. SSS1 showed one band at 81.7 kDa (lane 7, Figure 3.7) which was also detected in SSS2 with the molecular weight of 112, 90.2 and 81.7 kDa, the second band also coincided with a band detected in SSS3 (lane 9, Figure 3.7). The appearance of similar bands in these isoforms may be explained in 3 possibilities. Firstly, the unbounded peak SSS1 was the overloading of the column. This possibility was ruled out by reloading of the unbound peak to Phenyl-sepharose column which resulted in only the unbound peak.

Secondly, this may be a result of contamination of SSS1 in the SSS2 peak and contamination of SSS2 in SSS3 peak in the Phenyl-sepharose column which was not separable in Q-sepharose. This is quite unlikely since the ammonium sulfate concentrations used in elution were quite different for all isoforms and elution of each peak was prolonged until activity reached baseline. The last explanation is that the three isoforms were constituted of subunits as appeared on SDS-PAGE and some of the subunits were common to one or more isoforms. In the latter case, we can postulate that SSS1 consisted of one subunit with the molecular weight of 81.7 kDa, SSS2 consisted of 3 subunits of 112, 90.2 and 81.7 kDa and SSS3 consisted of 2 subunits of 90.2 and 79.8 kDa. Comparison with other reported molecular weight of SSS (Table 4.1), there are several values that correlate with our findings. For example, the molecular weight of SSS1 is close to those reported for SSI in maize kernel determined from cDNA sequence (Ozbun et al., 1971) and cassava 5-minute cultivar (Hirunyapaisarnsakul, 2000). Band 1 in SSS2 was more correlated to SSI in rice grain (Pisigan and Rosario, 1976) determined by Sephadex G-200, or SSII and SSSIII in potato tuber (Hawker et al., 1972; Edwards et al., 1995; Larsson et al., 1996). This postulation requires further conformation by using highly concentrated isoforms and determines their molecular weight by gel filtrations. However, this is quite difficult since SSS rapidly lost its activity and only minute amount of isoforms was obtained in the purification process. Another method of acquiring pure enzyme is through expression from cloned SSS genes. This is an attempt being persued by other group in the Cassava Consortium under BIOTECH. With co-operations among the members of this consortium, it is hoped that useful information in starch biosynthesis process in KU 50 can be obtained for the improvement of starch quality.

Table 4.1 Isoforms of starch synthase in various plant tissues

Source	Number of isoform	Molecular weight	Reference
Spinach leaf	4	-	Ozbun et al., 1972
Maize kernel	2	SSI = 76 kDa *	Ozbun et al., 1971
		SSII = 188 kDa *	Foster et al., 1996
			Gao et al., 1998
Potato tuber	2	SSII = 90 kDa *	Hawker et al., 1972
		SSIII = 140 kDa *	Edwards et al., 1995
			Larsson et al., 1996
Grape leaf	1	-	Hawker and Downton, 1974
Maize leaf	2	-	Hawker and Downton, 1974
Rice grain	2	SSI = 110 kDa **	Pisigan and Rosario, 1976
		SSII = 69 kDa **	
Sorghum seed	3	-	Boyer, 1985
Castor bean endosperm	1	-	Goldner and Beevers, 1989
Teosinte seed	2	-	Boyer and Fisher, 1984
Cassava parenchyma	1-3	79 kDa ***	Hirunyapaisarnsakul, 2000
		76 kDa ***	
		53.4 – 53.8 kDa ****	
Cassava parenchyma from cassava cultivar Kasetsart 50	3		This work

* determined by cDNA

** determined by Sephadex G-200

*** determined by SDS-polyacrylamide gel electrophoresis

**** determined by both * * and * * *

4.3.3 Effect of pH and temperature on starch synthase activity

Each SSS isoforms was incubated at various pH's and its activity measured. It was found that SSS1, SSS2 and SSS3 were the most active at pH 9.5, 7.0 and 9.5, respectively. SSS1 and SSS3 showed activity above 50% in the pH range 7 – 11, whereas SSS2 showed activity above 60% in the pH range 5.5 – 11. Below pH 5.5, all isoforms rapidly lost there activity with SSS2 show higher activity than the other isoforms. When the reaction was incubated at various temperatures, SSS1, SSS2 and SSS3 showed the highest activity at 37 °C, 35 °C and 37 °C, respectively. Activity of all three isozymes dropped lower than 50% at 20°C and higher than 45°C. When the isoforms were incubated at various temperatures for 30 minutes.

All isoforms were stable up to 37°C and activity dropped below 60%. SSS3 seemed to be more heat stable than SSS1 and SSS2 at 40 °C. When stability of all isoforms at 37°C were followed up to 36 hours at was observed that SSS3 was more stable than SSS1 and SSS2. SSS1 was the least stable isoforms. Therefore, all isoforms were most stable around 35 – 37 °C which was the most suitable temperature to carry out other experiments. Table 4.2 compared our results to those reported in other plants. It appeared that most SSS had the optimum pH in the more alkaline pH and optimum temperature and stability around 37 °C.

Table 4.2 Summary of the reported effect of pH and temperature on SSS activity in several tissues and plants

Source	Optimum pH	Optimum temperature (°C)	Temperature stability (°C)	Reference
Spinach leaf				Nakamura
SSI	8.0 – 8.5	30	-	et al., 1988
SSII	8.0 – 8.5	30 – 37	-	
SSIII	8.5	30	-	
SSIV	8.0	37	-	
Waxy maize kernel				Edwards et
SSI	8.0 – 8.5	30	-	al., 1999
SSII	8.5	37	-	
Maize kernel				Cao et al.,
SSI	8.0	42	42	2000
SSII	8.5	37	37	
Potato				Edwards et
SSII	-	-	35	al., 1999
Cassava SS				Hirunyapai-
(5 minutes cultivar)	8.5	28	45	sarnsakul, 2000
(Kasetsart 50 cultivar)				This work
SSS1	9.5	37	37	
SSS2	7.0	35	37	
SSS3	9.5	37	37	

4.3.4 Effect of different carbohydrates as primers

Starch synthase catalyzes the transfer of glucosyl units from nucleotide glucose donor to non-reducing ends of growing polysaccharides via new α(1→4) linkages. The glucose acceptor, called primer, must have the non-reducing end of glucose unit such as amylose, amylopectin, glycogen, and malto-oligosaccharide. The carbohydrates used to study the effect of primer of starch synthase can be defined into 3 groups.

1. Branched polysaccharide: amylopectin, rabbit liver glycogen and oyster glycogen.
2. Starch from different plants: cassava starch, rice starch, potato starch and corn starch.
3. Unbranched polysaccharides: maltopentaose, maltohexaose and amylose.

With the first group of primers, all three starch synthase showed highest activity with rabbit liver glycogen (RLG). SSS1 showed highest activity with oyster glycogen (OG) followed by rabbit liver glycogen (RLG), potato amylopectin (AP) and potato amylose (AM), respectively. SSS2 showed highest activity with rabbit liver glycogen (RLG) followed by potato amylopectin (AP), oyster glycogen (OG), and potato amylose (AM), respectively. SSS3 showed highest activity with potato amylopectin (AP) followed by rabbit liver glycogen (RLG), oyster glycogen (OG), and potato amylose (AM), respectively. Within the group of branched polysaccharide primers, all three isoforms prefer the polymers with highly branch chains, like glycogen and amylopectin. Our observations were similar to other starch synthase isoforms previously reported as shown in table 4.3. Most report showed preference of isoforms for primer in the order

RLG > OG > AP respectively. There were a few starch synthase isoforms, such as teosinte seed SSII, and *waxy* maize SSII, which showed preference in the order AP > RLG > OG. SSII of spinach preferred OG > RLG > AP same as SSS1 In our study.

Among the starch primers, all three isoforms showed high activity on starch with higher amylopectin content (see table 1.3) SSS1 was more sensitive to starch with higher content of amylose and showed lower activity than SSS2 and SSS3 in potato and corn starch.

For unbranched polysaccharide primers, all three starch synthases showed low activity especially SSS1, in the order potato amylose > maltohexaose > maltopentaose. This result indicated that the length of glucose units affected the ability of the oligomers to act as primers for starch synthase activity. Overall, all three isoforms preferred branched polysaccharides than unbranched with SSS1 showed highest sensitivity to unbranched primers.

Table 4.3 The effect of primers on starch synthase of various plant tissues

Source	Activity for primer (higher > lower)	Reference
Maize		Boyer et al., 1981
Leaf SSI	RLG > OG > AP	Pollock and Preiss, 1980
Kernel SSI	RLG > OG > AP	
Castor bean endosperm SSI	RLG > AP > OG > AM	Goldner and Beevers, 1989
Sorghum seed		Boyer, 1985
SSII	RLG > OG > AP	
SSIII	RLG > OG > AP	
Teosinte seed		Boyer and Fisher, 1984
SSI	RLG > OG > AP	
SSII	AP > RLG > OG	
ae maize kernel SSI	RLG > OG > AP	Boyer and Preiss, 1979
Spinach leaf		Ozbun et al., 1972
SSI	RLG > OG > AP	
SSII	OG > RLG > AP	
SSIII	RLG > AP > OG	
SSIV	RLG > OG >AP	
Waxy maize kernel		Ozbun et al., 1971
SSI	RLG > OG > AP	
SSII	AP > RLG > OG	
Cassava SS	RLG > OG > AP > AM	Hirunyapaisarnsakul, 2000
Cassava parenchyma from cassava cultivar Kasetsart 50		This work
SSS1	OG > RLG > AP > AM	
SSS2	RLG > AP > OG > AM	
SSS3	AP > RLG > OG > AM	

4.3.5 Effect of sulfhydryl reagents on starch synthase activity

When cassava starch synthase was incubated with various sulfhydryl reagents, it was found that the reagents had the effect on SSS1, SSS2 and SSS3 activities. DTT, a SH-group stabilizing agent which protects SH- group from oxidizing agents, activated SSS1 and SSS2 activity with increasing concentration. However, DTT did not show significant effect on SSS3. Perhaps, -SH group(s) in SSS3 may already be in a better protected environment compared to SSS2 and SSS3. For NEM, PCMB, and IAA included in the reaction mixture, all three starch synthase activities were inhibited at concentration as few as 5 mM. These reagents are SH- modifying reagents by alkylation of SH- group on cysteine residue. This result indicated that the SH- group in cassava starch synthase was important for its activity. Whether, the cysteine residues are within the active site, effect of enzyme activity up on modification of cysteine residues to bee carried out.

4.3.6 Kinetic constants of cassava starch synthase

As the starch synthase employed ADP-glucose and primer as substrates, experiments were carried out to investigate the K_m and V_{max} of ADP-glucose and its primers. Rabbit liver glycogen was used in this experiment because it was determined to be the most preferred primer for cassava starch synthase (section 3.3.4).

The K_m and V_{max} values which were calculated from Linweaver-Burk plot were not accurate because the initial velocity values from saturation curve were mostly at the end of the linera part. K_m and V_{max} values were estimated from the saturation curves. We found that K_m and V_{max} values of ADP-glucose from both methods were quite close, whereas K_m of rabbit liver glycogen were quite different. Therefore, the K_m values determined in these experiments can not be taken as accurate

values but they were in the range of the real values and were used for comparative purposes. The accurate values can be determined by experiments either on lower substrate concentrations or increase the amount of enzymes.

The K_m and V_{max} values were calculated by Lineweaver-Burk plot, the K_m values of SSS1, SSS2 and SSS3 for ADP-glucose were calculated to be 0.09, 06 and 0.08 mM and V_{max} were 14.50, 8.40 and 8.54 nmol of glucose incorporated/min, respectively. K_m values of SSS1, SSS2 and SSS3 for rabbit liver glycogen were about 1.46, 0.26 and 0.13 mg/ml and V_{max} were 13.15, 12.50 and 14.28 nmol of glucose in corporate/min, respectively (Table 3.3). This values were compared to those of many starch synthase isoforms previously reported (Table 4.5). V_{max} values cannot be compared since it depended on each preparation of enzyme. It seemed that K_m for ADP-glucose were not very variable among the SSS isoforms in difference plant and tissues but significant difference were observed with K_m for RLG. SSS2 and SSS3 in our report seemed to have lower K_m for RLG than SSS1, similar to most reported starch synthase except maize kernel SSI, SSII, spinach leaf SSI and SSS in cassava 5 minutes cultivar. These latter SSS were similar to Kasesart 50 SSS1 which showed higher K_m for glycogen. This may reflect some difference in their function such as addition of glucose units to branched polysaccharides or less branched polysaccharides. This will require comparison their K_m's for difference types of primers. Table 4.4 summarized the 4 kinetic parameters reported for SSS in other plants. The K_m values for ADP-glucose and glycogen were all in the same range.

From the reported result in this study, SSS existed as 3 isoforms in the parenchyma of cassava tubers cultivar KU 50. These isoforms showed difference in their biochemical characteristics which may reflect some difference in their function in the starch biosynthesis. To be able to elaborate more on the significance of our findings,

analysis of the data together with other starch biosynthetic enzymes and gene expressions carried out in our laboratory and other in the Cassava Consortium needs to be carried out.

Table 4.4 The K_m of ADP-glucose and rabbit liver glycogen and V_{max} of starch synthase catalyzed reaction

Source	ADP-glucose		Glycogen		Reference
	K_m *	V_{max} ***	K_m **	V_{max} ***	
Maize kernel SSI	0.10	-	0.60	-	Boyer and Preiss, 1979
ae maize kernel SSI	0.10	-	-	-	Boyer and Preiss, 1979
Potato SSII	0.25	-	-	-	Hawker et al., 1972
Spinach leaf					Ozbun et al., 1972
SSI	0.20	-	1.02	-	
SSII	0.29	-	0.8	-	
SSIII	0.15	-	-	-	
SSIV	0.25	-	0.88	-	
Waxy maize kernel					Ozbun et al., 1971
SSI	0.10	-	-	-	
SSII	0.12	-	-	-	
Maize kernel					Cao et al., 2000
SSI	0.11	2.23	12.8	2.79	
SSII	0.42	6.74	18.5	12.3	
Potato SSII	0.07	7.5	-	-	Edwards et al., 1999
Cassava					Hirunyapaisarnsakul,
(5 minutes cultivar)	0.10	0.90	1.31	0.77	2000
(Kasetsart 50 cultivar)					This work
SSS1	0.09	14.50	1.46	13.15	
SSS2	0.06	8.40	0.26	12.50	
SSS3	0.08	8.54	0.13	14.28	

*Unit = mM

**Unit = mg/ml

***Unit = nmol of glucose incorporate/min/mg protein

CHAPTER V

CONCLUSIONS

1. Cassava starch synthase was purified from parenchyma of cassava tubers of KU50 cultivar by 20 – 60% saturated ammonium sulfate, followed by column chromatographies on pheyl sepharose and Q-sepharose. Three activity peak were found and purified up to 426, 513 and 142 fold with 20, 4 and 5% recovery, respectively.
2. The optimum pH for cassava starch synthase were 9.5, 7.0 and 9.5 for SSS1, SSS2 and SSS3, respectively
3. The optimum temperature for cassava starch synthase were 37, 35 and 37 °C for SSS1, SSS2 and SSS3, respectively. The enzyme was stable at temperature up to 37 °C.
4. SSS1 showed highest preference for oyster glycogen as primer followed by rabbit liver glycogen, potato amylopectin, cassava starch, rice starch, corn starch, potato starch, potato amylose, malto-oligosaccharide, respectively.
5. SSS2 showed highest preference for rice starch as primer followed by rabbit liver glycogen, potato amylopectin, cassava starch, corn starch, oyster glycogen, potato starch, potato amylose, malto-oligosaccharide, respectively.
6. SSS3 showed highest preference for potato amylopectin as primer followed by rabbit liver glycogen, rice starch, corn starch, potato starch, oyster glycogen, cassava starch, potato amylose, malto-oligosaccharide, respectively.
7. Cassava starch synthase isoforms contained SH-group which was involved in its activity.

8. K_m and V_{max} of SSS1 for ADP-glucose were 0.09 mM and 14.50 nmol of glucose incorporated/min, respectively. The K_m and V_{max} for rabbit liver glycogen were 1.46 mg/ml and 13.15 nmol of glucose incorporated/min, respectively.
9. K_m and V_{max} of SSS2 for ADP-glucose were 0.06 mM and 8.40 nmol of glucose incorporated/min, respectively. The K_m and V_{max} for rabbit liver glycogen were 0.26 mg/ml and 12.50 nmol of glucose incorporated/min, respectively.
10. K_m and V_{max} of SSS3 for ADP-glucose were 0.08 mM and 8.54 nmol of glucose incorporated/min, respectively. The K_m and V_{max} for rabbit liver glycogen were 0.13 mg/ml and 14.28 nmol of glucose incorporated/min, respectively.

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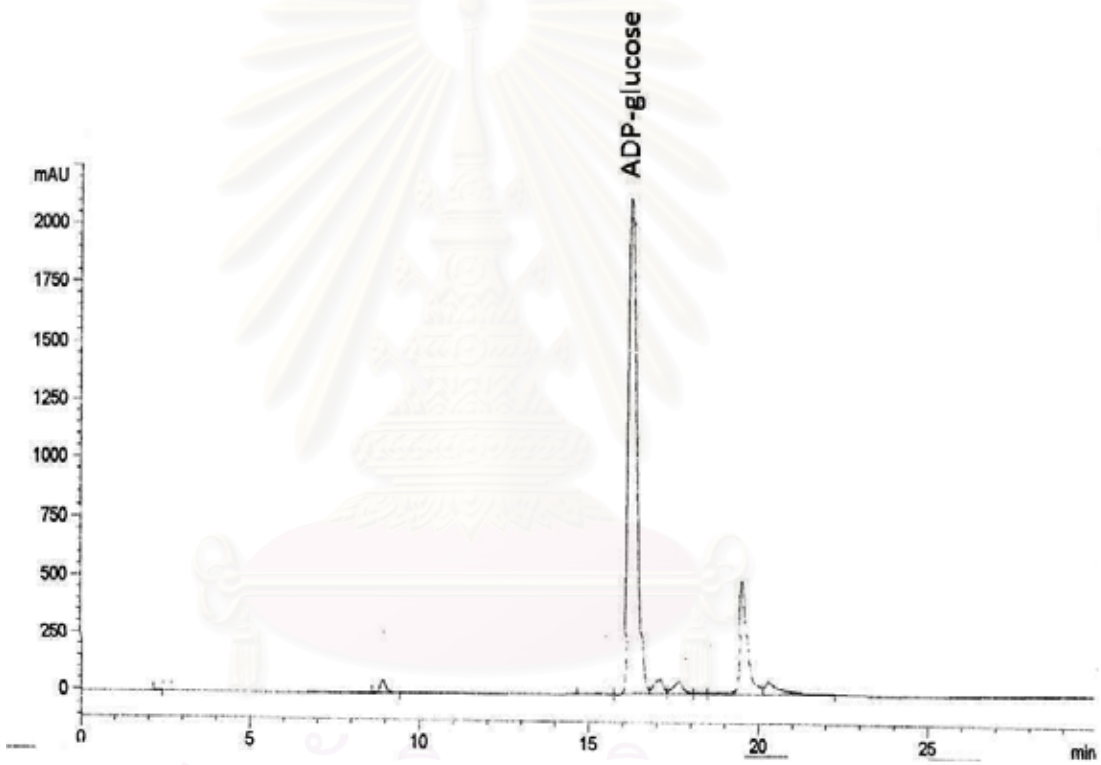
APPENDICES

APPENDIX A

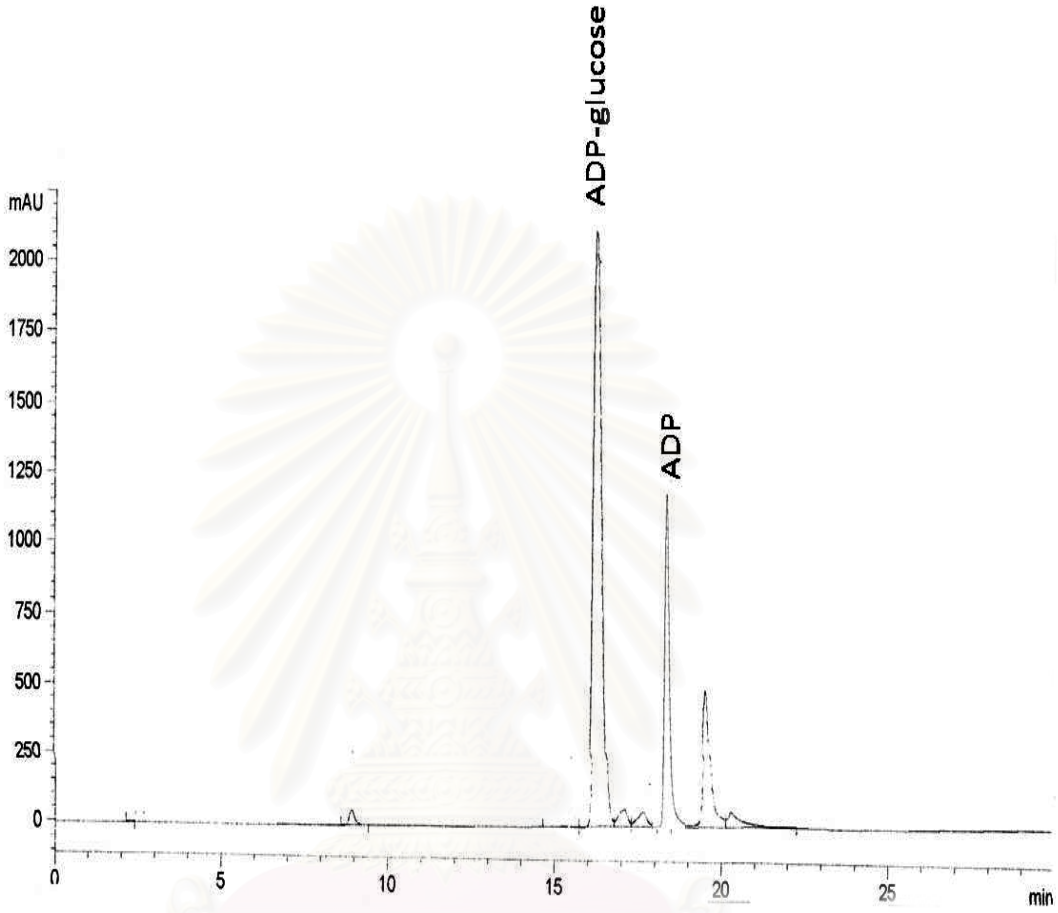
1. HPLC profile of ADP and ADP-glucose

1.1 Typical HPLC profile of blank and control in determination of starch synthase activity.



ADP-glucose RT = 16.25 min

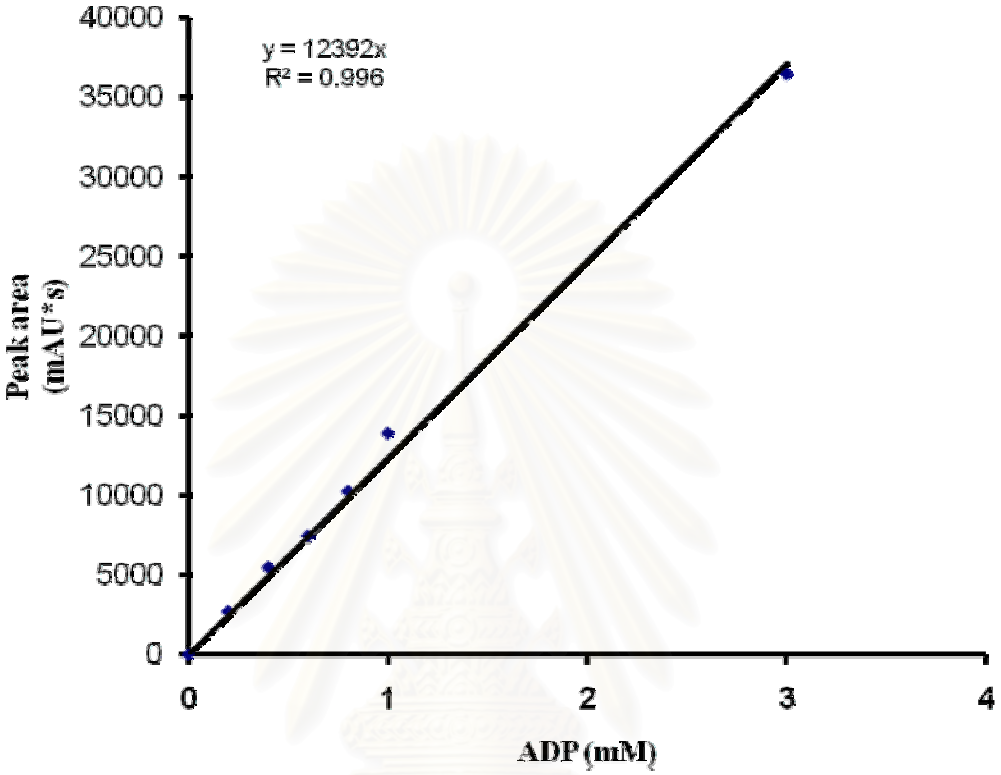
1.2 Typical HPLC profile of reaction mixture with starch synthase activity



ADP-glucose RT = 16.25

ADP RT = 18.75

2. Calibration curve of ADP concentration and peak area



3. Calculation of starch synthase activity

3.1 [ADP] (mM) = peak area of ADP/12392

3.2 Injection volumns = 20 µl; [ADP_{inj}] (nmol) = [ADP] (mM) x 20

3.3 Reaction mixture = 200 µl; [ADP_{rx}] (nmol) = [ADP_{inj}] x 10

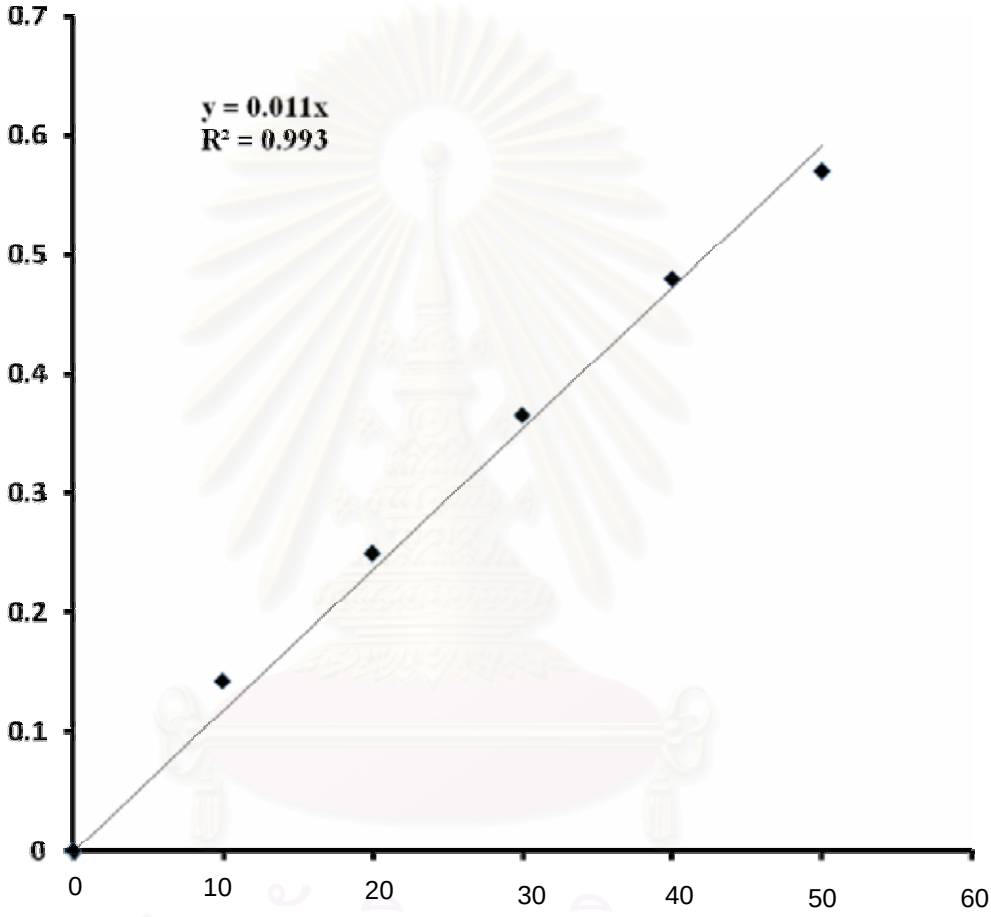
3.4 Activity (nmol ADP per minute) = [ADP_{rx}]/15

3.5 Total activity = activity (units) x (total volumn (ml)/ 100 µl)

3.6 Spectific activity = total activity (units)/total protein (mg)

APPENDIX B

Calibration curve of protein concentration



APPENDIX C

Preparation for polyacrylamide gel electrophoresis

1. Stock reagent

30% Acrylamind, 0.8% bis-acrylamide, 100ml

Acrylamide	29.2	g
N,N'-methylene-bis-acrylamide	0.8	g

Adjust volumn to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

Adjust pH to 8.8 with 1 M HCl and adjust volumn to 100 ml with distilled water.

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.20	g

Adjust pH to 8.8 with 1 M HCl and adjust volumn to 100 ml with distilled water.

0.5 M Tris-HCl pH6.8

Tris (hydroxymethyl)-aminomethane	6.06	g

Adjust pH to 6.8 with 1 M HCl and adjust volumn to 100 ml with distilled water.

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.10 g

Adjust pH to 6.8 with 1 M HCl and adjust volumn to 100 ml with distilled water.

Solution B (1.5M Tris-HCl pH 8.8)

2mM Tris-HCl pH 8.8	75 ml
Distilled water	25 ml

Solution B-SDS (1.5M Tris-HCl pH 8.8, 0.4% SDS)

2mM Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5M Tris-HCl pH 6.8)

1 M Tris-HCl pH 6.8	50 ml
Distilled water	50 ml

Solution C-SDS (0.5M Tris-HCl pH 6.8)

1 M Tris-HCl pH 6.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

5x Sample buffer

1 M Tris-HCl pH 6.8	3.1 ml
Glycerol	5 ml
1% Bromphenol blue	0.5 ml
Distilled water	1.4 ml

Non-denaturing electrophoresis buffer, 1 litter

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g

Dissolved in distilled water to 1 litter without pH adjustment. (Final pH should be 8.3)

SDS electrophoresis buffer, 1 litter

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g
SDS	1 g

Dissolved in distilled water to 1 litter without pH adjustment. (Final pH should be 8.3)

2. Preparation of non-denaturing PAGE

10% Separating gel

30% Acrylamide solution	3.3 ml
Solution B	2.5 ml
Distilled water	4.2 ml
10% $(NH_4)_2S2O_8$	100 µl
TEMED	10 µl

3% stacking gel

30% Acrylamide solution	0.4 ml
Solution C	1.0 ml
Distilled water	2.6 ml
10% $(NH_4)_2S2O_8$	50 µl
TEMED	10 µl

3. Incubation buffer for non-denaturing glycogen PAGE

0.1 M Bicine, pH 8.5

5 mM ADP-glucose

0.5 M Sodium citrate

0.5 mg/ml BSA

25 mM Potassium acetate

133 mM Ammonium sulfate

7 mM Magnesium chloride

10 mg/ml Rabbit liver glycogen

25 mM β-Mercatoethanol

APPENDIX D

Universal buffer

Universal buffer, 1 L

Citric acid	6.008	g
KH_2PO_4	3.893	g
H_3BO_3	1.769	g
Diethylbarbituric acid	5.266	g

100 ml of this mixture is titrated with 0.2 M NaOH to give the required pH.

BIOGRAPHY

Mr. Thanade Paoin was born in February 10th, 1981 in Bangkok. He finished Matthayom 6 at Yothin burana school, Bangkok and enrolled in the Faculty of Art and Science, Kasetsart University in 1998. He graduated with the B.Sc. in General science in 2001 and continued for M.Sc. in Biotechnology in 2003. He has publication:

1. Paoin, T., Boonseng, O., and Limpasani, T. 2006. Isolation and characterization of isoforms of soluble starch synthase from cassava *Manihot esculenta* crantz cultivar kasetsart 50 tuber. Proceeding of the 18th annual meeting of the Thai Society for Biotechnology "Biotechnology Benefits & Bioethics". The Montein Riverside Hotel, Bangkok, Thailand. pp. 146-147.