การเตรียมสตาร์ชซินเทสในหัวมันสำปะหลัง *Manihot esculenta* Crantz ให้บริสุทธิ์
และศึกษาสมบัติของเอนไซม์

นายวรพงษ์ หิรัญยไพศาลสกุล

สถาบันวิทยบริการ
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PURIFICATION AND CHARACTERIZATION OF STARCH SYNTHASE

FROM CASSAVA *Manihot esculenta* Crantz TUBERS

Mr.Worapong Hirunyapaisarnsakul

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วรพงษ์ หิรัญยไพศาลสกุล : การเตรียมสตาร์ชซินเทสในหัวมันสำปะหลัง *Manihot esculenta* Crantz ให้บริสุทธิ์และศึกษาสมบัติของเอนไซม์ (PURIFICATION AND CHARACTERIZATION OF STARCH SYNTHASE FROM CASSAVA *Manihot esculenta* Crantz TUBERS) อ. ที่ปรึกษา : ผศ. ดร. ทิพาพร ลิมปเสนีย์, อ. ที่ปรึกษาร่วม : ศ. ดร. มนตรี จุฬาวัฒนทล, 99 หน้า, ISBN 974-13-0763-2.

มันสำปะหลังเป็นพืชเศรษฐกิจที่มีปริมาณการส่งออกสูง แป้งมันสำปะหลังสามารถนำไปใช้ประโยชน์ได้ในอุตสาหกรรมต่างๆ แป้งประกอบด้วยโมเลกุลของอะไมโลสและอะไมโลเพคติน เอนไซม์ที่เกี่ยวข้องกับการสังเคราะห์อะไมโลสและอะไมโลเพคตินในพืชมีหลายชนิด สตาร์ชซินเทส เป็นเอนไซม์ตัวหนึ่งที่เกี่ยวข้องกับการสังเคราะห์แป้ง สตาร์ชซินเทส แบ่งได้เป็น 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 ในหัวมันสำปะหลัง โดยการสกัดแยกและทำให้บริสุทธิ์โดยการตกตะกอน 20-60 เปอร์เซ็นต์ แอมโมเนียมซัลเฟตอิ่มตัว และวิธีคอลัมน์โครมาโตกราฟีโดยใช้ Phenyl Sepharose, Sephadex G-200 และ Q-Sepharose ตามลำดับ สตาร์ชซินเทสที่เตรียมได้มีความบริสุทธิ์เพิ่มขึ้น 220 เท่า ผลการทดลองจากคอลัมน์โครมาโตกราฟีพบว่า สตาร์ชซินเทสมียอดของแอคติวิตี 1 ยอด ที่มีน้ำหนักโมเลกุลเท่ากับ 53.4 กิโลดาลตัน เมื่อวิเคราะห์โดยอิเล็กโทรโฟเรซีสแบบสองทางบนโพลีอะคริลาไมด์ พบแถบโปรตีน 2 แถบในโพลีอะคริลาไมด์แบบไม่เสียสภาพ ซึ่งเมื่อนำไปวิเคราะห์ต่อด้วยอิเล็กโทรโฟเรซีสแบบเสียสภาพที่มีเอสดีเอส พบแถบโปรตีน 3 แถบที่มีน้ำหนักโมเลกุล 79,76 และ 53.4กิโลดาลตัน และมีค่า pI เท่ากับ 6.91, 6.41 และ 6.41 ตามลำดับ ซึ่งคาดว่าเป็นไอโซฟอร์มของสตาร์ชซินเทส สตาร์ชซินเทสที่เตรียมได้สามารถเร่งปฏิกิริยาได้ดีที่ค่าความเป็นกรด-ด่างเท่ากับ 8.5 ที่อุณหภูมิ 28 องศาเซลเซียส rabbit liver glycogen เป็นตัวรับหน่วยกลูโคส (primer) ได้ดีกว่า oyster glycogen, อะไมโลเพคติน, อะไมโลส, แป้งจากพืชต่างๆ และ short chain glucose oligomers ตามลำดับ สตาร์ชซินเทสให้ค่า K_m ต่อ ADP-glucose และ rabbit liver glycogen เท่ากับ 0.1mM และ 1.31mg/ml ตามลำดับ การศึกษาผลของ thiol reagents ต่อสตาร์ชซินเทสพบว่า สตาร์ชซินเทสถูกยับยั้งโดย thiol modifying reagents

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WORAPONG HIRUNYAPAISARNSAKUL : PURIFICATION AND CHARACTERIZATION OF STARCH SYNTHASE FROM CASSAVA *Manihot esculenta* Crantz TUBERS. THESIS ADVISOR : ASSIST. PROF. TIPAPORN LIMPASENI, Ph.D. THESIS CO-ADVISOR: PROF. MONTRI CHULAVATNATOL, Ph.D. 99 pp. ISBN 974-13-0763-2.

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(l, 4)-glucosidic linkage in amylose 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, Sephadex G-200 and Q-Sepharose column chromatographies. The chromatographic profiles showed a single peak of starch synthase activity with molecular weight of 53.4 kDa on Sephadex G-200. The enzyme preparation obtained was purified up to 220 folds with 2.8% recovery. The purified cassava soluble starch synthase showed optimum pH and temperature at 8.5 and 28°C, respectively. The Q-Sepharose preparation of starch synthase showed 2 bands on non-denaturing polyacrylamide gel which appeared on second dimension electrophoresis as 3 bands with molecular weight of 79, 76 and 53.8 kDa. The pI of these proteins were 6.91, 6.41 and 6.41, respectively. These data suggested the possible existence of 3 isoforms of cassava starch synthase. The enzyme utilized rabbit liver glycogen as primer better than oyster glycogen, amylopectin, amylose, starches, and short chain glucose oligomers, respectively. The K_m's for ADP-glucose and rabbit liver glycogen were 0.10 mM and 1.31 mg/ml respectively. It can be inhibited by thiol modifying reagents, indicating the involvement of SH-group on cassava starch synthase activity.

Department………………………………	Student's signature…………………………….
Field of study…………………………..	Advisor's signature…………………………….
Academic year………………………...	Co-advisor's signature…………………………..

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LIST OF ABBREVIATIONS

A	Absorbance
BSA	bovine serum albumin
DTT	dithiothreitol
g	gravitational acceleration
HCl	hydrochloric acid
IEF	isoelectric focusing
KCl	potassium chloride
kDa	kilo Dalton
MW	molecular weight
NaOH	sodium hydroxide
$(NH_4)_2SO_4$	ammonium sulfate
$(NH_4)H_2PO_4$	ammonium dihydrogen phosphate
$(NH_4)_2HPO_4$	di-ammonium hydrogen phosphate
$(NH_4)_2S_2O_8$	ammonium persulfate
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethyl ethylene diamine
v/v	volume by volume
w/v	weight by volume
HCN	hydrogencyanide
nm	nano-metre
SS	starch synthase
SBE	starch branching enzyme
DBE	debranching enzyme
GBSS	granule-bound starch synthase
SSS	soluble starch synthase
ADP-glucose	adenosine-5'-diphosphate glucose
ADP	adenosine-5'-diphosphate
PCMB	ρ-Chloromercuribenzoic acid

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EDTA	Ethelenediaminetetraacetic acid
NEM	N-Ethylmaleimide
IAA	Iodoacetic acid
PMSF	Phenylmethylsulfonyl fluoride
M	molar
KOH	potassium hydroxide
GSH	glutathione reduced form
HPLC	High Performance Liquid Chromatography
TCA	trichloroacetic acid
SAX	strong anion exchanger

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CHAPTER 1
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 during the 16th and 17th centuries it was dispersed widely by the Portuguese in tropical and sub-tropical areas of Africa, Asia and the Caribbean (Table 1.1). Moreover, 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 harvest when market, processing or other conditions are more favorable (1).

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 (2).

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Figure 1.1 Cassava tree and its underground tuberous roots.

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The utilization of cassava as major food crops for a long time in tropics can cause the 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 (1). Leaves, however, may be high in hydrocyanic acid which can lead to goiter, neurological disorders, tropical ataxic neuropathy, respiratory poisoning and sometimes death but the HCN can be reduced to safe levels in most cases when the liquid is squeezed out after grinding and through evaporation during cooking (3).

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, pharmaceutical, bio-degradable products, butanol and acetone, manufacture of explosives, and coagulation of rubber latex.

In Thailand, cassava is produced in large scale and most of it is exported widely in the world (Tables 1.2 and 1.3). Europe is the largest market for cassava products, chips and pellets using as livestock feed since after World War II. Until 1994, Thailand encountered some problems on the quota and tariff in exporting cassava to Europe. Quotations of cassava pellets in the EC (the most important cassava product traded internationally and the main cassava import market) are determined by the domestic prices of grains, especially barley, and the prices of protein-rich meals. The price falling in EC market (Table 1.4) was caused the diverse of cassava products and markets. New market in Asia, Japan, Korea and Taiwan, began to import pellets for animal feed but there exists competition with

grains. Cassava production is projected to increase because both yield improvements and area expansion. To reduce dependence on the EC feed market and add value to cassava production must be diverted into starch and starch-based products.

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Table 1.1 World cassava production (4)

	1997	1998	1999 *
WORLD	**165.3**	**161.0**	**166.7**
Africa	85.8	88.1	85.5
Congo Dem. Rep.	16.2	15.6	15.0
Ghana	7.0	7.2	8.4
Madagascar	2.4	2.4	2.4
Mozambique	5.3	5.6	5.6
Nigeria	32.1	32.7	30.4
Tanzania	5.7	6.2	6.0
Uganda	2.3	2.6	2.3
Asia	47.5	45.2	50.2
China	3.6	3.`4	3.6
India	6.0	6.1	6.2
Indonesia	15.1	14.7	15.4
Philippines	2.0	1.8	1.8
Thailand	18.1	16.3	20.3
Viet Nam	2.0	2.0	2.0
Latin America	31.8	27.5	30.8
Brazil	24.3	19.7	22.5
Colombia	1.7	1.6	2.0
Paraguay	3.2	3.3	3.3

Unit: million tons

* Preliminary data

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Table 1.2 World trade in cassava (4)

	1997	1998	1999 [*]
Exports	**6.4**	**4.9**	**5.5**
Thailand	5.3	3.9	4.6
Indonesia	0.2	0.2	0.2
China [1]	0.4	0.3	0.2
Others	0.5	0.5	0.5
Imports	**6.4**	**4.9**	**5.5**
EC [2]	3.6	2.9	3.6
China [1]	0.6	0.5	0.6
Japan	0.3	0.3	0.3
Korea. Rep.	0.5	0.5	0.3
Others	1.4	0.7	0.7

Unit: million tons

[*] Preliminary data

[1] Including Taiwan Province.

[2] Excluding trade between EC members

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Table 1.3 The export of cassava pellets and chips from Thailand (5)

Country	Jan-Sep 1999		Jan-Sep 2000		%Change		Market share (2000)
	Volume (ton)	Value Million US$	Volume (ton)	Value Million US$	volume	Value Million US$	
EC							
Netherlands	1,785,878	136.20	1,160,103	77.15	-35	-43	40.12
Spain	645,197	48.59	991,448	64.58	54	33	34.29
Portugal	270,282	21.60	286,601	19.75	6	-9	9.91
Germany	-	-	162,670	9.04	-	-	5.63
Belgium	123,381	9.70	54,315	8.42	-56	-13	1.88
Italy	32,941	2.58	37,134	2.73	13	6	1.28
Total EC	2,857,679	218.67	2,692,271	181.67	-6	-17	93.11
Total Non EC	164,311	14.01	199,313	10.20	21	-27	6.89
Total	3,021,990	232.68	2,891,584	191.86	-4	-18	100.00

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Table 1.4 Prices of cassava, soybean meal and barley in EC (4)

	Cassava pellets [1]	Soybean meal [2]	Cassava soybean meal mixture [3]	Barley [4]	Barley per cassava mixture (ratio)
1990	167	208	175	225	1.29
1991	178	197	186	222	1.19
1992	183	204	187	235	1.26
1993	137	208	151	197	1.30
1994	144	192	154	182	1.18
1995	177	197	181	209	1.15
1996	152	268	175	194	1.11
1997	108	276	142	161	1.13
1998	107	170	120	145	1.21
1999 [5]	102	146	111	144	1.29

Unit: US$ per ton

[1] F.o.b. Rotterdam (barge or rail) including 6% levy

[2] Argentina 45/46 % proteins) c.i.f. Rotterdam

[3] Consisting of 80% of cassava pellets and 20% of soybean meal

[4] Selling price of barley in Spain

[5] January-September average

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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 1.5. Percent of amylose and amylopectin in reserve plant starch (6)

Starch	Amylose %	Amylopectin %
Rice	18.5	81.5
Waxy rice	0	100
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

Amylose is a homopolymer of glucose units which are linked with $\alpha(1\rightarrow4)$ glucosidic bond with a very few $\alpha(1\rightarrow6)$ branches (about 1 branch per 1000 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 (7).

Amylopectin is chemically similar to glycogen in that both are $\alpha(1\rightarrow4)$-linked, $\alpha(1\rightarrow6)$-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 (7). 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 granule 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 9nm (8). 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 peck together in order arrays to give the crystalline lamellae (9) (Figure 1.2). 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 1.3). Each amylopectin contains one C-chain, and thus one reducing group (7).

Table 1.6. Major properties of the separated starch component (10)

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 β-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

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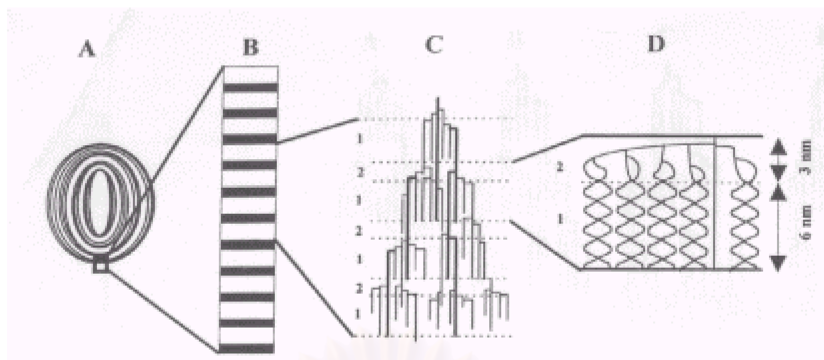

Figure 1.2 Starch granule organization (11)

A: A schematic view of a 1.5μm 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.

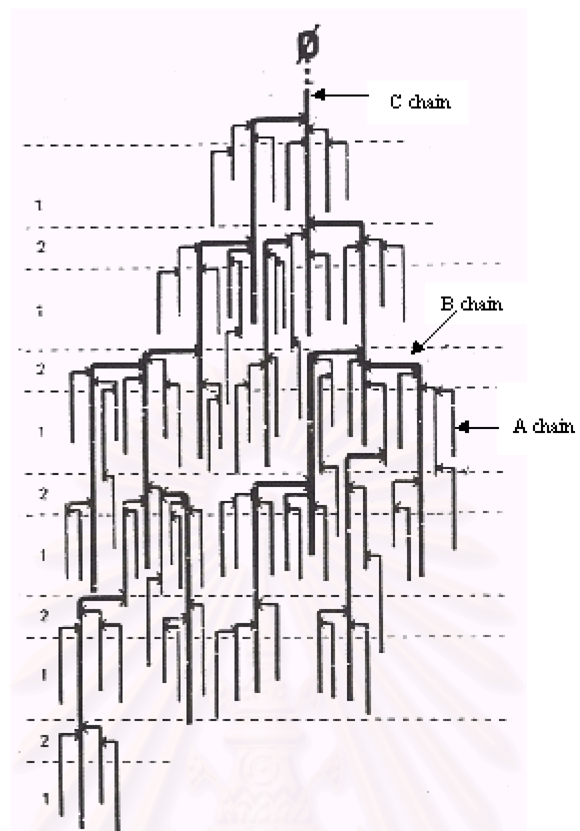

Figure 1.3 The branch structure of amylopectin (7)

1 = compact, crystalline region

2 = less compact, amorphous region

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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-1-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 (12). Starch biosynthesis depends on amylose and amylopectin biosynthesis.

1.3.1 Amylose biosynthesis

Amylose molecules appear to exist as single helix within the starch granule, interspersed with amylopectin in amorphous regions. Amylose synthesis in storage organs is a specific function of the granule-bound starch synthase I (GBSSI) class of isoforms of starch synthase which catalyzes the transferring of glucosyl units from ADP-glucose to non-reducing ends of growing polysaccharides via new $\alpha(1\rightarrow4)$ linkages. The glucose acceptor or primer for GBSSI activity is the short glucans and malto-oligosaccharides (13). Amylose synthesis occurs within the matrix of the starch granule (14). The space available in the matrix, which created by the synthesis of amylopectin, may be an important of 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 SS 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 $\alpha(1\rightarrow4)$ linkages and transfer of the released reducing end to a C_6 hydroxyl, creating a new $\alpha(1\rightarrow6)$ linkage. The other two enzymes which are, potentially, involve in amylopectin biosynthesis are debranching enzyme (DBE) and disproportionating enzyme (D-enzyme) (12). DBE catalyzes the hydrolysis of $\alpha(1\rightarrow6)$ linkages and D-enzyme catalyzes the transfer a segment of one linear chain to another. 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. 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 (12). 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 (12).

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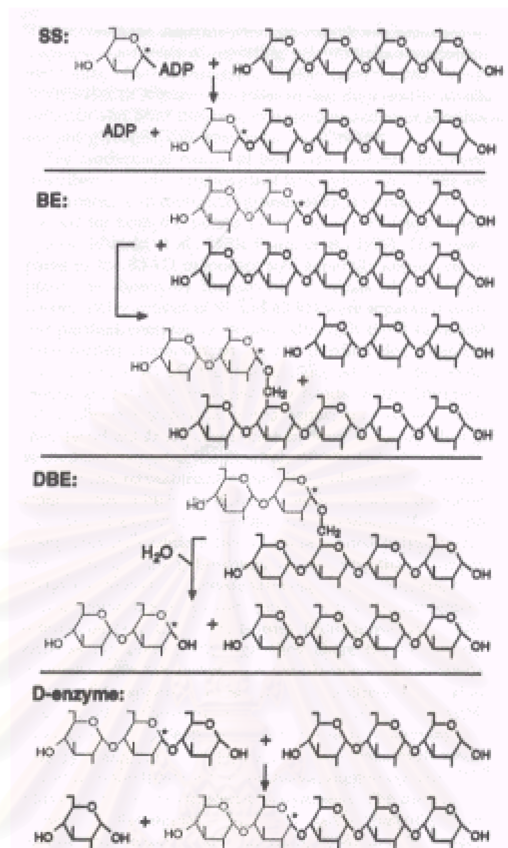

Figure 1.4 Reaction of enzyme involve in amylopectin synthesis (12)

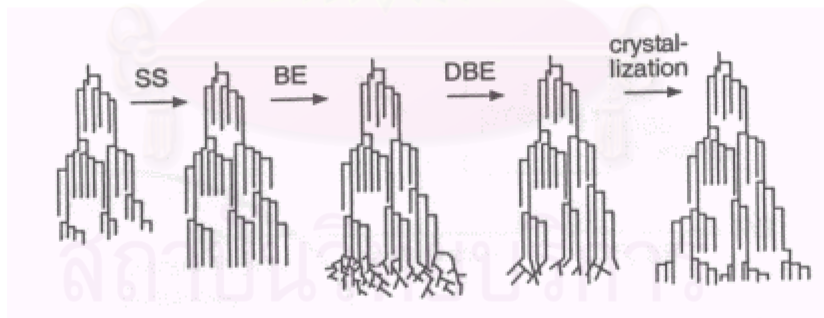

Figure 1.5 Amylopectin biosynthesis model (12)

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1.4 Starch synthase

Starch synthase (EC 2.4.1.21) was first observed by Leloir *et al.* (15). 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 $\alpha(1\rightarrow4)$ linkages. This reaction was first described using UDP-glucose as the nucleotide glucose donor. Subsequently, Recondo and Leloir showed that ADP-glucose was a better substrate both in terms of V_{max} and affinity (16). 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 60kDa protein in maize, 60kDa protein in rice, 68kDa protein in amaranth, 60kDa protein in pea and 60kDa 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 (14). 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 stage of development, 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 (14).

GBSSI used ADP-glucose and malto-oligosaccharide as substrates while amylopectin acted as an effector increasing the rate at which it elongated malto-oligosaccharides. The affinity of GBSS I for amylopectin as an effector was greater than its affinity for amylopectin as a substrate (15). 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 processing elongation of malto-oligosaccharide (15).

Moreover, a novel GBSS was discovered in waxy wheat, in which amylose was observed in pericarp, embryo and aleurone layer. Novel GBSS was different from GBSSI by molecular weight, isoelectric point and N-terminal amino acids. Its molecular weight was 56kDa and the N-terminal was similar to GBSSI by 40-50% and cross-react with GBSSI antisera of maize and potato. Therefore, the 56kDa protein was suggested to be GBSSI class enzyme and it was a waxy protein isoform, and its expression was controlled by development stage and may be tissue specific (17,18).

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 (19).

In cassava, the size of waxy protein (GBSSI) was predicted to be about 58.61kDa (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%) (20). 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 (21).

4.1.2 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 (22), 2 isoforms in maize kernel (23), 3 isoforms in potato tuber (24), 1 isoform in grape leaf (25), 2 isoforms in maize leaf (25), 2 isoforms in rice grain (26), 3 isoforms in sorghum seed (27), and 1 isoform in castor bean endosperm (28). Some isoform(s) of starch synthase can catalyze the synthesis of α-(1$\rightarrow$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 (29,30), maize (31), and pea (32).

Maize SSSs have been studied for about 30 years. The 2 isoforms of SSSs were discovered in maize kernel (23). 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 (33). SSSI showed the molecular weight about 76kDa and was 90% associated with starch granule (31). 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 (34). Moreover, the antiserum against SSSI eliminated approximately 60% of the total SSS activity (35). 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 (23). The optimum temperature is 37°C and optimum pH is about 8.5 (33). The study in maize kernels homozygous for the recessive *dull* allele (*du*) found lower of SSS activity (36). The *du1*- mutation defined a gene with an important function in starch synthesis enzymes, especially for SSSII and SBEIIa. The *Du1* gene was predicted coding for SSSII because its similarity of predicted amino acid sequence to potato SSIII and glycogen synthase (37). The other information to confirmed *Du1* gene code for SSSII was the *Du1* antisera eliminated 20-30% of total SSS activity from the kernel extract. Otherwise, the C-terminal of *Du1* gene product has the conserved sequence for SS and glycogen synthase (35). The *du1* antisera detected a soluble endosperm protein more than 200kDa that was lacking in *du*- mutants. It was related the 188kDa protein which predicted by cDNA of *Du1* gene (37).

In potato, the major isoform of SS in the soluble fraction from potato tuber, referred to 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 (38). The molecular weight of SSIII was determined of which was about 140kDa 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 (39). 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 (39).

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1.5 Objectives

Because cassava starch is used as a raw material in many industries and required the different starch quality, the study of starch biosynthesis enzymes in cassava are important to improve cassava starch quality and quantity for many local industries that can reduce dependence on the EC feed market. There is no report about soluble starch synthase in cassava.

The objectives of this thesis are:

1. To purify and characterize starch synthase from cassava tubers.
2. To study kinetic constants of the purified cassava starch synthase.
3. To study the effect of primers on cassava starch synthase activity.
4. To study the effect of thiol group reagents on cassava starch synthase activity

CHAPTER II

MATERIAL AND METHODS

2.1 Plant material

Cassava tubers were purchased from Pak Klong Ta Lad market, Bangkok, Thailand.

2.2 Chemical

Chemical	Company
Acrylamide	Merck, Germany
ADP-glucose	Sigma, USA
Aquasorb	BML, Thailand
Ammonium dihidrogen phosphate	May&Baker, England
di-Ammonium hydrogen phosphate	May&Baker, England
Ammonium persulfate	Merck, Germany
Ammonium sulphate	Merck, 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
ρ-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
Glucose	Sigma, USA
Chemical	Company
Glutathione, Reduced form	Sigma, USA
Glycine	Sigma, USA

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Glycogen, Oyster	Sigma, USA
Glycogen, Rabbit liver	Sigma, USA
Iodoacetic acid (IAA)	Aldrich, USA
Isoelectric focusing calibration kit pH 3-10	Pharmacia Biotech, 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	Pharmacia, Sweden
Sodium laulyl sulphate (SDS)	BDH, England
Standard low molecular weight marker proteins	Pharmacia, USA
N,N,N',N'-Tetramethylene ethylene diamine (TEMED)	BDH, England
Trichloroacetic acid	Fluka, Switzerland
Tris (hydroxymethly) aminomethane	Fluka, Switzerland

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

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 II	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 (Figure 2.1) were peeled and the cortex (Figure 2.2) was removed. The four kilograms of parenchyma (Figure 2.3) was chopped and homogenized in a blender containing 1 liter of ice-cold 50mM Tris-acetate pH 8.5 with 10mM EDTA, 2mM DTT (23). The buffer was added 1mM PMSF and 20% glycerol to prevent some protease activity and stabilize enzyme. The homogenate was filtered and centrifuged at 15,000x g for 30 minutes at 4°C to remove starch and lipid. The supernatant was collected as crude enzyme and kept at 4°C for further works.

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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 15,000 x g for 60 minutes at 4°C. Solid ammonium sulfate was further added to the supernatant to give 60% saturation. The pellet was collected by centrifugation at 15,000 x g for 60 minutes at 4°C and dissolved in 50mM Tris-acetate pH 8.5 containing 10mM EDTA and 2mM DTT (23).

2.4.3 Phenyl Sepharose column chromatography

Phenyl Sepharose high performance column (1.7 x 7cm.) was washed with 2 column volumes of sodium hydroxide 0.5N followed with 500ml distilled water. The column was then equilibrated with elution buffer (50mM Tris-acetate pH 8.5, 10mM EDTA and 2mM DTT) containing 1M ammonium sulfate. The sample from ammonium sulfate precipitation step was centrifuged to remove undissolved particles. The pellet was re-dissolved and determined for starch synthase activity. The supernatant was loaded onto the column and eluted with 10 column volumes of elution buffer at flow rate of 20ml/hr controlled by peristaltic pump. The enzyme was step-wise eluted with 0.5M, 0.2M, 0.1M, and 0M.ammonium sulfate in elution buffer, respectively. Fractions of 4ml were collected using fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280nm 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.

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2.4.4 Sephadex G-200 column chromatography

Sephadex G-200 was swelled in distilled water for 24 hours at 50°C. The swelled gel was degassed and packed into a glass column (2.4 x 90 cm.) using peristaltic pump at flow rate of 25ml/hr. The Sephadex G-200 column (2.4 x 75cm.) was equilibrated with elution buffer for 5 column volumes at flow rate of 20ml/hr to allow stabilization of bed volume of the column. The column was calibrated with molecular weight marker proteins (catalase MW = 232kDa, bovine serum albumin MW = 68kDa, ovalbumin MW = 43kDa, chymotrypsinogen MW = 27kDa, and cytochrome C MW = 11.7kDa) at flow rate of 20ml/hr at air-condition room temperature. Blue dextran 2000 and potassium dichromate were used to determine the void volume and the total volume of the column. An aliquot (5ml) of the concentrated enzyme from Phenyl Sepharose column chromatography was loaded onto the column and eluted with elution buffer at a flow rate of 20ml/hr. Fractions of 2ml were collected using fraction collector. The elution profile was monitored for protein and enzyme activity as previously described (section 2.4.3). The fractions showing enzyme activity were pooled for further purification step.

2.4.5 Q-Sepharose column chromatography

Q-Sepharose column (1.7 x 7cm.) was washed with 2 column volumes of 0.5N sodium hydroxide followed with 500ml of distilled water. The column was then equilibrated with 100ml of elution buffer. The pooled fraction from Sephadex G-200 column chromatography was loaded onto the column and washed with 10 column volumes of elution buffer to remove unbound proteins. The enzyme was eluted with 20 column volumes of a linear gradient of 0-0.5M potassium chloride in elution buffer at flow rate of 20ml/hr. Fractions of 2ml were collected using fraction collector. The elution profile was monitored for protein and enzyme activity as previously described in section 2.4.3. The fractions showing enzyme activity was pooled and kept in 20% (v/v) glycerol. The pooled fractions were

dialyzed in elution buffer including 20% (v/v) glycerol. The dialyzed enzyme was collected in aliquots and kept in –20°C for further characterization.

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Figure 2.1 Cassava tuber

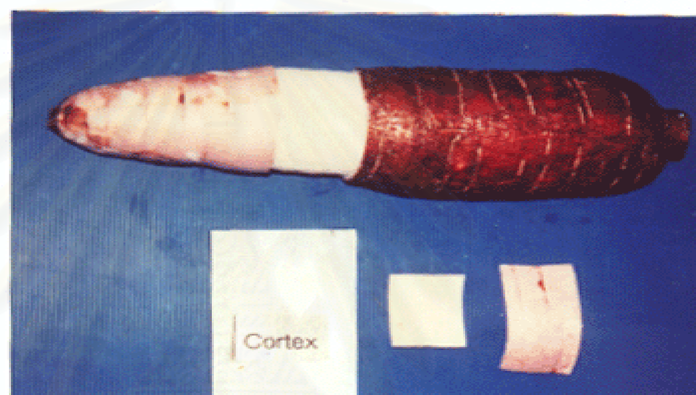

Figure 2.2 Cassava cortex

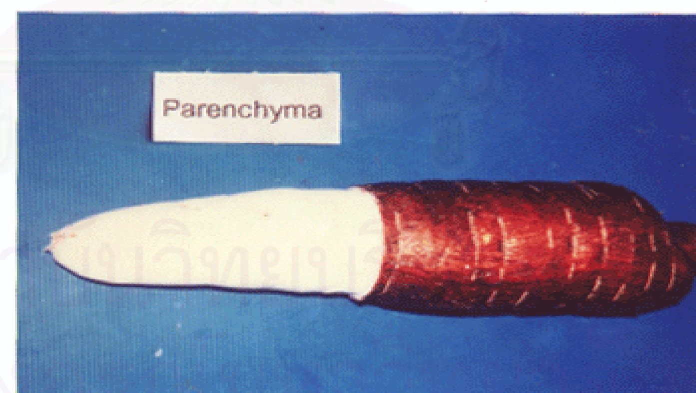

Figure 2.3 Cassava parenchyma

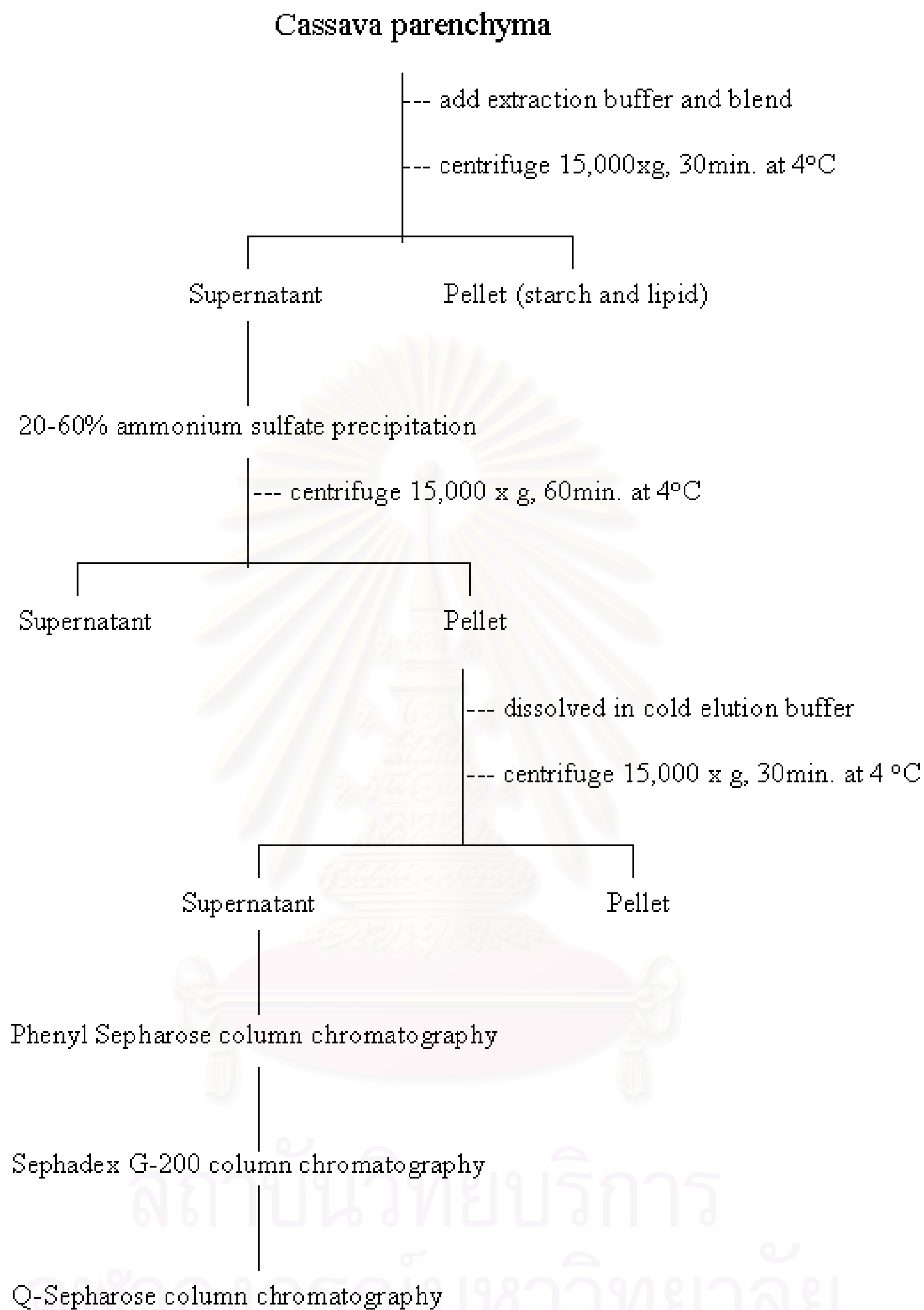

Figure 2.4 Flow chart of purification of cassava starch synthase.

2.5 Determination of starch synthase activity

Starch synthase activity was determined by the developed methods of Hawker (40) and Viola (41). ADP-glucose was used as a nucleotide sugar donor for this reaction.

In the reaction, when 1 nmole of glucose units was incorporated into primer, 1nmole of ADP was released and represented the amount of incorporated glucose units. The assay mixture contained 100mM Bicine-KOH pH 8.5, 25mM potassium acetate, 10mM glutathione (reduced form), 5mM EDTA, 1mM ADP-glucose, 20mg/ml glycogen, and enzyme in a final volume of 200μl. After incubation at 28°C for 15 minutes, the reaction was stopped by heating in boiling water for 5 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was separated and assay for amount of ADP produced by using HPLC system (Hewlett-Packard LC 1050 series with UV detector, USA.). Aliquot (20μl) of supernatant was injected onto Sphereclone-SAX (10μ, 250 x 4.6 mm: Phenomenex, USA.). Mobile phases were (A) 10mM ammonium phosphate, pH 3.0 and (B) 450mM Ammonium phosphate, pH 4.5. Flow rate was 1ml per minute. The column was equilibrated with 100% A and 0% B before injected. After injection, the gradient of mobile phase was established as followed: isocratic 0% B for 1 minute, linear increases to 5% B for 5 minutes, isocratic 5% B for 2 minutes, linear decreases to 0% B for 0.5 minute and isocratic 0% B for 2 minutes. The detector was set at 254nm. Under these conditions, ADP-glucose was eluted at 6.8 minutes and ADP was eluted at 7.1 minutes. Unit activity of starch synthase was defined as 1 nmole of glucose incorporated in 1 minute at 28°C. Specific activity was defined as unit activity per mg protein. The calculation of activity and specific activity was described in Appendix A.

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2.6 Determination of protein concentration

Protein concentration was determined by the method of Bradford (42) using bovine serum albumin as standard (see Appendix B). The 100μl of sample was mixed with 1ml of Coomassie blue reagent and left for 5 minutes before measured absorbance at 595nm. The 1 liter of Coomassie blue reagent was the mixture of 100mg Coomassie blue G250, 50ml ethanol, 100ml 85% phosphoric acid, and 850ml distilled water.

2.7 Polyacrylamide gel electrophoresis (PAGE)

2.7.1 Non-denaturing gel electrophoresis

The enzyme from each step of purification was analyzed by non-denaturing polyacrylamide gel electrophoresis to determine the native protein pattern. The system was modified from the method of Cameo and Blaquier (43). The separating gel (10 x 8.6 x 0.075cm) contained 10% (w/v) acrylamide in 0.025M Tris and 0.192M Glycine pH 8.3. The stacking gel (10 x 2 x 0.075cm) contained 3% (w/v) acrylamide in 0.125M Tris-HCl pH 6.8. Preparation of solutions and polyacrylamide gel was described in Appendix C. The enzyme from each step was mixed with 5 x sample buffer by ratio 5:1 and loaded onto the gel. Electrophoresis was performed at a constant current of 10mA. Following electrophoresis, the gel was stained for protein as described in section 2.7.3.

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2.7.2 Two-dimension SDS-polyacrylamide gel electrophoresis

The SDS-polyacrylamide gel electrophoresis system was performed according to the modified method described by Laemmli (44). The slab gel system consisted of a stacking gel (10 x 2 x 0.1cm) of 3% (w/v) acrylamide and a separating gel (10 x 8.6 x 0.1cm) of 10% (w/v) acrylamide. The gel preparation was described in Appendix C. The enzyme from Q-Sepharose column chromatography step was analyzed for protein pattern in native state by non-denaturing gel electrophoresis on a slab gel system as described in section 2.7.1. The gel, which contained enzyme pattern in native state, was cut and soaked in sample buffer (1% SDS, 4% glycerol, 1% β-mercaptoethanol in 0.062M Tris-HCl pH 6.8) for 30 minutes. Afterward, the soaked gel was transferred onto the SDS-polyacrylamide gel. Electrophoresis was performed at a constant current of 10mA. The standard molecular weight markers used were phosphorylase b (MW = 94kDa), bovine serum albulin (MW = 67kDa), ovalbumin (MW = 43kDa), carbonic anhydrase (MW = 30kDa), soybean trypsin inhibitor (MW = 20.1kDa) and α-lactalbumin (MW = 14.4kDa). Following electrophoresis, the gel was stained for protein to determine molecular weight of starch synthase as described in section 2.7.3.

2.7.3 Determination of protein pattern on polyacrylamide gel

The gel from electrophoresis methods was stained for 30 minutes with staining solution (0.1% (w/v) Coomasie Brilliant Blue R-250 in 40% (v/v) methanol and 7% (v/v) acetic acid) at room temperature with moderate shaking. Destaining was performed by immersing the gel for 30 minutes in destaining solution I (40% methanol and 7% acetic acid) followed with destaining solution II (5% methanol and 7% acetic acid) overnight.

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2.8 Native IEF and two-dimension gel electrophoresis

The native isoelectric focusing polyacrylamide gel on mini gel system was used for determining the pI value of starch synthase. The gel (10 x 9.4 x 0.075cm) was prepared as described in Appendix D. The IEF system was run at pH range 3.0-10.0. The enzyme from Q-Sepharose column chromatography step was mixed with sample buffer (60% glycerol, 4% ampholyte) at ratio of 2:1. The IEF condition was performed by the method of Robertson (45), the cathode solution was 25mM NaOH and the anode solution was 20mM acetic acid. Electrophoresis was performed at room temperature for 1.5 hour at 200V constant voltage, then increased to 400V constant voltage for additional 1.5 hour. After electrophoresis was completed, the gel was fixed by immersion in 10% trichloroacetic acid (TCA) for 10 minutes followed with 1% TCA for at least 2 hours to remove ampholyte. The fixed gel was stained for protein as described in section 2.7.3. The standard pI calibration kit (pI 3.0-10.0) was used as standard pI markers.

For two-dimension SDS-PAGE, 1 lane of IEF gel was cut and incubated in equilibration buffer (5% β-mercaptoethanol, 62.5mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol, 0.1% bromphenol blue) for 30 minutes. The gel was loaded onto SDS-polyacrylamide gel and electrophoresis performed as described in section 2.7.2. After electrophoresis, the gel was stained for protein as described in section 2.7.3.

2.9 Characterization of starch synthase

2.9.1 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 100mM of MOPS-NaOH, Bicine-NaOH and Glycine-NaOH were used as reaction buffer for pH 6.5-7.9, 7.6-8.9 and 8.6-10.5 respectively. After the reaction was stopped by heating in boiling water for 5 minutes, ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

2.9.2 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, 37, 40, 45, 50, 55 and 60°C for 15 minutes. After the reaction was stopped by heated in boiling water for 5 minutes, the ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

2.9.3 Temperature stability of starch synthase

The temperature stability of starch synthase was studied. The enzyme was incubated at 25, 30, 35, 40, 45, 50, 55 and 60°C for 15 minutes. Afterward, the activity of the incubated enzyme was assayed at 28°C for 15 minutes. The reaction was stopped by heated 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, 6, 12, 18, 24, 30, 36, 42 and 48 hours and collected to assay as described previously. After the reaction was stopped by heated in boiling water for 5 minutes, the ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

2.9.4 Effect of primer on starch synthase activity

The effect of various primers on starch synthase was studied. The reaction mixture was prepared as described in section 2.5. The rabbit liver glycogen was replaced by 20mg/ml of oyster glycogen, amylopectin, amylose, cassava starch, rice starch, potato starch, and corn starch. In addition, the 20mM of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and was also used instead of rabbit liver glycogen in assay mixture. The reaction was stopped by heated in boiling water after incubated at 28°C for 15 minutes. The ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

2.9.5 Effect of sulfhydryl group reagents on starch synthase activity

The effect of the sulfhydryl group reagents on starch synthase activity were studied. Different concentrations (2mM, 5mM, and 10mM) of DTT, NEM, IAA, and PCMB were added to different reaction mixtures. The reactions were stopped by heated in boiling water after incubated at 28°C for 15 minutes. The ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

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2.9.6 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 amount of excess rabbit liver glycogen. The reaction was

incubated at 28°C for 15 minutes, and was stopped by heated in boiling water and the ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

2.9.7 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 20.0mg/ml with fixed concentration of excess ADP-glucose. The reaction was stopped by heated in boiling water after incubation at 28°C for 15 minutes. The ADP was detected and measured as described in section 2.5. The enzyme activity unit was calculated as described in Appendix A.

CHAPTER III

RESULTS

3.1 Purification of starch synthase form cassava tubers

3.1.1 Preparation of crude enzyme

Crude cassava starch synthase was prepared from cassava parenchyma tissue as described in section 2.4.1. From 4 kilograms of cassava parenchyma, crude cassava starch synthase fraction was obtained with 4,273mg proteins and 60,255 units of starch synthase activity in total volume of 2,285ml. The specific activity of the enzyme in the crude preparation was 14.1nmol of glucose incorporated/min/mg protein.

3.1.2 Ammonium sulfate precipitation

Crude cassava starch synthase was further purified by ammonium sulfate precipitation as described in section 2.4.2. 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 most starch synthase activity in the 20-30, 30-40, 40-50, and 50-60% with highest activity in 30-40%. Therefore, to harvest most of starch synthase, protein fractionation was performed in the range of 20-60% saturated ammonium sulfate precipitation. The protein remained was 2,565mg with starch synthase activity recovered at 17,372 units (about 29% recovery from crude enzyme). The specific activity of the enzyme from this step was 6.8nmol of glucose incorporated/min/mg protein.

3.1.3 Phenyl Sepharose column chromatography

Cassava starch synthase from 20-60% saturated ammonium sulfate precipitation was dissolved 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 3.1. The unbound proteins were eluted from Phenyl Sepharose with elution buffer containing 1M ammonium sulfate. The other proteins, which were bound to the column, were eluted by step-wise method with elution buffer containing 0.5, 0.2, and 0.1M ammonium sulfate. Starch synthase bound to Phenyl Sepharose column and was eluted by elution buffer without ammonium sulfate. The fractions with starch synthase activity were pooled and concentrated by aquasorb to reduce volume. The protein remained from this step was 84.96mg with 4,594 activity units of starch synthase. The specific activity of the enzyme from this step was 54.1nmol of glucose incorporated/min/mg protein. Starch synthase activity was purified for about 4 folds and recovery was about 7.6% compared to crude enzyme.

3.1.4 Sephadex G-200 column chromatography

The concentrated protein from Phenyl Sepharose column was further purified by Sephadex G-200 column chromatography as described in section 2.4.4. The chromatographic profile was shown in Figure 3.2. The column was calibrated with various standard molecular weight markers: catalase, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C and molecular weight calibration curve was constructed (Figure 3.3). The molecular weight of starch synthase determined from the curve was 53.4kDa. The fractions containing starch synthase activity were pooled. Starch synthase activity was recovered at 4,046 activity units with 9.67mg protein. The specific activity of the enzyme from this step was 418.2nmol of glucose incorporated/min/mg protein. The enzyme was purified for about 30 folds with about 6.7% recovery compared to crude enzyme.

3.1.5 Q-Sepharose column chromatography

The pooled starch synthase activity fraction from Sephadex G-200 was loaded onto Q-Sepharose column chromatography as described in section 2.4.5. The chromatographic profile is shown in Figure 3.4. 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 0M to 0.5M potassium chloride. The enzyme was eluted in the range of 0.3M to 0.4M potassium chloride. The activity fractions were collected and glycerol was added to final concentration of 20% (V/V) and dialyzed against elution buffer containing 20% glycerol. The dialyzed enzyme has 0.54mg protein with 1,647 activity units. The specific activity of the enzyme was 3,100nmol of glucose incorporated/min/mg protein. From this step, the enzyme was purified to 220 folds with about 2.8% recovery. The enzyme from this step was kept in aliquot at –20°C for further characterization.

3.1.6 Summary of cassava starch synthase purification

Cassava starch synthase was extracted and purified by ammonium sulfate precipitation and column chromatographs as described previously. The summary of purification of this enzyme is shown in Table 3.1 and Figure 3.5. In the final step of purification, cassava starch synthase was purified to about 220 times compared to crude enzyme with about 2.8% recovery of starch synthase activity.

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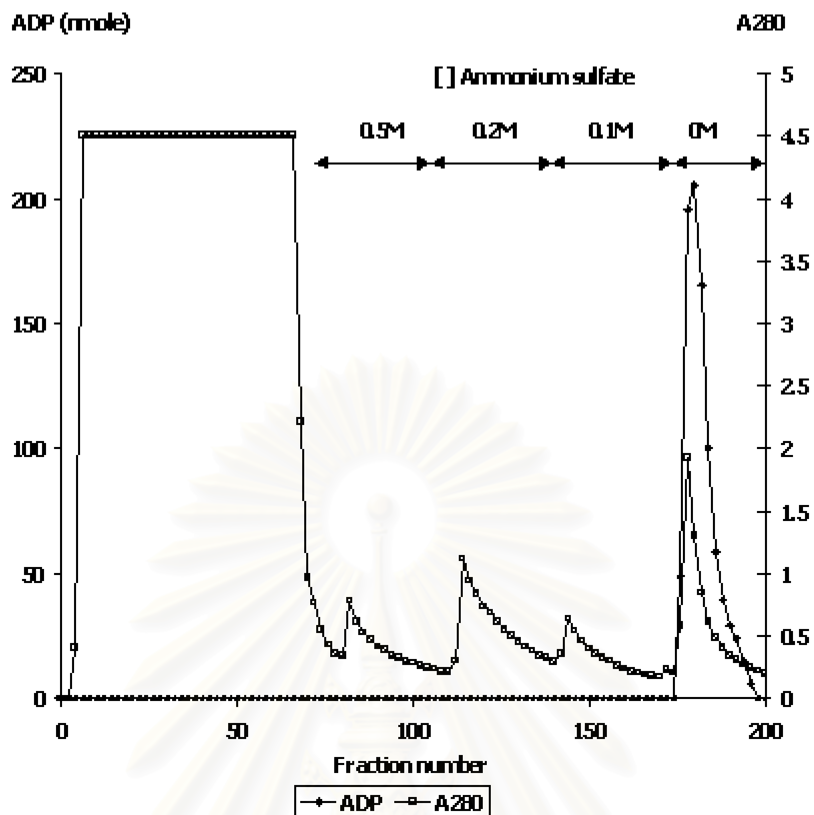

Figure 3.1 Chromatographic profile on Phenyl Sepharose High Performance of 20-60% saturated ammonium sulfate precipitated fraction from cassava parenchyma.

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Figure 3.2 Chromatographic profile on Sephadex G-200 column of concentrated enzyme from Phenyl Sepharose High Performance column.

Fraction size = 2 ml

1 = Blue dextran

2 = Catalase MW = 232 kDa

3 = Bovine serum albumin MW = 68 kDa

4 = Ovalbumin MW = 43 kDa

5 = Chymotrypsinogen MW = 27 kDa

6 = Cytochrome C MW = 11.7 kDa

7 = Potassium dichromate

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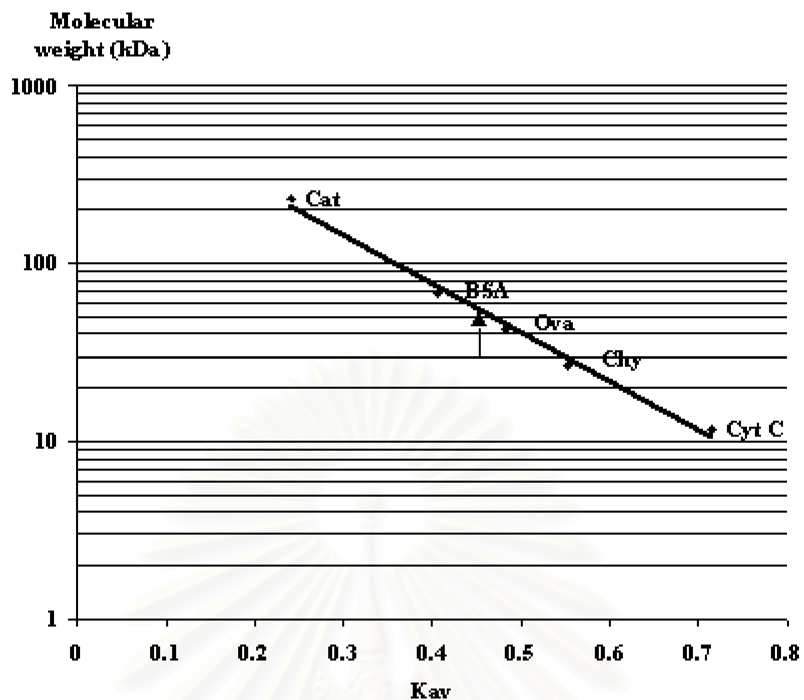

Figure 3.3 Calibration curve for native molecular weight determines by chromatography on Sephadex G-200 column.

Cat	= Catalase	MW = 232 kDa
BSA	= Bovine serum albumin	MW = 68 kDa
Ova	= Ovalbumin	MW = 43 kDa
Chy	= Chymotrypsinogen	MW = 27 kDa
Cyt C	= Cytochrome C	MW = 11.7 kDa

Arrow indicates the K_{av} of starch synthase

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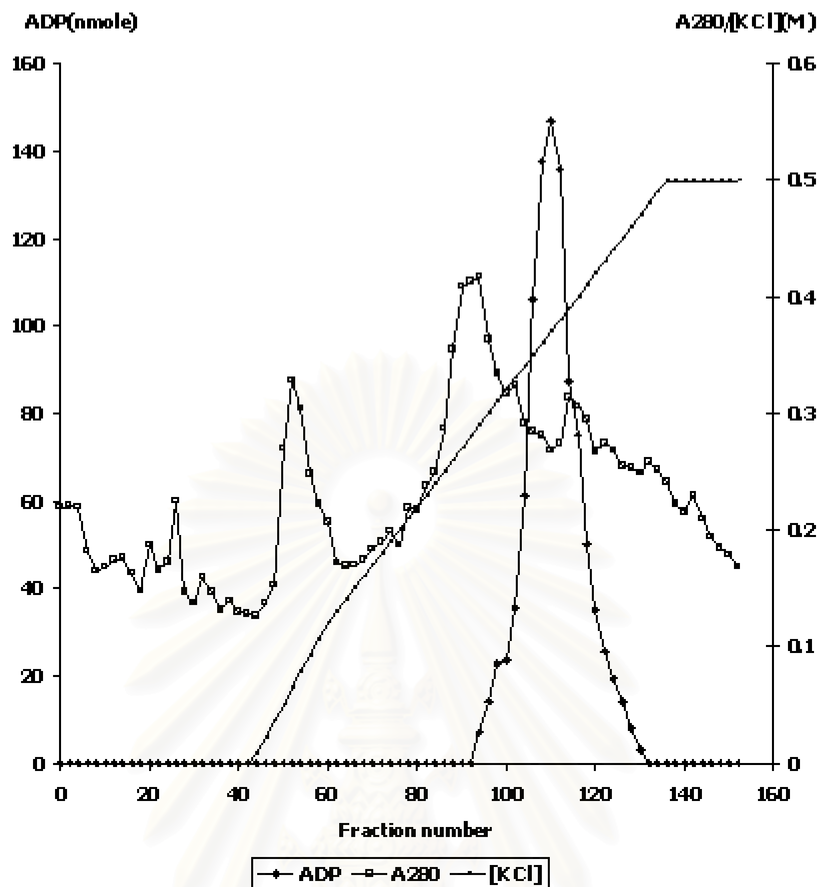

Figure 3.4 Chromatographic profile on Q-Sepharose column of the enzyme from Sephadex G-200 column

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Table 3.1 Purification Table of cassava starch synthase

Fraction	Volume (ml)	Total Protein (mg)	Total Activity	Specific Activity	Purification fold	Recovery(%)
Crude	2,285	4,273	60,255	14.1	1	100
Am	540	2,565	17,372	6.8	0.5	28.8
Phenyl	96	84.96	4,594	54.1	3.8	7.6
G-200	59	9.67	4,046	418.2	29.7	6.7
Q-Sep	36	0.54	1,674	3,100	219.9	2.8

Activity unit : The nmole of glucose incorporated in 1 minute at 28°C

Specific activity : The activity unit per mg protein

Am = The enzyme from 20-60% saturated ammonium sulfate precipitation

Phenyl = The enzyme from Phenyl Sepharose column chromatography

G-200 = The enzyme from Sephadex G-200 column chromatography

Q-Sep = The enzyme from Q-Sepharose column chromatography

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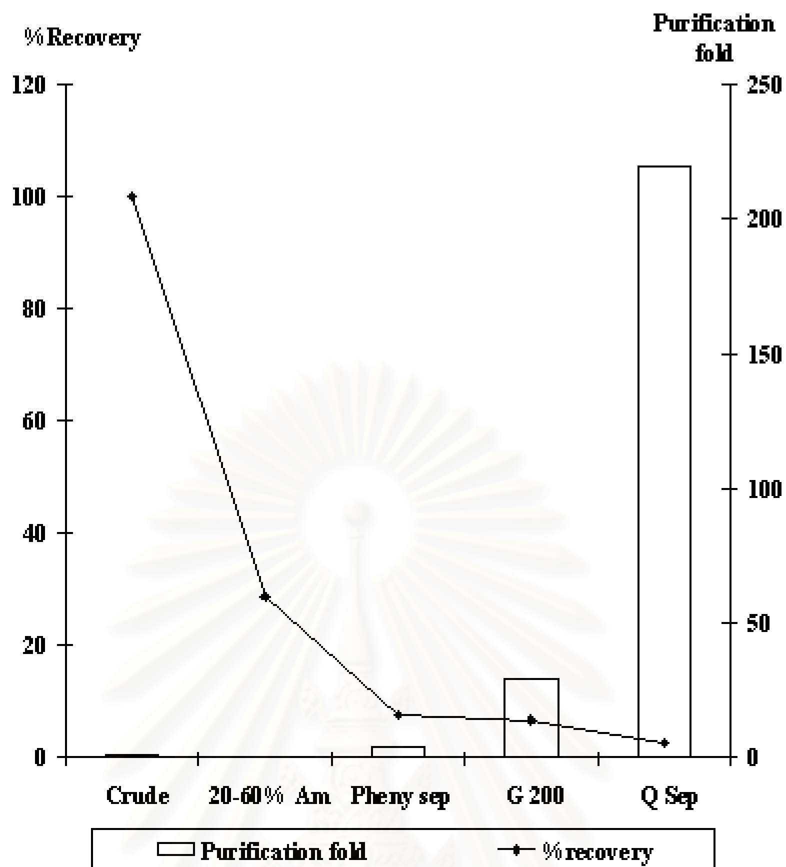

Figure 3.5 Summary of cassava starch synthase purification

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3.2 Determination of protein pattern on polyacrylamide gel electrophoresis

3.2.1 Non-denaturing polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed on non-denaturing PAGE and stained for protein as described in section 2.7.1. The results were shown in Figure 3.6. The protein pattern of starch synthase from Q-Sepharose column (lane5) showed two protein bands, which could implicate the existence of 2 isoforms of the enzyme.

3.2.2 Two-dimension SDS polyacrylamide gel electrophoresis

As the Q-Sepharose purified enzyme on non-denaturing polyacrylamide pattern showed two protein bands, their molecular weight were determined on SDS-polyacrylamide gel electrophoresis by two-dimension electrophoresis of the non-denaturing gel. The gel strip from non-denaturing PAGE was aligned on two-dimension SDS-PAGE and electrophoresis was performed as described in section 2.7.2. The result was shown in Figure 3.7. Starch synthase fraction showed 3 protein bands with the molecular weight in denaturing state of 79, 76,and 53.8kDa. The slow moving band on non-denaturing polyacrylamide gel electrophoresis appeared as 79 and 76kDa and the fast moving band corresponded to 53.8kDa. The 53.8kDa complied with the result from Sephadex G-200, while the 79 and 76kDa matched the position at the upper shoulder of the starch synthase activity peak. This further implicated the possible existence of 3 isoforms.

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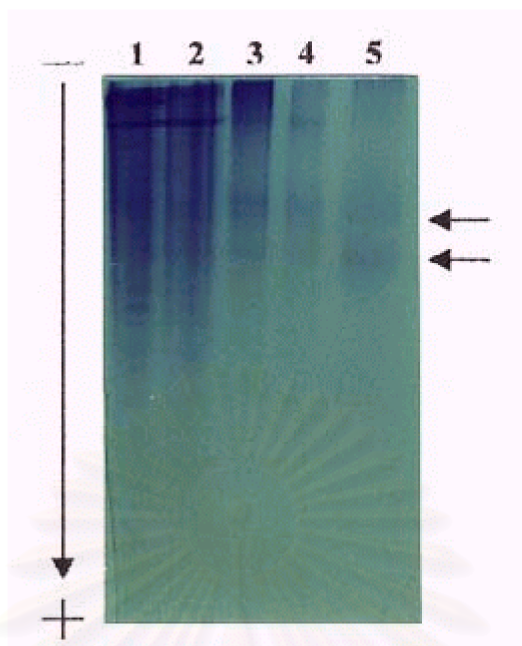

Figure 3.6 Non-denaturing polyacrylamide gel for enzyme purification

Lane 1 = Crude enzyme (40μg)

Lane 2 = 20-60% saturated ammonium sulfate precipitation (40μg)

Lane 3 = Phenyl Sepharose column chromatography (10μg)

Lane 4 = Sephadex G-200 column chromatography (5μg)

Lane 5 = Q-Sepharose column chromatography (5μg)

Arrow lines indicate the positions of starch synthases as determined from two-dimension gel electrophoresis in Figure 3.7.

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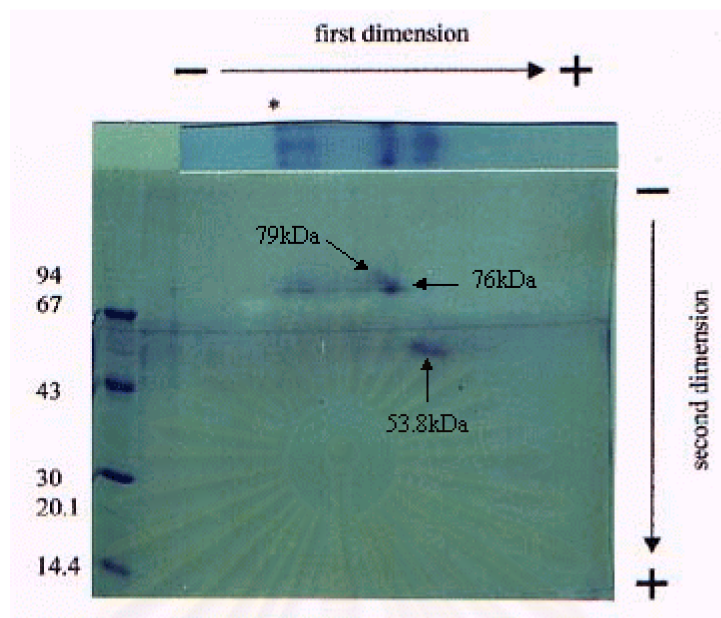

Figure 3.7 Two-dimension SDS-PAGE after non-denaturing PAGE

Phosphorylase B	MW = 94 kDa
Bovine serum albumin	MW = 67 kDa
Ovalbumin	MW = 43 kDa
Carbonic anhydrase	MW = 30 kDa
Soybean trypsin inhibitor	MW = 20.1 kDa
α-Lactabumin	MW = 14.4 kDa

* position of interface between stacking and separating gel

A, B, and C indicate the positions of the 79, 76, and 53.8 kDa proteins, respectively

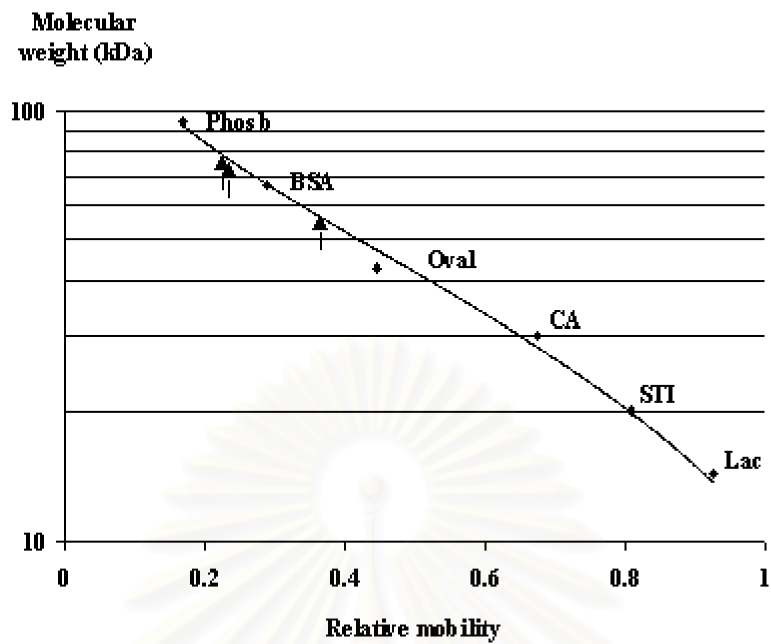

Figure 3.8 Calibration curve for molecular weight on two-dimension SDS-PAGE

Phos B	= Phosphorylase B	MW = 94 kDa
BSA	= Bovine serum albumin	MW = 67 kDa
Oval	= Ovalbumin	MW = 43 kDa
CA	= Carbonic anhydrase	MW = 30 kDa
STI	= Soybean trypsin inhibitor	MW = 20.1 kDa
Lac	= α-Lactabumin	MW = 14.4 kDa

Arrows indicate the positions of starch synthase

3.3 Native IEF and two-dimension polyacrylamide gel electrophoresis

As the enzyme showed two bands on the non-denaturing polyacrylamide gel and three bands on SDS-polyacrylamide gel, the pI of these bands cannot be identified on regular IEF on gel bond. Two-dimensional gel electrophoresis of the starch synthase was performed on IEF gel running on mini gel system followed by SDS-PAGE. The enzyme was determined for pI value on mini gel system as described in section 2.8. The result was shown in Figure 3.9. Two protein bands appeared at pI 6.41 and 6.91. On second dimension SDS-polyacrylamide gel, the protein at pI 6.41 appeared as two protein bands at 76 and 53.8kDa position and the protein at pI 6.91 appeared at 79kDa position. (Figure 3.10)

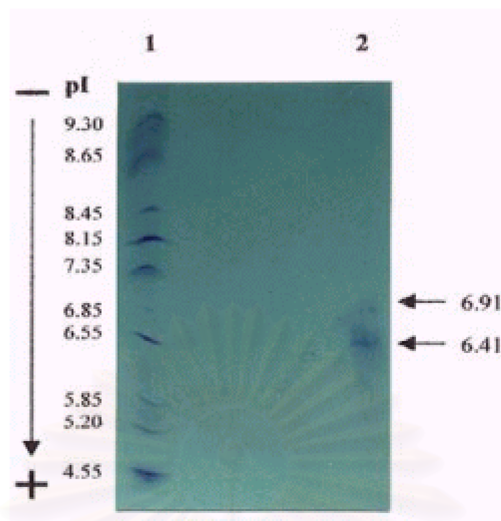

Figure 3.9 IEF of starch synthase on polyacrylamide mini gel system

Lane1 = Standard pI markers

Soybean trypsin inhibitor	pI = 4.55
β-Lactoglobulin A	pI = 5.20
Bovine serum albumin B	pI = 5.85
Human carbonic anhydrase B	pI = 6.55
Myoglobin acidic	pI = 6.85
Myoglobin basic	pI = 7.35
Lentil lectin acidic	pI = 8.15
Lentil lectin middle	pI = 8.45
Lentil lectin basic	pI = 8.65
Trypsinogen	pI = 9.30

Lane 2 = Starch synthase fraction from Q-Sepharose column (10μg)

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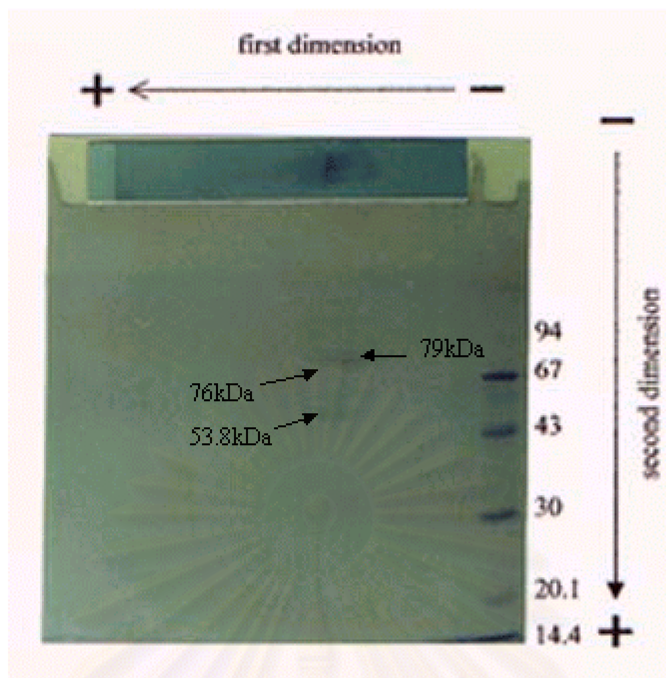

Figure 3.10 Two-dimension on SDS-PAGE after IEF on polyacrylamide mini gel system

Phosphorylase B	MW = 94 kDa
Bovine serum albumin	MW = 67 kDa
Ovalbumin	MW = 43 kDa
Carbonic anhydrase	MW = 30 kDa
Soybean trypsin inhibitor	MW = 20.1 kDa
α-Lactabumin	MW = 14.4 kDa

A, B, and C indicate the positions of the 79, 76, and 53.8 kDa proteins, respectively.

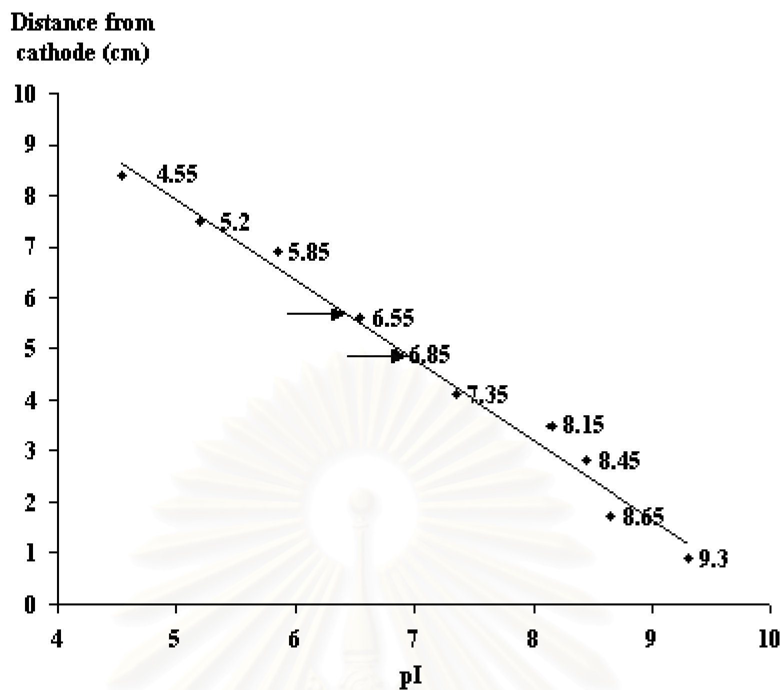

Figure 3.11 Calibration curve of pI on polyacrylamide mini gel system

Soybean trypsin inhibitor	pI = 4.55
β-Lactoglobulin A	pI = 5.20
Bovine serum albumin B	pI = 5.85
Human carbonic anhydrase B	pI = 6.55
Myoglobin acidic	pI = 6.85
Myoglobin basic	pI = 7.35
Lentil lectin acidic	pI = 8.15
Lentil lectin middle	pI = 8.45
Lentil lectin basic	pI = 8.65
Trypsinogen	pI = 9.30

Arrows indicate the positions of starch synthases

3.4 Characterization of cassava starch synthase

The cassava starch synthase purified from Q-Sepharose column chromatography was used for the characterization studies.

3.4.1 Optimum pH of cassava starch synthase activity

Cassava starch synthase was assayed at various pHs as described in section 2.9.1. The result was shown in Figure 3.12. MOPS/NaOH, Bicine/NaOH and Glycine/NaOH were used for pH range 6.5-7.9, 7.6-8.9 and 8.6-10.5, respectively. The enzyme showed highest activity or optimum pH at 8.5 and this was defined as 100% activity. At pH below 7.0, the starch synthase activity was reduced to about 30% and at pH higher than 10.0, the activity was reduced to about 20%.

3.4.2 Optimum temperature of cassava starch synthase activity

Cassava starch synthase was assayed at various temperatures at pH 8.5 as described in section 2.9.2. The result was shown in Figure 3.13. The enzyme showed the highest activity at 28°C and was defined as 100% activity. At 10 and 40°C, the starch synthase activity was decreased to about 40%. Starch synthase showed a little activity at 50°C and completely lost at 55°C.

3.4.3 Temperature stability of cassava starch synthase

Cassava starch synthase was preincubated at various temperatures for 15 minutes before assayed as described in section 2.9.3. The result was shown in Figure 3.14. The starch synthase activity of non-preincubated enzyme was defined as 100% activity. Its activity was significantly reduced when the temperature was over 45°C.

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The enzyme was further incubated at 45°C and checked for its activity for every 6 hours. The result was shown in Figure 3.15. The starch synthase activity at start was defined as 100% activity. After 6 hours, the enzyme activity started to reduce and the activity reduced to 50% after 30 hours.

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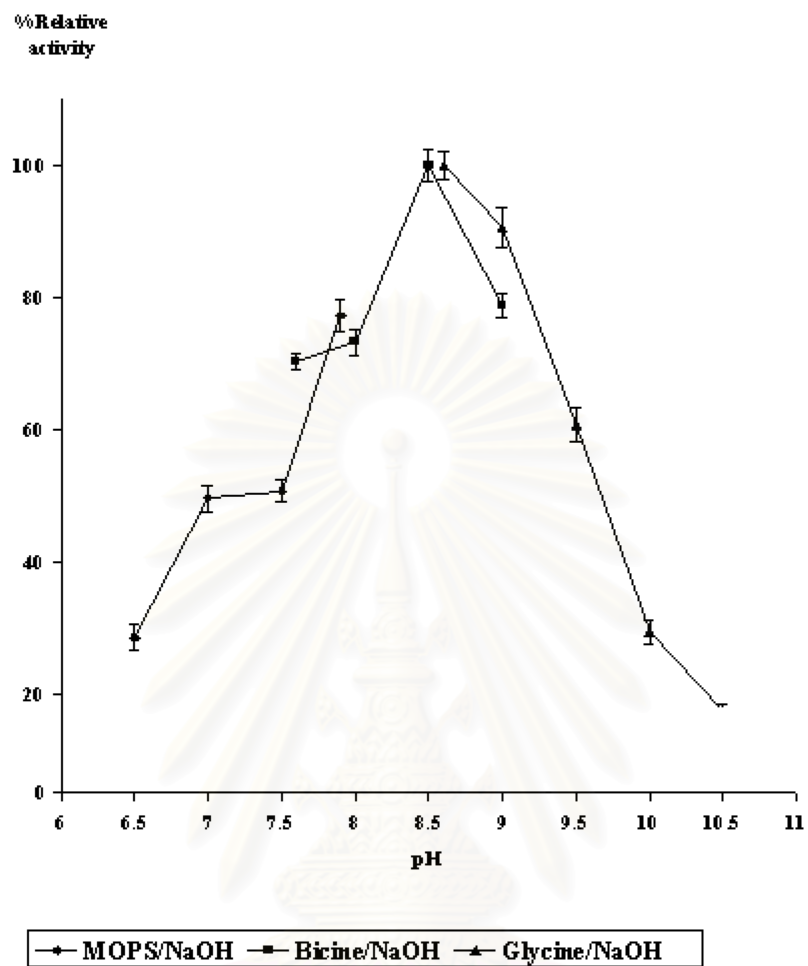

Figure 3.12. Effect of pH on cassava starch synthase activity

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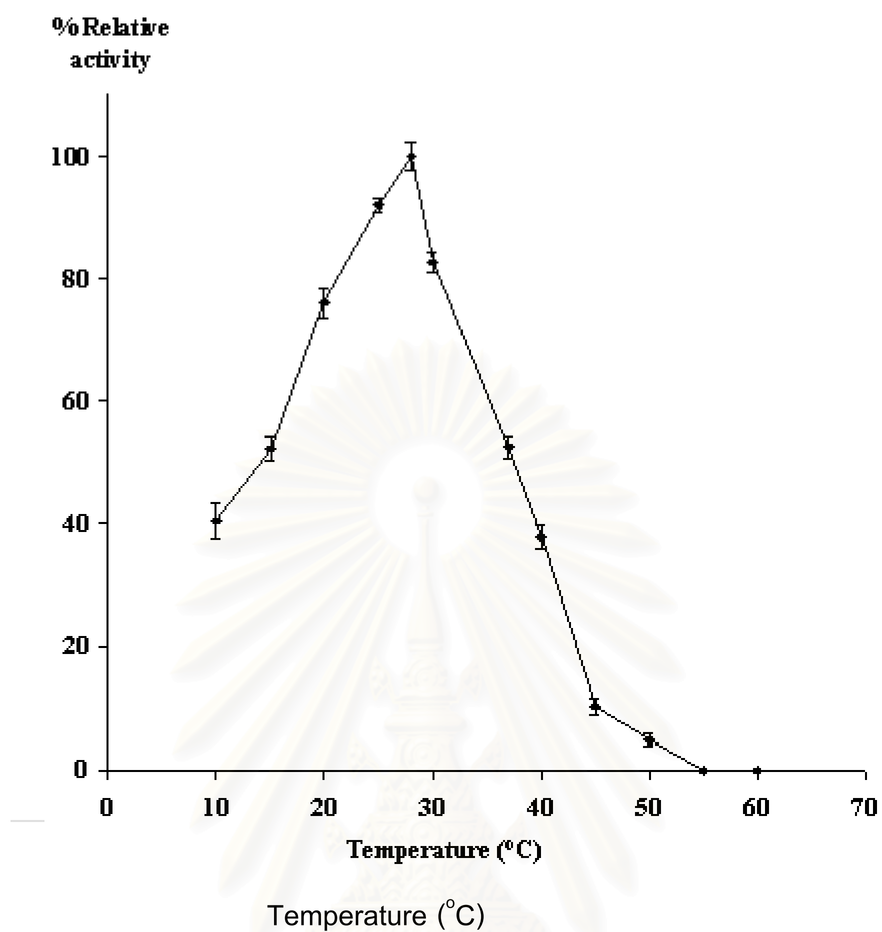

Figure 3.13. Effect of temperature on cassava starch synthase activity

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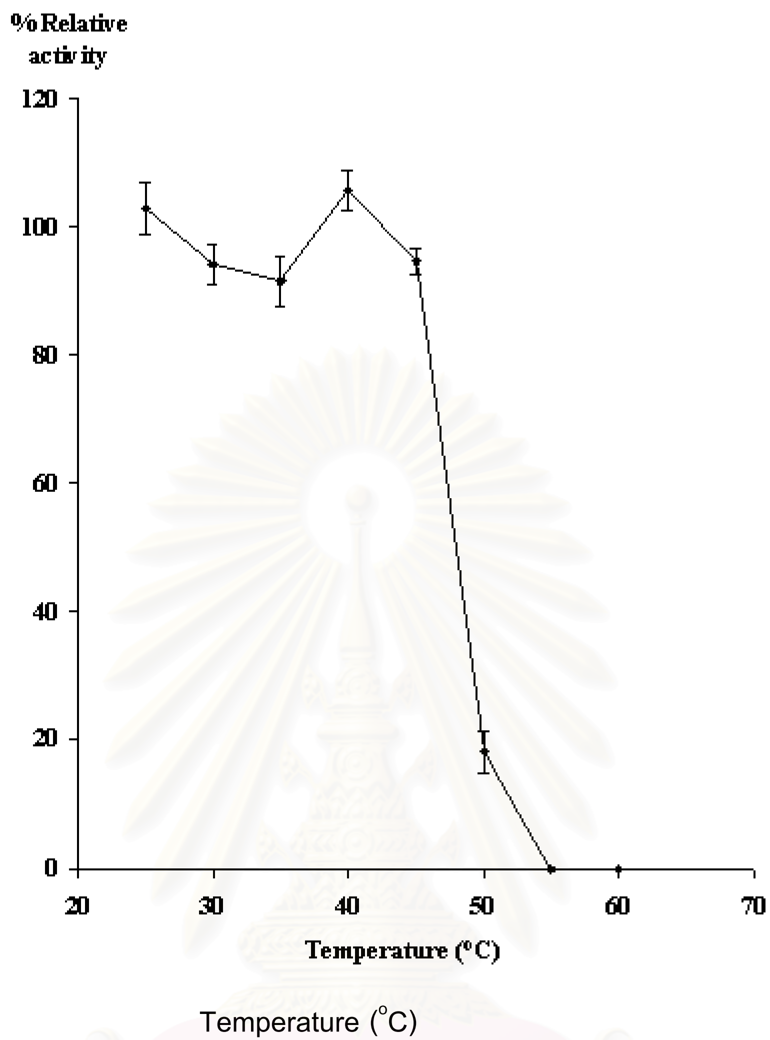

Figure 3.14. Temperature stability of cassava starch synthase

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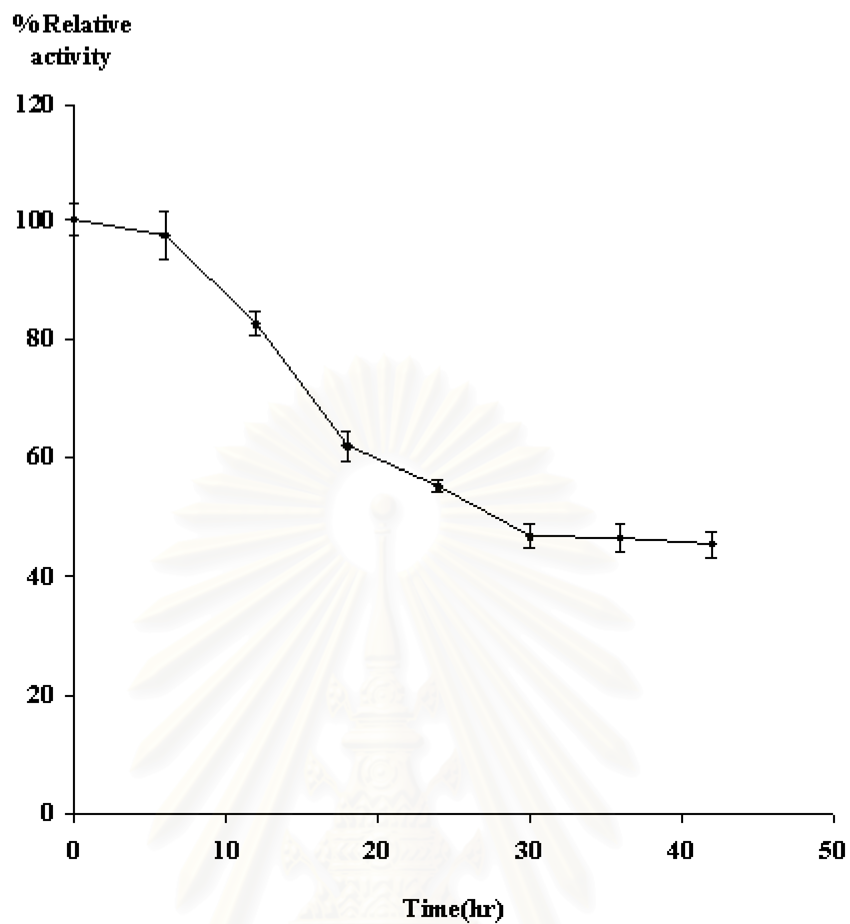

Figure 3.15. Enzyme stability at 45°C

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3.4.4 Effect of primer on cassava starch synthase activity

Cassava starch synthase was assayed using various primers as described in section 2.9.4. The primers can be defined into 3 groups. First, the homopolymers of glucose units, i.e. rabbit liver glycogen, oyster glycogen, amylopectin, and amylose. The second group is starch from differrent sources: cassava, rice, potato, and corn. And the last group is glucose and malto-oligosaccharides which have 2 to 7 glucose units. The results of the effect of primers were shown in Table 3.2 and Figure 3.16. The starch synthase activity when rabbit liver glycogen was used as primer was defined as 100% activity. For homopolymers of glucose units, the enzyme can use glycogen as a primer better than amylopectin and amylose. The rabbit liver glycogen gave the highest activity when it was used as primer for cassava starch synthase activity. For starch group, rice starch gave higher activity than other starches. The enzyme can also add the glucose units from ADP-glucose to malto-oligosaccharides which possesed at least three glucose units.

Table 3.2 Effect of primer on cassava starch synthase activity

Primer	Activity unit	%relative
Rabbit liver glycogen *	53.31	100
Oyster glycogen *	33.17	62.2
Corn amylopectin *	17.83	33.4
Corn amylose *	8.94	16.8
Cassava starch *	8.34	15.6
Rice starch *	18.95	35.5
Potato starch *	7.85	14.7
Corn starch *	7.65	14.3
Glucose **	0	0
Maltose **	0	0
Maltotriose **	2.47	4.63
Maltotetraose **	5.30	9.94
Maltopentaose **	4.90	9.18
Maltohexaose **	5.16	9.68
Maltoheptaose **	5.00	9.38

Activity unit = 1 nmole of glucose incorporated in 1 minute at 28°C

* Concentration 20 mg/ml

** Concentration 20 mM

Number of experiment (n) = 3

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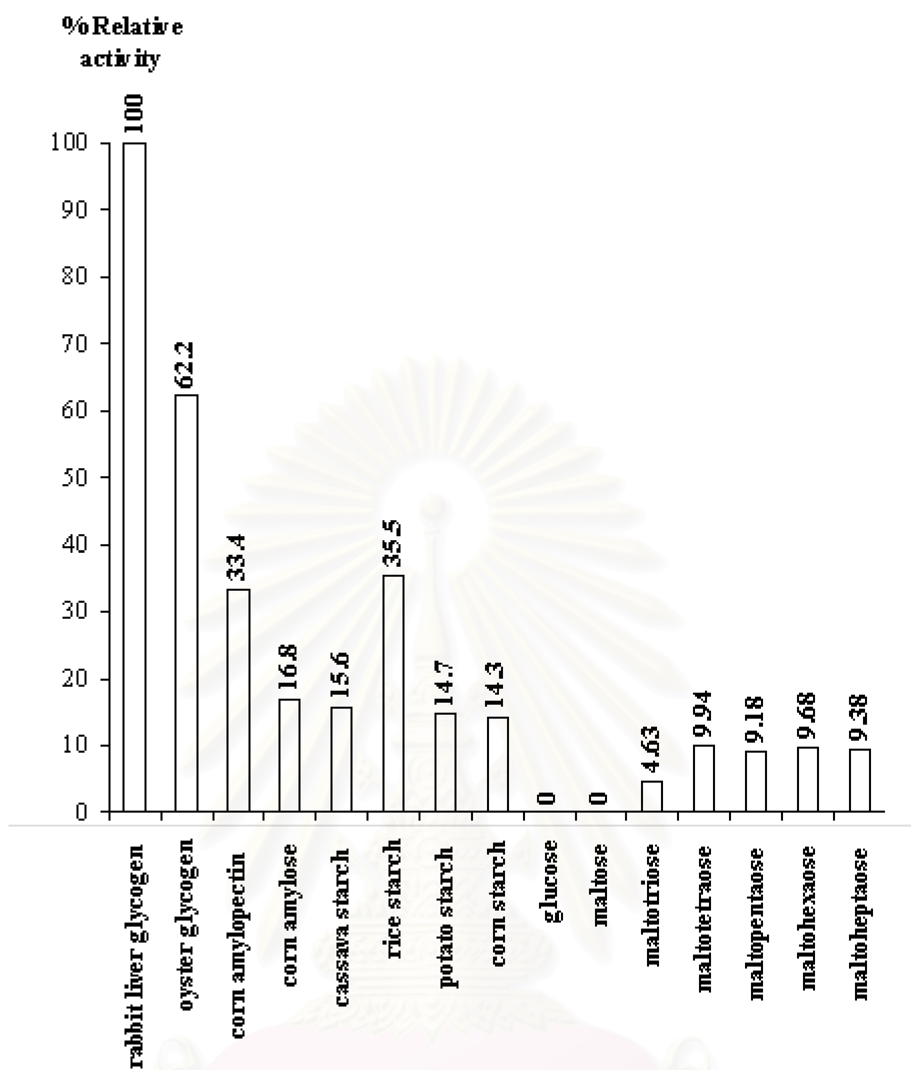

Figure 3.16. Summary of effect of primers on cassava starch synthase activity

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3.4.5 Effect of thiol group reagents on starch synthase activity

Cassava starch synthase was assayed with added thiol group reagents to determine the effect of SH- group on its activity as described in section 2.9.5. The results were shown in Table 3.3. Dithiothreitol (DTT) at 2-10mM showed no significant effect on enzyme activity. N-ethylmaleimide (NEM), ρ-chloromercuribenzoic acid (PCMB), and iodoacetic acid (IAA), which are SH- modifying reagents, showed the inhibitory effects on cassava starch synthase activity.

Table 3.3. The effect of thiol group reagent

Thiol group reagent	Activity unit	%Relative activity
None	53.31	100
2 mM DTT	58.12	109
5 mM DTT	57.37	107.6
10 mM DTT	53.39	100.1
2 mM NEM	19.2	36
5 mM NEM	2.88	5.4
10 mM NEM	2.31	4.33
2 mM PCMB	0	0
5 mM PCMB	0	0
10 mM PCMB	0	0
2 mM IAA	0	0
5 mM IAA	0	0
10 mM IAA	0	0

Activity unit = 1 nmole of glucose incorporated in 1 minute at 28°C

Number of experiment (n) = 3

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3.4.6 Kinetic constant for ADP-glucose

Cassava starch synthase was assayed at 28°C for 15 minutes as described in section 2.9.6. The saturation curve of ADP-glucose was shown in Figure 3.17 and the Lineweaver–Burk plot was shown in Figure 3.18. From both curves, the K_m for ADP-glucose was 0.10mM and the V_{max} is 54.64nmol of glucose incorporated/min.

3.4.7 Kinetic constant for rabbit liver glycogen

Cassava starch synthase was assayed at 28°C for 15 minutes as described in section 2.9.7. The rabbit liver glycogen was used as primer because it showed the highest activity when used as primer as the result in section 3.2.4. The saturation curve of rabbit liver glycogen was shown in Figure 3.19 and the Lineweaver–Burk plot was shown in Figure 3.20. From both curves, the K_m for rabbit liver glycogen was 1.31mg/ml and the V_{max} was 46.73nmol of glucose incorporated/min.

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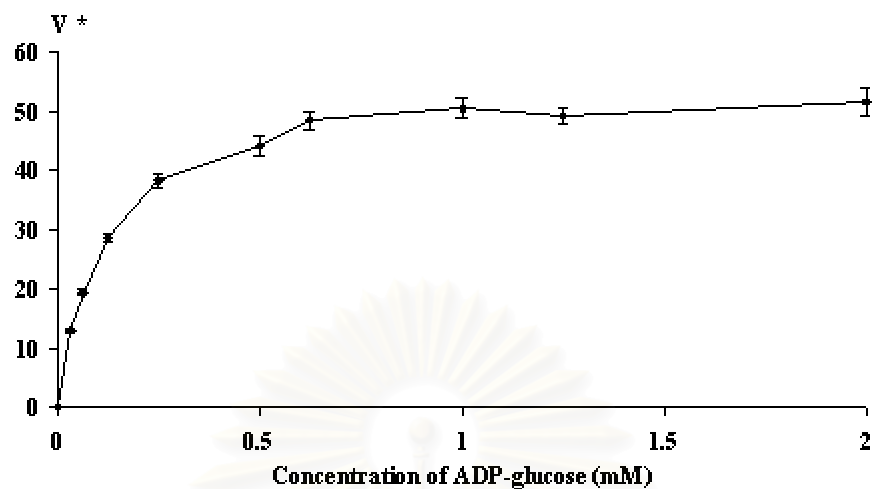
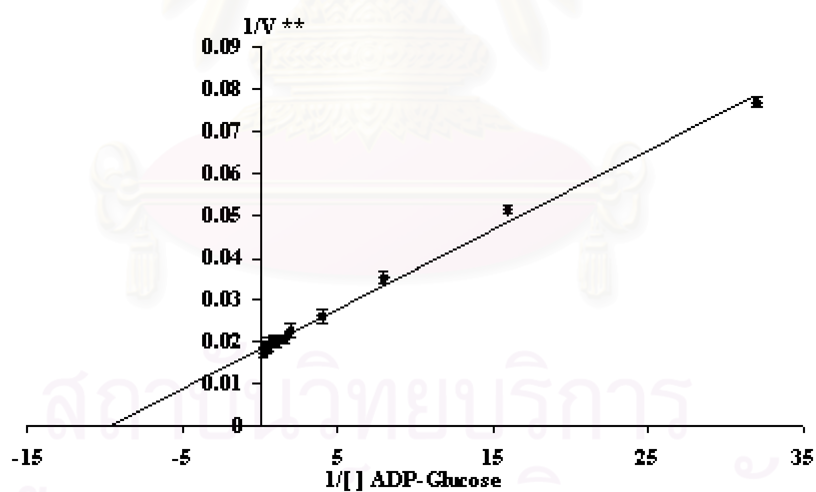


Figure 3.17 Saturation curve of ADP-glucose for cassava starch synthase activity (n = 5)



* **unit** = nmol of glucose incorporated/min.

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* **unit** = nmol of glucose incorporated/min.

Figure 3.18 Lineweaver-Burk plot of ADP-glucose for cassava starch synthase activity

** **unit** = 1/nmol of glucose incorporated/min.

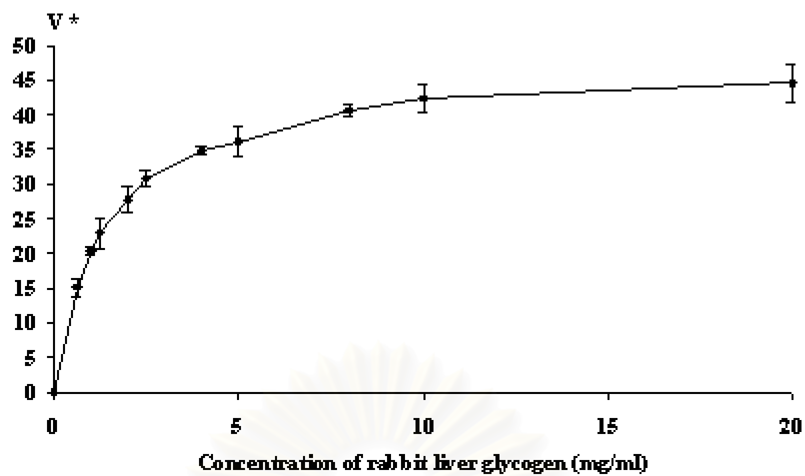

Figure 3.19 Saturation curve of rabbit liver glycogen for cassava starch synthase activity (n = 5)

* unit = nmol of glucose incorporated/min.

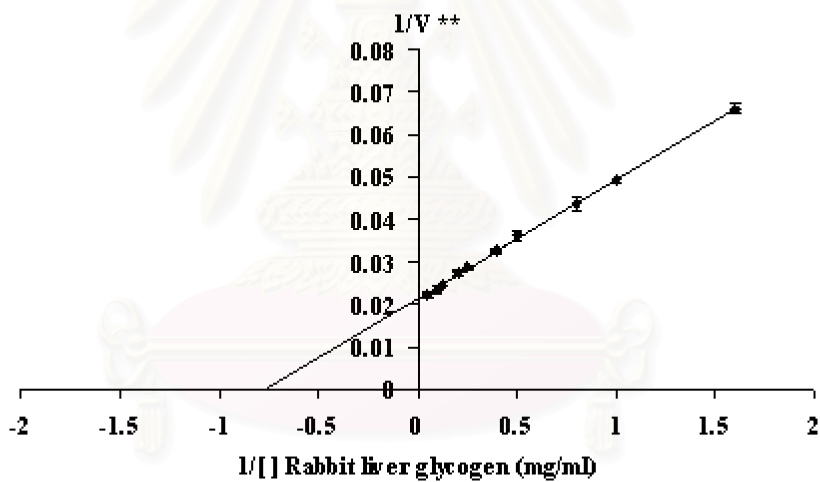

Figure 3.20 Lineweaver-Burk plot of rabbit liver glycogen for cassava starch synthase activity

** unit = 1/nmol of glucose incorporated/min.

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CHAPTER IV

DISCUSSION

Cassava is one of the most economically important crops which is produced in large scale and most of it is exported from Thailand. The problems of cassava trade in Thailand are the falling price and the reduction of demand of cassava products in EC market. To solve this problem and increase the value, cassava should be converted to starch because it is used in many 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.

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 1974, Hawker *et al.* (40) presented the assay method using radioactive ADP[U-^{14}C]-glucose in Bicine buffer pH 8.0 including glutathione (reduced form), EDTA, potassium acetate, and glycogen. After incubation at 37°C for 15 minutes, the reaction was stopped by adding 1% KCl in 75% methanol to precipitate glycogen which was incorporated with [^{14}C]glucose. The precipitate was washed three times with 1%KCl in 75% methanol, dissolved in distilled water and counted for amount of incorporated [^{14}C]glucose. Since then, this method was the conventional method used and widely accepted for assaying starch synthase activity. In 1999, Viola *et al.* (41) 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) 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 0.7-1.0nmol of ADP (41). Therefore, the HPLC method was employed throughout the work reported here. Experiment was also performed to ensure that there was no hydrolytic activity of ADP-glucose itself by autolysis and heat because ADP was not detected in the blank (no enzyme) and control (enzyme heated before reaction). Incubation of ADP-glucose and enzyme without primer also showed negligible trace of ADP-glucose hydrolysis.

4.2 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 (19,30,34,35,39). There were only a few reports on the attempt to purify soluble starch synthases by conventional method from plant tissues (33,46). They were unable to purify starch synthase to homogeneity. In our work, we tried to purify starch synthase from cassava tubers by column chromatographies and was able to obtain purification fold up to 220 times over crude enzyme.

Starch synthase was extracted from parenchyma of cassava tubers by the modified method of Ozbun *et al.* (23). The purification procedures used were ammonium sulfate precipitation followed by column chromatographies: Phenyl Sepharose, Sephadex G-200, and Q-Sepharose. In the ammonium sulfate precipitation, about half of the proteins was removed but about two thirds 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. Preliminary experiment was tried not using ammonium sulfate precipitation but directly applied the crude enzyme to DEAE-cellulose. It was found that there was great loss of starch synthase as well with less removal of other proteins. 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. Therefore, it was decided that ammonium sulfate precipitation and Phenyl Sepharose column were more appropriate in the first two steps to avoid desalting and concentration of samples, although activity loss was unavoidable. The sample from ammonium sulfate precipitation was directly applicable to Phenyl Sepharose column without desalting since the column was eluted with high concentration of ammonium sulfate first, followed by lower concentrations of ammonium sulfate.

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Sephadex G-200 and Q-Sepharose contributed greatly to the purification procedures, with less loss of starch synthase activity compared to the amount of protein removed. Sephadex G-200 retained most of the enzyme activity while about 88% of proteins were removed. In addition, Q-Sepharose further removed contaminating proteins, resulting in the total of 220 folds purification from the crude preparation. However, the yield obtained was rather low due to rapid loss of enzyme activity. The purified fraction was kept in 20% (v/v) glycerol which helped in conserving the activity for longer period for characterization.

4.3 Characterization of cassava starch synthase

4.3.1 Determination of molecular weight

From Sephadex G-200 column chromatography and its molecular weight calibration curve, the molecular weight of starch synthase as determined from the highest activity point of the peak was 53.4kDa. The enzyme preparation was subjected to non-denaturing polyacrylamide gel electrophoresis and 2 bands of similar intensity on protein stain were observed with some protein remaining at the interface of stacking and separating gel. When the lane containing Q-Sepharose enzyme preparation on the non-denaturing polyacrylamide gel electrophoresis was cut and subjected to second dimension electrophoresis on SDS-polyacrlamide gel, we were able to identify two intense spots on the SDS-polyacrylamide gel corresponded to the 2 bands on non-denaturing polyacrylamide gel electrophoresis. The fast migrating band on non-denaturing polyacrylamide gel appeared at molecular weight of 53.8kDa on SDS polyacrylamide gel. The slow migrating band on non-denaturing polyacrylamide gel corresponded to the spot on SDS-polyacrylamide gel with molecular weight of 76 and 79kDa. The smear protein band on the interface of the stacking and separating polyacrylamide gel was likely to be coagulation of 79 and 76kDa proteins because it was separated on second dimension SDS polyacrylamide gel at these positions. The sample itself formed precipitate after repeated freeze and thaw.

The molecular weight of 53.8kDa corresponded well with the molecular weight obtained from the fraction with highest activity in the starch synthase activity peak on Sephadex G-200 column. The molecular weight of the other bands 76 and 79kDa although did not coincide with the highest activity fraction, were still within the molecular weight range of the pooled starch synthase activity peak of about 100 - 21kDa from Sephadex G-200 column. The 3 bands on second dimension SDS-polyacrylamide gel should not represent subunits of the enzyme since dimer molecular weight of any combinations did not fit in the activity peak on Sephadex G-200 column.

Therefore, it was postulated at this stage that the starch synthase purified in this experiment may contain 3 isoforms with molecular weight of 79, 76 and 53.4-53.8kDa. Previous report on the study of starch synthase showed the presence of isoforms of starch synthase with varied molecular weight (Table 4.1). Since the specific activity of starch synthase in the Q-Sepharose fraction was very high with 220 folds purification, it was unlikely that the most intense band on the polyacrylamide gel represented other contaminating protein rather than an isoform of starch synthase. There were reports on a granule bound starch synthase in seed of wheat (*Triticum monococcum* L.) with molecular weight of 56kDa and an isoform of starch synthase which was both granule bound and soluble with that 77kDa in pea embryos.

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Table 4.1 Isoforms of starch synthase in various plant tissues.

Source	Number of isoform	Molecular weight	Reference
Spinach leaf	4	-	22
Maize kernel	2		23
		SSI = 76 kDa *	31
		SSII = 188 kDa *	37
Potato tuber	2		24
		SSII = 90 kDa *	29
		SSIII = 140 kDa *	38
Grape leaf	1	-	25
Maize leaf	2	-	25
Rice grain	2	SSI = 110 kDa **	26
		SSII = 69 kDa **	
Sorghum seed	3	-	27
Castor bean endosperm	1	-	28
Teosinte seed	2	-	47
Cassava parenchyma	1-3	79kDa ***	This work
		76 kDa ***	
		53.4-53.8 kDa ****	

* determined by cDNA

** determined by Sephadex G-200

*** determined by SDS-polyacrylamide gel electrophoresis

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

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4.3.2 Determination of pI

Since the starch synthase preparation contained more than one band on non-denaturing polyacrylamide gel electrophoresis, determination of its pI's on IEF gelbond unit was difficult because we cannot correlate the pI values to each starch synthase bands. Experiment was, thus, performed on two-dimension gel by the method of Robertson (1987) (45). This method had an advantage over using IEF gel bond unit because the gel from mini gel can be cut and the protein pattern determined by two-dimension non-denaturing or SDS-polyacrylamide gel electrophoresis. In our experiment, the two-dimension SDS-polyacrylamide gel electrophoresis was used for determining the position of starch synthase on mini gel system IEF. Starch synthase was subjected to IEF gel, run on mini protein gel chamber, so that the gel could be cut and subjected to second dimension on SDS-polyacrylamide gel to identify the starch synthase bands by the molecular weight. The result from IEF showed 2 protein bands with pI 6.41 and 6.91 (figure 3.9). When the IEF gel strip was subjected to second dimension electrophoresis on SDS-polyacrylamide gel, the pI 6.91 protein band appeared as single band protein at molecular weight 79kDa while the pI 6.41 protein band showed up as two protein bands on second dimension SDS-polyacrylamide gel at molecular weight 76 and 53.8kDa. It is possible that there were 3 isoforms of starch synthase in the Q-sepharose preparation: isoform I molecular weight 79kDa and pI 6.91, isoform II molecular weight 76kDa and pI 6.41, and isoform III molecular weight 53.8kDa and pI 6.41. On the other hand, either the 79kDa or 76kDa proteins may be contaminating proteins. If the latter explanation applied, the 79kDa which was less intense was more likely to be the contaminated one.

However, to prove the explanation of isoforms, the three protein bands should be further purified and each form studied for starch synthase activity. The enzyme could be further purified by chromatofocusing followed by preparative gel electrophoresis to separate the isoforms of the enzyme. Chromatofocusing column should be able to separate the

79kDa protein which had the pI of 6.91 while the 76 and 53.8kDa would be co-eluted at pI 6.41. The latter two proteins should be separable by preparative gel electrophoresis.

4.3.3 Effect of pH's and temperature on starch synthase activity

The enzyme was incubated at various pH's and its activity measured. It was found that the enzyme was most active at pH 8.5, and the activity decreased by 50% at pH lower than 7.0 and higher than 9.5. When the reaction was incubated at various temperatures, starch synthase activity showed the highest activity at 28°C and dropped by 50% at lower than 15°C and higher than 37°C. When the enzyme was incubated at various temperatures for 15 minutes, starch synthase activity was activated at 25 and 45°C and dropped by 50% between 45-50°C. When incubated at 45°C, the temperature at which starch synthase was still stable at various times to investigate the stability, it was found to maintain activity with minor activity loss for 6 hours. After which the enzyme activity dropped steadily to 50% at 30 hours of incubation and remained stable up to 40 hours which the experiment terminated. This result may be supportive of the speculation on the existence of isoforms of the enzyme. After 6 hours, one or two isoforms might be completely denatured but the other isoform(s) was still stable. After 30 hours, the remaining isoform(s) might be further denatured leaving the isoform that was heat stable and the activity was maintained at 45°C up to 40 hours.

In other plants, the optimum pH's and optimum temperatures of starch synthase differed for different isoforms. However, the range of optimum pH was about 8.0-8.5 and optimum temperature was about 30-42°C (see table 4.2), which was within the same range with our findings.

Table 4.2 The effect of pH and temperature on starch synthase activity of various plant tissues

Source	Optimum pH	Optimum temperature (°C)	Temperature stability (°C)	Reference
Spinach leaf				18
SSI	8.0-8.5	30	-	
SSII	8.0-8.5	30-37	-	
SSIII	8.5	30	-	
SSIV	8.0	37	-	
waxy maize kernel				19
SSI	8.0-8.5	37	-	
SSII	8.5	30	-	
Maize kernel				33
SSI	8.0	42	42	
SSII	8.5	37	37	
Potato				19
SSII	-	-	35	
Cassava SS	8.5	28	45	This work

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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 $\alpha(1\rightarrow4)$ linkages. The glucose unit's 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. Glucose homopolymer: amylose, amylopectin, rabbit liver glycogen, oyster glycogen
2. Starch: cassava starch, rice starch, potato starch, corn starch
3. Glucose, maltose and malto-oligosacharides: glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose

With the first group of primers, starch synthase showed highest activity with rabbit liver glycogen (RLG) followed by oyster glycogen (OG), amylopectin (AP), and amylose (AM), respectively. It seemed that the branched polymers such as glycogen and amylopectin acted as primers better than the linear polymer, amylose. Among the branched polymers, starch synthase seemed to prefer the polymers with highly branch chains. The observations on the effect of different primers on cassava starch synthase were similar to some starch synthase isoforms previously reported as shown in table 4.3, such as maize SSI, sorghum seed SSII and III, teosinte seed SSI, and spinach leaf SSI and IV. There were a few other starch synthase isoforms, such as maize kernel SSII, castor bean SSI, teosinte seed SSII, spinach SSII, and *waxy* maize SSII, which showed preference for oyster glycogen and amylopectins.

Among the starch primers, starch synthase showed highest activity when rice starch was used as primer. On the other hand, using corn starch as primer gave the lowest starch synthase activity. Amylopectin content seemed to be the determining factor for this result because rice starch has higher amylopectin content than the other starch used in this experiment while the corn starch has lowest amylopectin. (see Table 4.3)

For the last group of primer, starch synthase can use malto-oligosaccharide as primer but cannot use glucose and maltose. Maltotriose showed the activity of about half of the other malto-oligosaccharide. This result indicated that the length of glucose units affected the ability of the oligomers to act as primers for starch synthase activity.

Table 4.3 Amylopectin and amylose content in various plants starch

Starch	% Amylopectin	% Amylose
Rice *	86.4	13.6
Cassava *	83.4	16.6
Potato **	80	20
Corn *	76.9	23.1

* data from Nilmanee,2000 (49)

** data from Young,1984 (6)

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Table 4.4 The effect of primers on starch synthase of various plant tissues

Source	Activity for primer (higher > lower)	Reference
Maize		36, 46
Leaf SSI	RLG > OG > AP	
Kernel SSI	RLG > OG > AP	
SSII	AP > RLG > OG	
Castor bean endosperm SSI	RLG > AP > OG > AM	28
Sorghum seed		27
SSII	RLG > OG > AP	
SSIII	RLG > OG > AP	
SSIV	RLG > OG > AP	
Teosinte seed		47
SSI	RLG > OG > AP	
SSII	AP > RLG > OG	
ae maize kernel SSI	RLG > OG > AP	48
Spinach leaf		22
SSI	RLG > OG > AP	
SSII	OG > RLG > AP	
SSIII	RLG > AP > OG	
SSIV	RLG > OG > AP	
waxy maize kernel		23
SSI	RLG > OG > AP	
SSII	AP > RLG > OG	
Cassava SS	RLG > OG > AP > AM	This work

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4.3.5 Effect of thiol reagents on starch synthase activity

When cassava starch synthase was incubated with various thiol reagents, it was found that some thiol reagents had the effect on starch synthase activity. DTT, a SH- group stabilizing agent which protects SH- group from oxidizing agents, either slightly activated or did not affect starch synthase activity. For NEM, PCMB, and IAA included in the reaction mixture, the starch synthase activities were inhibited with the most inhibitory effect from PCMB and IAA. 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.

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 primer. Rabbit liver glycogen was used in this experiment because it was determined to be the most preferred primer for cassava starch synthase (section 3.4.4).

The K_m for ADP-glucose and rabbit liver glycogen of the purified starch synthase which determined from the Lineweaver-Burk plot were 0.1mM and 1.31mg/ml, respectively. This value was comparable to that of many starch synthase isoforms previously reported (see table 4.4). However, V_{max} value cannot be compared since it depended on each preparation of enzyme. Starch synthase isoforms reported in each plants (Table 4.4) seemed to have very close K_m values. Therefore, it was not surprising that we obtained a single K_m value for our enzyme preparation which we postulated to contain more than one isoforms.

Table 4.5 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	-	0.595	-	48
ae maize kernel SSI	0.10	-	-	-	48
Potato SSII	0.25	-	-	-	24
Spinach leaf					22
SSI	0.20	-	1.02	-	
SSII	0.29	-	0.8	-	
SSIII	0.15	-	-	-	
SSIV	0.25	-	0.88	-	
waxy maize kernel					23
SSI	0.10	-	-	-	
SSII	0.12	-	-	-	
Maize kernel					33
SSI	0.11	2.23	12.8	2.79	
SSII	0.42	6.74	18.5	12.3	
Potato SSII	0.07	7.5	-	-	19
Cassava	0.10	0.90	1.31	0.77	This work

* Unit = mM

** Unit = mg/ml

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

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CHAPTER V

CONCLUSIONS

1. The cassava starch synthase was purified from parenchyma of starch tubers by 20-60% saturated ammonium sulfate, followed by column chromatographies on Phenyl Sepharose, Sephadex G-200, and Q-Sepharose. The enzyme was purified 220 folds with 2.8% recovery. The native molecular weight of the cassava starch synthase was estimated by Sephadex G-200 to be 53.4kDa.

2. Two-dimensional electrophoresis indicated the possible existence of 3 isoforms of cassava starch synthase. Non-denaturing polyacrylamide gel and isoelectrofocusing gel showed up as 3 bands on second-dimension SDS-polyacrylamide gel electrophoresis with molecular weight 79, 76, and 53.8kDa corresponding to the pI values of 6.91,6.41, and 6.41, respectively.

3. The optimum pH and temperature for cassava starch synthase were 8.5 and 28°C, respectively. The enzyme was stable at temperature up to 45°C.

4. The enzyme showed highest preference for rabbit liver glycogen as primer followed by, oyster glycogen, amylopectin, amylose, rice starch, cassava starch, potato starch, malto-oligosaccharide, respectively.

5. Cassava starch synthase contained SH- group which was involved in its activity.

6. The K_m and V_{max} of ADP-glucose was 0.10mM and 54.64 nmol of glucose incorporate/min, respectively. The K_m and V_{max} of rabbit liver glycogen was 1.31mg/ml and 46.73 nmol of glucose incorporate/min, respectively.

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APPENDICES

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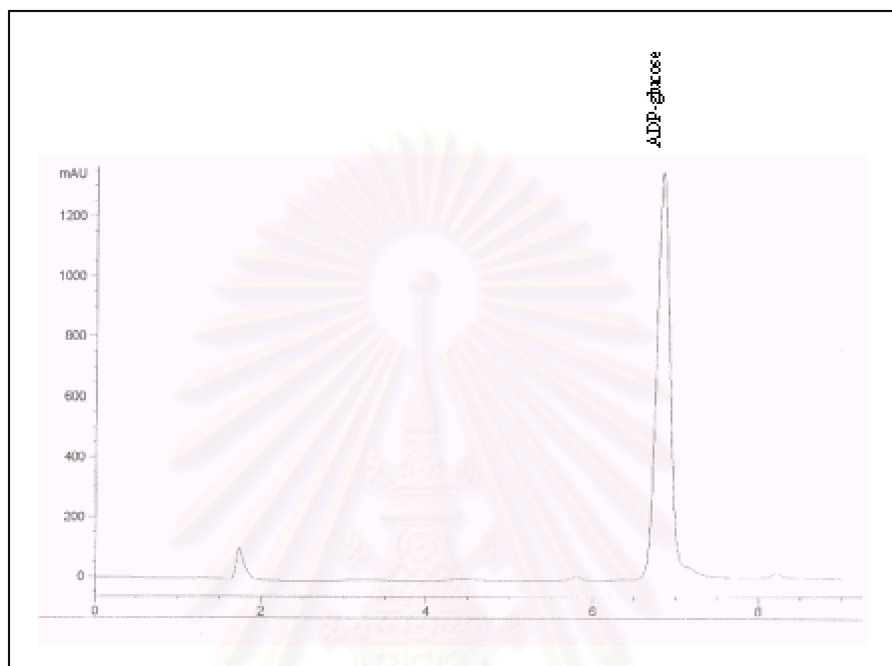
APPENDIX A

1. HPLC profile of ADP and ADP-glucose

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



ADP-glucose: RT = 6.81 min. Area = 16,000

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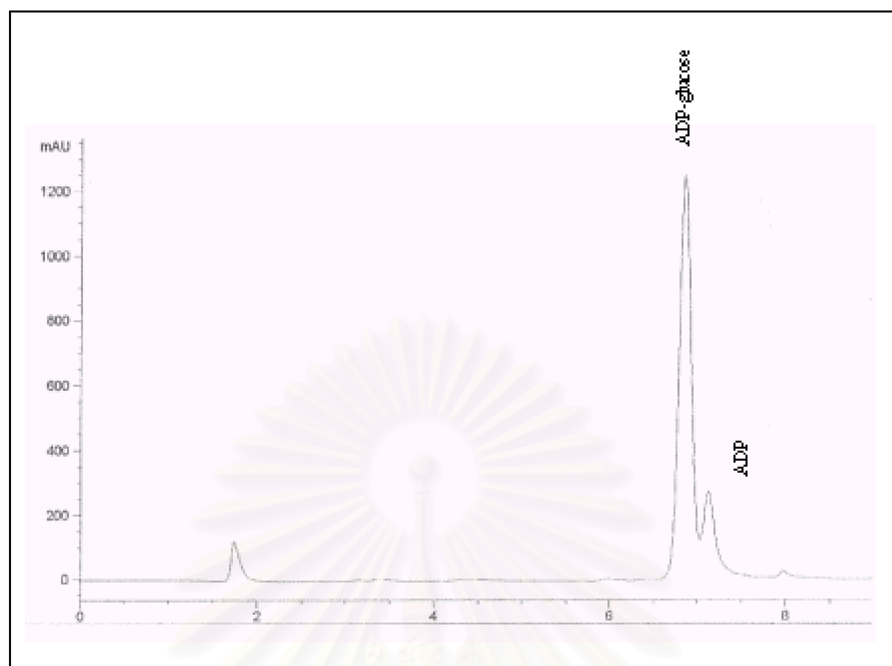
1.1.2 Typical HPLC profile of reaction mixture with starch synthase activity



ADP-glucose: RT = 6.83 min. Area = 12,439

ADP: RT = 7.12 min. Area = 3,162

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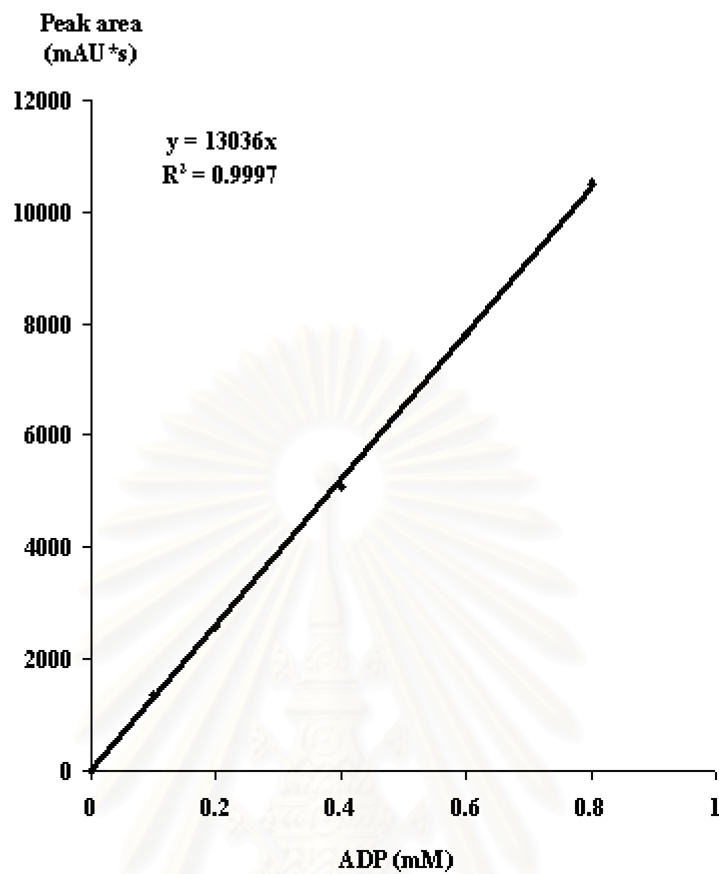
2. Calibration curve of ADP concentration and peak area



3. Calculation of starch synthase activity

3.1 [ADP] (mM) = peak area of ADP / 13036

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

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

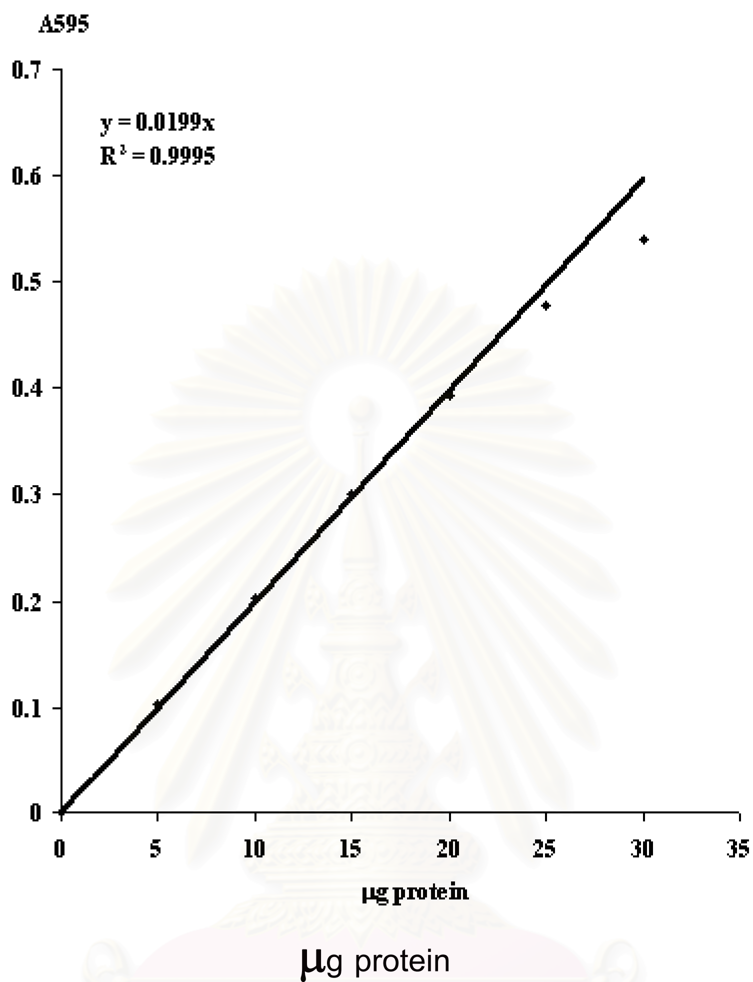
3.4 Activity (nmol ADP per minute) = $[ADP_{rx}]$ / 15

3.5 Total activity = activity (units) x (total volume (ml) / 50 μl)

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

APPENDIX B

Calibration curve of protein concentration



μg protein

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APPENDIX C

Preparation for non-denaturing polyacrylamide gel electrophoresis

1. Stock reagents

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

acrylamide	29.2 g
N.N -methylene-bis-acrylamide	0.8 g

Adjusted volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with 1M HCl and adjusted volume to 100 ml with distilled water

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2 g

Adjusted pH to 8.8 with 1M HCl and adjusted volume to 100 ml with distilled water

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1g

Adjusted pH to 6.8 with 1M HCl and adjusted volume to 100 ml with distilled water

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Solution B (1.5M Tris-HCl pH 8.8)

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

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

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

Solution C (0.5M Tris-HCl pH 6.8)

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

Solution C –SDS (0.5M Tris-HCl pH 6.8, 0.4% SDS)

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

5x Sample buffer

1M 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 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g

Dissolved in distilled water to 1 litre without pH adjustment.

(Final pH should be 8.3).

SDS electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine, SDS 0.1%)

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

Dissolved in distilled water to 1 litre without pH adjustment.

(Final pH should be 8.3).

2. Preparation of Non-denaturing PAGE

10.0% Separating gel

solution	3.3 ml
Solution B	2.5 ml
Distilled water	4.2 ml
10% $(NH_4)_2S_20_8$	50 μl
TEMED	10 μl

3.0% stacking gel

30% Acrylamide solution	0.4 ml
Solution C	1.0 ml
Distilled water	2.6 ml
10% $(NH_4)_2S_20_8$	30 μl
TEMED	5 μl

3. Preparation of SDS-PAGE

10 % separating gel

Prepare as described for non-denaturing gel but using with solution B

containing SDS instead solution B.

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3 % stacking gel

Prepare as described for non-denaturing gel but using

with solution C

containing SDS instead solution C.

APPENDIX D

Preparation of mini gel system IEF

30% acrylamide solution	2.0 ml
distilled water	9.7 ml
ampholyte solution pH 3-10	288 μl

mixed well and degassed before added:

10% $(NH_4)_2S_20_8$	50 μl
TEMED	20 μl

BIOGRAPHY

Mr.Worapong Hirunyapaisarnsakul was born in November 11th, 1975 in Bangkok. He finished Matthayom 6 at Rajavivit Matthayom School, Bangkok and enrolled in the Faculty of Science, Chulalongkorn University in 1993. He graduated with the B.Sc. in Biochemistry in 1997 and continued for M.Sc. in Biotechnology in that year.

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