การทำให้บริสุทธิ์และลักษณะสมบัติของไอโซฟอร์มของเอนไซม์สร้างโซ่กิ่งในหัวมันสำปะหลัง *Manihot esculenta* CRANTZ

นาย สุรชัย ใหญ่เย็น

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PURIFICATION AND CHARACTERIZATION OF ISOFORMS OF STARCH BRANCHING ENZYME FROM TUBER OF CASSAVA *Manihot esculenta* CRATNZ

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สุรชัย ใหญ่เย็น : การทำให้บริสุทธิ์และลักษณะสมบัติของไอโซฟอร์มของเอนไซม์สร้างโซ่กิ่งในหัวมันสำปะหลัง *Manihot esculenta* CRANTZ (PURIFICATION AND CHARACTERIZATION OF ISOFORMS OF STARCH BRANCHING ENZYME FROM TUBER OF CASSAVA *Manihot esculenta* CRANTZ.) อ.ที่ปรึกษา : รศ.ดร. ทิพาพร ลิมปเสนีย์, อ.ที่ปรึกษาร่วม: ศ.ดร. มนตรี จุฬาวัฒนทล

มันสำปะหลังเป็นพืชเศรษฐกิจที่มีการส่งออกปริมาณสูง แป้งมันสำปะหลังสามารถนำไปใช้ประโยชน์ในอุตสาหกรรมต่างๆ เช่น ผลิตผงชูรส แอลกอฮอล์ และ กระดาษ แป้งจะประกอบด้วยอะไมโลส และ อะไมโลเพคติน เอนไซม์ที่ทำหน้าที่เกี่ยวข้องกับการสังเคราะห์ อะไมโลส และ อะไมโลเพคติน ในพืชมีหลายชนิด เอนไซม์สร้างโซ่กิ่งเป็นเอนไซม์ตัวหนึ่งที่เกี่ยวข้องกับการสังเคราะห์อะไมโลเพคติน เอนไซม์สร้างโซ่กิ่งเร่งปฏิกิริยาการสังเคราะห์ α-1,6-glucosyl-α-1,4-glucan ในการวิจัยเอนไซม์สร้างโซ่กิ่งในพืช เช่น ข้าวโพด มันฝรั่ง พบว่าเอนไซม์นี้มีหลายไอโซฟอร์ม ซึ่งสามารถแบ่งได้ 2 กลุ่ม คือ กลุ่มที่จำเพาะต่อการสร้างโซ่กิ่งในอะไมโลเพคติน เรียกว่า กลุ่ม A และกลุ่ม B ที่จำเพาะต่อการสร้างโซ่กิ่งในอะไมโลส การทดลองนี้ศึกษาเอนไซม์นี้ในหัวมันสำปะหลัง โดยการเปรียบเทียบอายุของหัวมันสำปะหลัง 2 สายพันธุ์คือ Rayong 1 และ KU 50 พบว่าที่ระยะ 6-9 เดือน มีอัตราการเพิ่มขึ้นของเอนไซม์สูง จึงเลือกหัวมันอายุ 9 เดือน ของสายพันธุ์ KU 50 ซึ่งเป็นสายพันธุ์ที่มีปริมาณแป้งสูง มาทำการสกัดและแยกไอโซฟอร์มให้บริสุทธิ์ โดยการตกตะกอนโปรตีนด้วย polyethylene glycol ที่ความเข้มข้น 10% และ คอลัมน์โครมาโตกราฟีชนิดแลกเปลี่ยนไอออนคือ DEAE-Toyopearl สามารถแยกได้ 3 ไอโซฟอร์ม และทำแต่ละไอโซฟอร์มให้บริสุทธิ์เพิ่มขึ้นด้วย Hitrap Q-sepharose ไอโซฟอร์มของเอนไซม์สร้างโซ่กิ่ง ที่เตรียมได้มีความบริสุทธิ์เพิ่มขึ้น125, 77 และ 250 เท่าตามลำดับ และมีน้ำหนักโมเลกุลเมื่อวิเคราะห์ด้วย Sephacryl HR S-200 เท่ากับ 57 กิโลดาลตันทุกไอโซฟอร์ม เมื่อวิเคราะห์ต่อด้วยอิเล็กโตโฟริซีสแบบเสียสภาพพบในไอโซฟอร์มที่ 1 มีโปรตีน 2 แถบ ที่มีน้ำหนักโมเลกุล 108,000, 57,000 ดาลตัน ส่วน ไอโซฟอร์ม 2 และ 3 มีแถบโปรตีน แถบเดียว มีน้ำหนัก 57000 และ 60,000 ดาลตัน มีค่า pI เท่ากับ 4.9 4.9 และ 5.0 ตามลำดับ ไอโซฟอร์ม 1, 2 และ 3 สามารถทำปฏิกิริยาได้ดีที่ค่าความเป็นกรด-ด่าง 8.0, 6.5 และ 7.5 ที่อุณหภูมิ 37, 37 และ 30 องศาเซลเซียส ตามลำดับ มีความเสถียรที่อุณหภูมิ 40 องศาเซลเซียสทุกตัว ทุกไอโซฟอร์มสามารถถูกยับยั้งด้วยไอออนของโลหะที่มีประจุเป็น +2 เอนไซม์มีค่า Km ต่อ อะไมโลส เท่ากับ 1.12 1.37 และ 2.17 มก./มล. ไอโซฟอร์ม ที่ 1 และ 2 มีความจำเพาะต่ออะไมโลสมากกว่าอะไมโลเพคติน ส่วนไอโซฟอร์มที่ 3 มีความจำเพาะต่ออะไมโลเพคตินมากกว่าอะไมโลส

ภาควิชา.....ชีวเคมี.....
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ลายมือชื่อนิสิต .....
ลายมือชื่ออาจารย์ที่ปรึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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KEY WORD: STARCH BRANCHING ENZYME / CASSAVA / PURIFICATION
SURACHAI YAIYEN : PURIFICATION AND CHARACTERIZATION OF ISOFORMS OF STARCH BRANCHING ENZYME FROM TUBER OF CASSAVA *Manihot esculenta* CRANTZ. THESIS ADVISOR : ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D. THESIS CO-ADVISER PROF. MONTRI CHULAVATNATOL, Ph.D.

Cassava starch is one of the major raw material used in many industries. Starch is composed of the polysaccharides, amylose and amylopectin, which were synthesized by a group of enzymes including starch branching enzyme (SBE). Starch branching enzyme involved amylopectin synthesis by catalyzing formation of α-1, 6-glucosyl-α-1,4-glucan. There are two classes of, class A preferentially branches amylopectin whereas class B preferentially branch amylose. In this study, starch branching enzyme was monitored in cassava tubers from two cultivars, Rayong 1 and KU 50. SBE activity rapidly increased around 9 months old. Purification and isoform studies were performed with tubers from 9 months old KU 50. The crude enzyme was purified by precipitation with 10% (w/v) polyethylene glycol, followed by DEAE-Toyopearl column which could separate SBE into 3 isoforms. Hitrap Q-sepharose column was further used to purify each isoform which resulted in purities of 125, 77 and 250. All isoform showed molecular weight of 57 kDa on Sephacryls HR S-200. Isoform 1 separated by SDS- PAGE, into 2 bands with molecular weight 108 and 57 kDa whereas isoform 2 and 3 showed single band with molecular weight of 57 and 60 kDa. The pI of the 3 isoform was 4.9, 4.9 and 5.0, respectively. Optimum pH's were 8.0, 6.5, 7.5 and optimum temperatures were 37, 37 and 30 °C for isoform 1, 2 and 3, respectively. All isoforms were stable up to 40 °C. The K_m's for amylose were 1.12, 1.37 and 2.17 mg/ml. Isoform 1 and 2 were more active than isoform 3 towards amylose as substrate, whereas isoform 3 was more active than isoforms 1 and 2 with amylopectin as substrate. All isoforms were absolutely inhibited by divalent metal ion.

Department .....Biochemistry..... Student's signature.....
Field of study.....-..... Advisor's signature.....
Academic year……2003..... Co-adviser's signature.....

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LIST OF ABBREVIATION

A	Absorbance
AMP	Adenosine-5’-monophosphate
BSA	Bovine serum albumin
DBE	Debranching enzyme
DTT	Dithiothreitol
EDTA	Ethelenediaminetetraacetic acid
FPLC	Fast performance liquid chromatography
g	gravitational acceleration
GBSS	Granule-bound starch synthase
HCl	Hydrochloric acid
HCN	Hydrogencanide
IAA	Iodoacetic acid
IEF	Isoelectric focusing
kDa	kilo Dalton
M	Molar
mM	Millimolar
MW	molecular weight
NEM	N-ethylmaleimide
NaCl	Sodiumchloride
NaOH	sodium hydroxide
pI	isoelectric point
SDS	Sodium dodecyl sulfate

SSs	Soluble starch synthase
TCA	Trichloroacetic acid
TEMED	N,N,N’,N’-tetramethyl ethylene diamine

CHAPTER 1

INTRODUCTION

1.1 Cassava

Cassava (*Manihot esculenta* Crantz.) is a tropical crop, is a dicotyledonous plant (Figure 1.1a) in the botanical family Euphorbiaceae. It shows extreme variation of shrub branch, root shape, coloration of petiole or external and internal parts of root, indicating large degree of hybridization (1). Cassava is a native of Brazil and has been dispersed to tropical and sub-tropical area of Africa, Asia and the Caribbean (Table 1.1) by the Portuguese (2). Cassava can be grown throughout the year because it has no critical period for propagation and harvesting. The propagation is easy by using seeds or stem cuttings. It can tolerate drought and can be grown on soils with low nutrient capacity. It but also responds well to irrigation or higher rainfall conditions. Cassava has high yield and high resistant to the damage from serious pests and diseases (3).

The utilization of cassava as major food crops for a long time in the tropics can cause the health problems due to deficiency of protein. Leaves are more nutritionally balance and can be eaten as fresh vegetable, ground fresh and frozen in plastic bag or dried and ground for sale in plastic bag (4). However it may be high in hydrocyanic acid which can lead to goiter, neurological disorders tropical ataxic neuropathy, respiratory poisoning and sometime death. The HCN can be reduced to safe levels in most cases when the liquid is squeezed out after grinding and through evaporation during cooking (5).

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 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 specialized food and industrial products, production of alcohol, butanol and acetone, starch for sizing paper and textiles, glues, MSG, sweeteners, pharmaceuticals, biodegradable products, manufacturing of explosive and coagulation of rubber latex.

In Thailand, cassava is produced in large scale and is exported world wide (Table 1.2)(6). Europe is the largest market of cassava, chips and pellets used as livestock feed after World War II. Until 1994, Thailand encountered some problem on the quota and tariff in exporting cassava to Europe. Quotation of cassava pellets in the EC (the most important cassava product traded internationally and the main cassava import market) are determined by the domestic price of grains, especially barley, and the prices of protein-rich meal (Table 1.3)(7). The price falling in EC market caused the diversion 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 due to yield improvements and area expansion. To reduce dependence on the EC feed market and adding value to cassava production must be diverted into starch-based products.

Cassava root contains high moisture and starch with little protein (Table 1.4)(8). The chemical components of cassava vary with species, soil types, climate and harvesting ages. Root tissue was divided into cortex and parenchyma (Figure 1.1 B). Up to 40% of the starch concentration can be deposited in root parenchymal tissue.

A

B

Figure 1.1 Cassava plant and tuber

A) Cassava (*Manihot esculenta* Crantz.) plant

B) Cassava root and it tissue component, cortex and parenchyma

Table 1.1 World cassava production (2)

	2000	**2001**	**2002**
	(million tons)		
World	**176.7**	**180.8**	**184.0**
Africa	**95.3**	**96.6**	**99.1**
CongoDem. Rep.	16.0	15.4	14.9
Ghana	8.1	9.0	9.9
Madagascar	2.5	2.5	2.5
Mozambique	5.4	5.4	5.6
Nigeria	32.0	32.6	33.6
Tanzania	5.8	5.6	5.8
Uganda	5.0	5.3	5.4
Asia	**50.4**	**52.3**	**51.5**
China	3.8	3.8	3..8
India	6.2	7.0	7.1
Indonesia	16.1	17.1	16.7
Philippine	1.8	1.7	1.7
Thailand	19.1	18.3	17.3
Vietnam	2.5	3.5	3.9
Latin America and Caribbean	**31.3**	**31.7**	**33.2**
Brazil	23.3	22.5	23.1
Columbia	1.8	2.0	2.2
Paraguay	2.7	3.6	4.1

Table 1.2 world trade in cassava (6)

	2000	2001	2002
	(million tons)		
Exports	**6.9**	**7.4**	**5.9**
Thailand	6.5	7.1	5.7
Indonesia	0.2	0.1	0.1
Others	0.2	0.2	0.1
Imports	**6.9**	**7.4**	**5.9**
EC [1]	3.7	2.7	1.5
China [2]	0.9	2.6	2.5
Japan	0.6	0.7	0.7
Korea Rep.	0.1	0.2	0.1
Malaysia	0.2	0.2	0.2
United States	0.1	0.1	0.1
Others	0.8	0.6	0.7

[1] Excluding trade between EC numbers

[2] Including Taiwan province

Table 1.3 Prices of cassava, Soybean meal and Barley in the EU (7)

	Cassava pelletes [1]	Soybean mea l [2]	Cassava soybean meal mixture[3]	Barley [4]	Cassava meal mixture/Barley
	(US$/ton)				(ratio)
1993	137	208	151	197	0.77
1994	14	192	154	182	0.85
1995	177	197	181	209	0.87
1996	152	268	175	194	0.9
1997	108	276	142	161	0.88
1998	107	170	120	145	0.83
1999	102	152	112	143	0.78
2000	84	189	105	144	0.73
2001	82	181	102	148	0.69
2002	90	175	107	140	0.79
2003 [5]	94	186	112	118	0.95

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

[2] Argentina (45/46 %protein) c.i.f Rotterdam until September 1999. As from October 1999 Argentina (44/45 %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-April average.

Table 1.4 A typical cassava root compositions (8).

Consumption	**Percent (%)**
Moisture	70
Starch	24
Fiber	2
Protein	1
Fat, mineral, sugar	3

In Thailand, a cassava-breeding program was started with the release of Rayong1 in 1975. Rayong1 was developed from a selection of local cultivars, and was the first variety bred as a sourced for industrial raw material. Since then, there have been many cassava varieties being produced with different physical and chemical properties (Table 1.5)(9). Cassava breeding in Thailand aims to improve starch yield and adaptability to a wide range of growing conditions. Starch yield is the function of starch content and root dry matter yield. There has been no systematic institutional breeding of cassava for improved cooking quality in Thailand. From the many varieties developed, there are only a few that are widely adopted (Table 1.6)(10).

The breeding programs which are still continuously developing cassava varieties are:

- Rayong Field Crops Research center (RAY-FCR), Department of Agricultural, Ministry of Agriculture
- Sriracha Reseach Center of Kasetsart University (KU), Ministry of Universities Affair
- Extension Station of the Thai Tapioca Development Institute Fund

Table 1.5 Cassavas varieties in Thailand (9)

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

Table 1.6 Comparison of cassava varieties in Thailand (10)

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

1.2 Starch

Starch is the most significant form of carbon reserve in plant, occurs largely as complex insoluble granule located in amyloplast. Starch is synthesized in leaves during the day from photosynthetically fixed carbon and is mobilized at night. It is also synthesized transiently in other organs such as meristems and root caps cells, but its major site of accumulation is in storage organs, including seeds, fruits, tubers and storage roots. Starch is synthesized in plastid, which are called amyloplast in storage organs committed primarily to starch production. The organelles developed directly from plastids contain internal lamella structure.

Starch can be chemically fractionated into two types of glucan polymers amylose and amylopectin. Starch contains varied ratio of amylose and amylopectin in different plants, resulting in different properties of plant starch (Table 1.7)(11).

Amylose consists of predominantly linear chain of α(1-4) linkaged glucose residues, each ~ 1000 residues long. Amylose is usually branched at a low level (approximately one branch per 1000 residues) by α(1-6) linkage and make up ~ 30% of starch. This proportion, however may vary considerably with the plant species. Once extracted from plants and insulation, amylose forms hydrogen bonds between molecules, resulting in rigid gel. However, depending on the concentration, degree of polymerization, and temperature, it may crystallize and shrink (retrogradation) after heating .

Amylopectin, which consists of highly branched glucan chains, accounted for ~70% of starch. Chains of roughly 20 α(1-4) linkage glucose residues are joined by α(1-6) linkages to other branches. The branches themselves form an organized structure. Some are not substituted on the six positions and are called A chains (Figure 1.2).

Table 1.7 Percent of amylose and amylopectin in reserve plant starch (12)

Starch	Amylose (%)	Amylopectin (%)
Rice	18.5	81.5
Waxy	0	100
Barley	22	78
Waxy	0	100
Wheat	28	72
Oat	27	73
Corn	28	72
Tapioca	16.7	83.3
Potato	20	80
Sweet potato	17.8	82.2

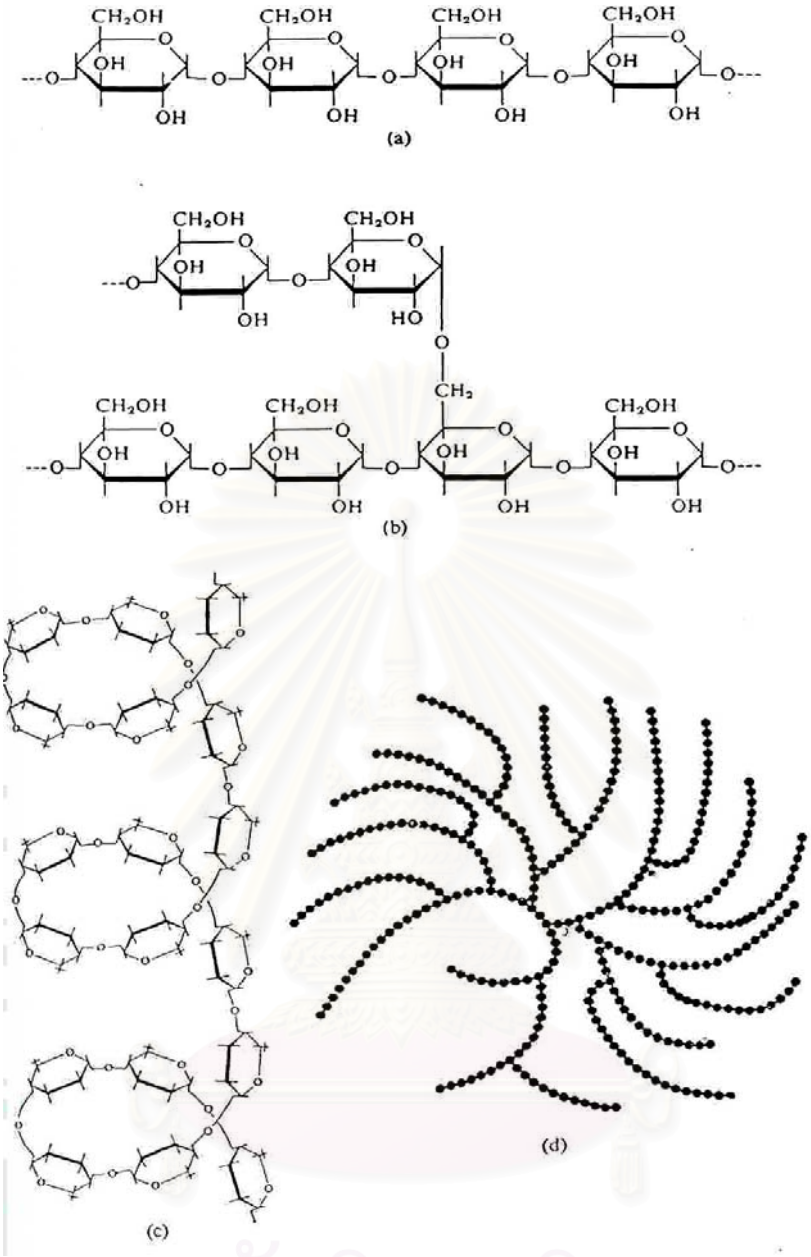


Figure 1.2 Structures of amylose and amylopectin

A) Amylose, showing the mode of linkage of the chain

B) Amylopectin, showing the branch point

C) The conformation of the chain in amylose

D) The branched structure of the amylopectin and glycogen type of molecule

These chains are α(1-6) linkage to inner branches (B chains), which may be branches at one or several points. A single chain in an amylopectin molecule has a free reducing end (C chains). The branches are not randomly arranged but are clustered at 7-10 nm interval (Figure 1.3). An average amylopectin molecule is 200 to 400 nm long (20 to 40 clusters) and ~ 15 nm wide. After extraction, amylopectin has more limited hydrogen bonding than amylose in solution and is more stable and giving high viscosity and elasticity to paste and thickeness (11). Table 1.8 summarized the different properties of amylase and amylopectin.

Starch structures, e.g. granular size and shape, amylose and amylopectin contents, molecular structure, phosphate monoester derivative, lipid and phospholipid content, affect functional properties. There are two-type of starch granules, the A-granules are large with diameters between 20-35 μm and are in disk shape. The B-granules are small with diameter between 2-5 μm and in a spherical shape (Figure 1.4). Development of the A-granules and B-granules in wheat has been reported Result showed that the A-granules appear in the early stage of endosperm development, whereas the B-granules appear about 10 to 12 days after flowering. Recent studies on structure of the A and B-granules of wheat, barley and triticale showed that the branch length distribution of the amylopectin in A- and B-granules varies. For all three varieties, B-granule starch amylopectin possess more short chains (DP 6 to 21) but less medium size (DP 22 to ~44) and long branch chain (DP > ~44) than do the amylopectin in A-granule starch. All the small B-granules studied consist of less amylose and display lower pasting viscosity than do the large A-granule. Small B-granules, with larger proportions of short branch chains, display lower gelatinization temperature than the large A-granules, except barley. The differences in the branch structure of amylopectin (Figure 1.2)

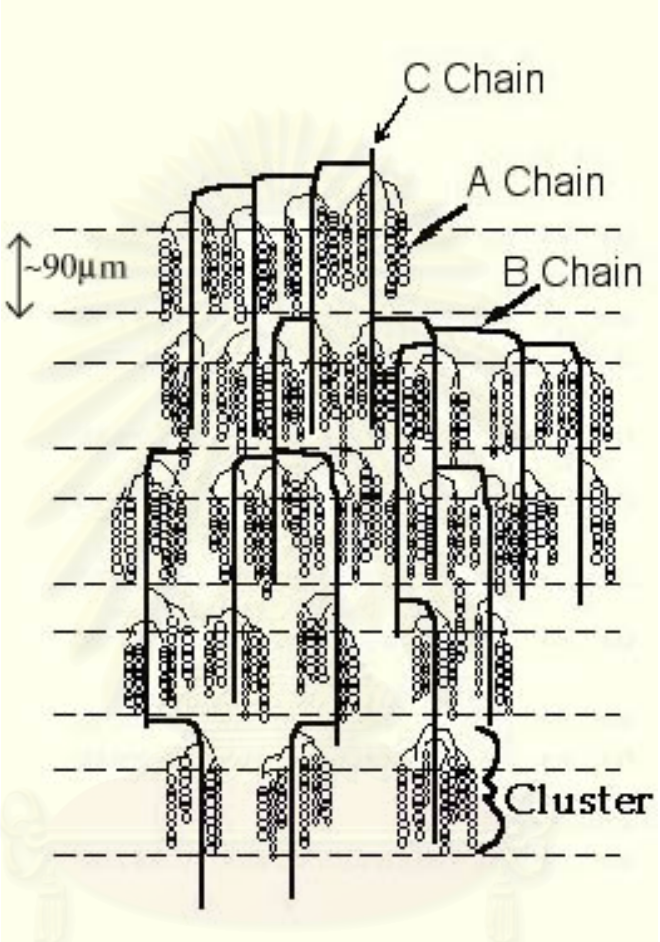
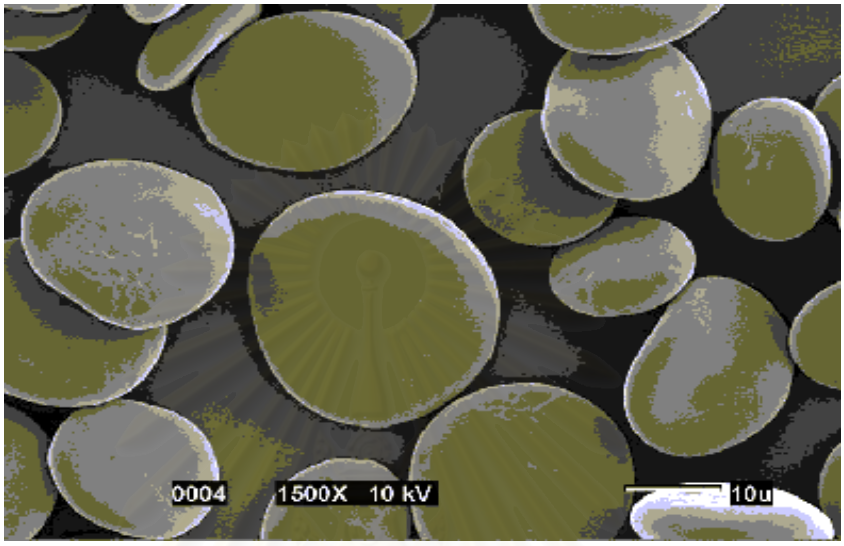


Figure 1.3 The branch structure of amylopectin

Table 1.8 Properties of the amylose and amylopectin components of starch (11)

Property	Amylose	Amylopectin
General structure	Essential linear	Branched
Color with iodine	Dark blue	Pur[le
λ-max of iodine complex	644 nm.	544-556 nm.
Iodine affinity	20.1%	1.05-1.25%
Average chain length (glucose residue)	100-10,000	20-30
Degree of polymerization (glucose residue)	100-10,000	10,000-100,000
Solubility in water	Variable	Soluble
Stability in aqueous solution	Retrogrades	Stable
Conversion to maltose by crystalline β-amylase	82%	59%

A.



B.

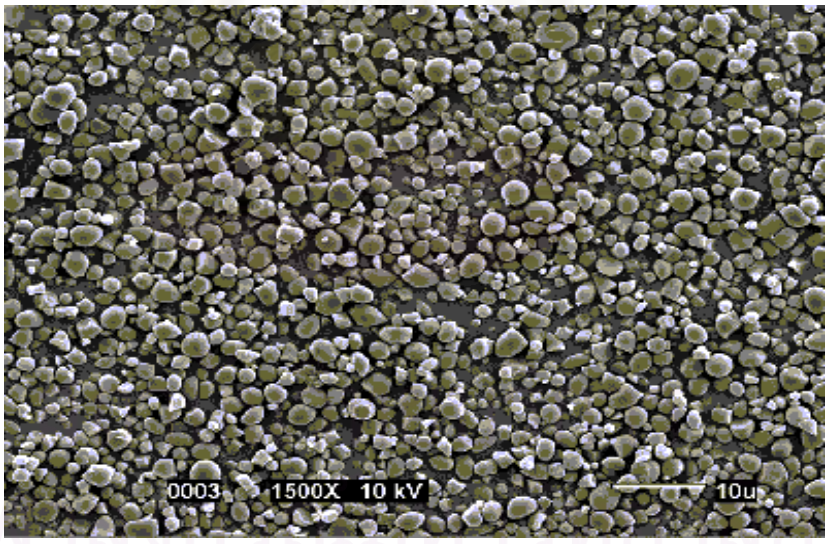


Figure 1.4 The starch granule in Wheat.

A. Large granule (A-granule)

B. Small granule (B-granule)

between the small and the large granules of wheat, barley, and triticale are the reverse of the small and the large granules of other starches, such as potato and maize. This difference may be related to the presence of the starch branching enzyme found primarily in the large granules.

Within starch granule, the amylopectin molecules are arranged radially and adjacent branches within the branch cluster may form double helices that can be packed regularly, giving a crystallinity to the starch granules. The degree of crystallinity is determined in part by the branch lengths in the amylopectin. The degree of branching and consequently the crystallinity of starch granules may vary considerably, even between different organs of the plant. The starch granule is not uniformly crystallinic, but also contains relatively amorphous regions (Figure 1.5). Amylose molecules form single helical structure and are thought to be packed into these amorphous regions, which are present throughout the granule(9).

Starch granules from storage organs and leaves have rather different macrostructures. Starch granules from storage organs show internal semicrystalline growth rings which are differentially sensitive to chemical and enzymatic attack. The denser, more resistant layers may be regions of closer packing of branches within the branch clusters of parallel amylopectin molecules. The formation of these rings may result from periodic differences in the rate of starch synthesis. Starch granules in leaves are generally smaller than those in storage organs and have a distinct macrostructure. They are thought to have a crystalline core with an amorphous outer mantle that consists of less branched glucan polymers. Most of the turnover in starch during day/night cycles involves the amorphous mantle of the granule (10). Other components within all starch granules are proteins (0.5% in cereal endosperm and 0.05% in potato tuber), which

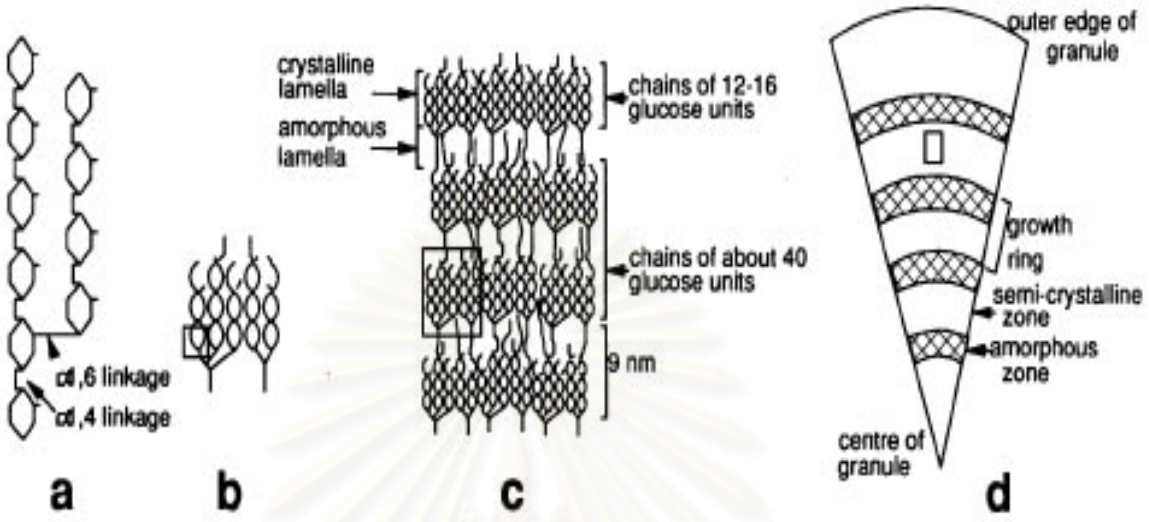


Figure 1.5 Schematic representation of levels of organization within the starch granule. The boxes within the diagrams in panel b, c and d represent the area occupied by the structure in the preceding panel (13).

a. Structure of two branches of an amylopectin molecule, showing individual glucose units.
b. A single cluster within an amylopectin molecule, showing association of adjacent branches to form double helices.
c. Arrangement of clusters to form alternating crystalline and amorphous lamellae. The lamellae are produced by the packing of double helices in ordered arrays. Chains of 12-16 glucose units span one cluster; chains of about 40 glucose units span two clusters.
d. Slice through a granule, showing alternating
e. zones of semicrystalline material, consisting of crystalline and amorphous lamellae, and amorphous material.

include the enzymes of starch biosynthesis and may contribute to the flavor of starch and lipid (1% in cereal endosperm and 0.1% in potato tuber). The chemical structure of amylose facilitates its association with lipid, so the lipid may be localized in specific regions within the starch granule.

1.3 Starch biosynthesis

There are many enzymes involved in starch biosynthesis (Figure 1.6)(15). 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 which is the rate-limiting step of starch biosynthesis and control starch quantity. Starch synthase (SS, EC 2.4.1.21) catalyzes the transfer of glucosyl units from nucleotide-glucose (such as ADP-glucose and UDP-glucose) donor to non-reducing ends of growing polysaccharides, such as amylose, amylopectin and glycogen, via $\alpha(1\rightarrow4)$linkages. Starch synthases are defined in two types: granule-bound starch synthase (GBSS) and soluble starch synthase (SSS). Starch branching enzyme (SBE, EC 2.4.1.18) introduces branching points in the amylopectin molecules by hydrolysis of the $\alpha(1\rightarrow4)$ glucan chains at 15-20 units from the non-reducing end. Debranching enzyme (DBE, EC 2.4.1.25) removes the outer chains from unorganized glucan created by the SBE and SS, but does not have access to branch points formed close to the organized. Disproportionating enzyme (D-enzyme, EC 2.4.1.25) catalyzes the reversible condensation of donor and acceptor 4-α-D-oligoglucan chain. The enzymes play role in the starch quality, physical and biochemical properties. Starch biosynthesis depends on amylose and amylopectin synthesis.

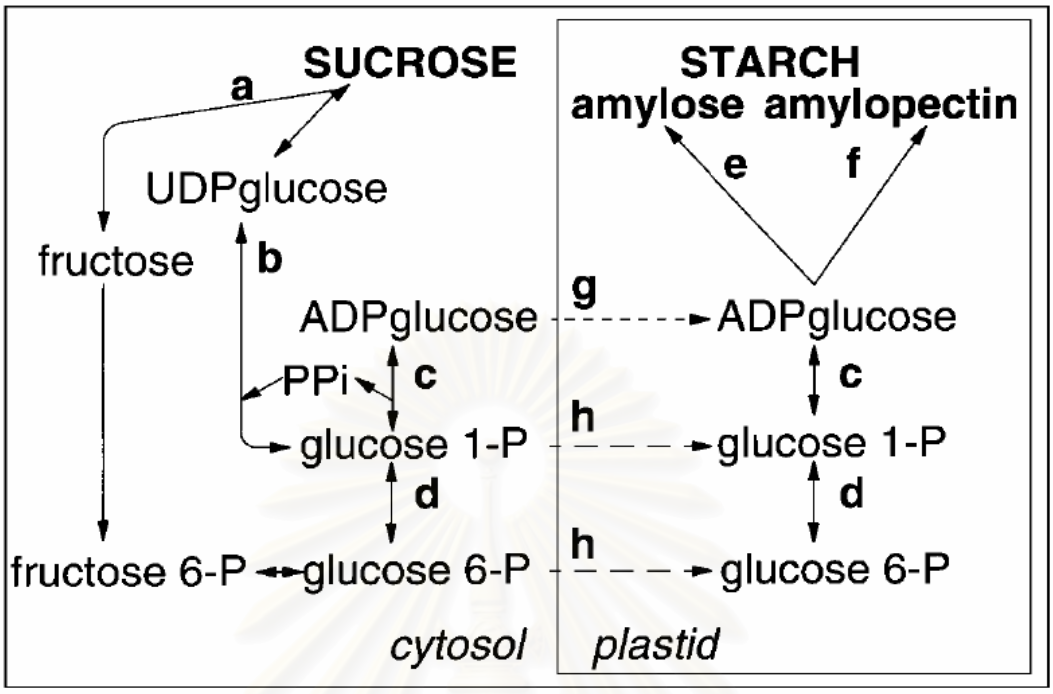


Figure 1.6 The major metabolites and enzyme involved in the conversion of sucrose to starch in storage organs. Carbons is shown entering the plastid either as a hexose phosphate (13). or as ADP-glucose: a. sucrose synthase; b. UDP-glucose pyrophosphorylase; c. ADP-pyrophosphorylase; d. phosphoglucomutase; e. starch synthase (GBSSI); f. starch synthase and starch branching enzyme; g. ADP-glucose transporter; h. hexose phosphate transporter. PPi: inorganic pyrophosphate.

1.3.1 Amylose synthesis

Amylose molecules appear to exist as single helices within the starch granule, intersperse with amylopectin in amorphous regions. Their precise location in relation to the ordered amylopectin matrix remains unclear. Amylose synthesis in storage organs is a specific function of the granule bound starch synthase I (GBSSI) which catalyzes the transfer of glucosyl unit 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 (14). It is evident that GBSSI must possess specific properties different from other isoforms, and detailed comparison of the structure-function relationships with those of other isoforms is likely to yield valuable information. The synthesis of the amylose *in vivo* is integrated in a complex way with the synthesis of the granules matrix: the non-uniform distribution of the amylose within granules with reduced GBSSI activity, the synthesis of amylopectin rather than amylose via GBSSI in the isolated starch granules, and the positive correlations observed in some species between the rate of starch synthesis and its amylose content (13).

1.3.2 Amylopectin synthesis

It is widely accepted that amylopectin is elaborated at the surface of the starch granule by the soluble starch synthase (SSS) and starch branching enzyme (SBE) in the soluble fraction of the amyloplast. Soluble starch synthase elongates very short chains at the peripheral of the granule. Initially, these chains are of insufficient length to act as substrates of SBE which acts preferentially upon chains in double helical conformation, and they remain un-branched. When they reach appropriate length for branching to occur, branches are created through the action of SBE by catalyzing the cleavage of

$\alpha(1\rightarrow4)$ linkage and transfer of the released reducing end to a C_6 hydroxyl, creating a new $\alpha(1\rightarrow6)$ linkage. The others two enzymes which are, potentially, involved in amylopectin biosynthesis are debranching enzyme (DBE) and disproportionating enzyme (D-enzyme) (15). DBE catalyzes the hydrolysis of $\alpha(1\rightarrow6)$ linkages and D-enzyme catalyzes the transfer segment of one linear chain to another. Amylopectins 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 (Figure 1.7). 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 DBE 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 (15). 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 (Figure 1.8)(15).

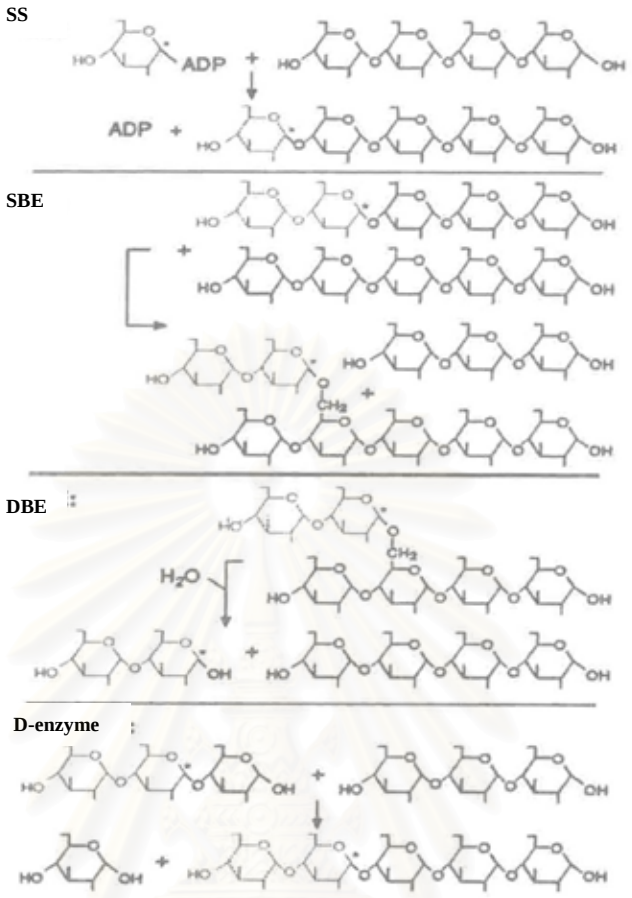


Figure 1.7 Reactions of enzymes involve in amylopectin synthesis (15)

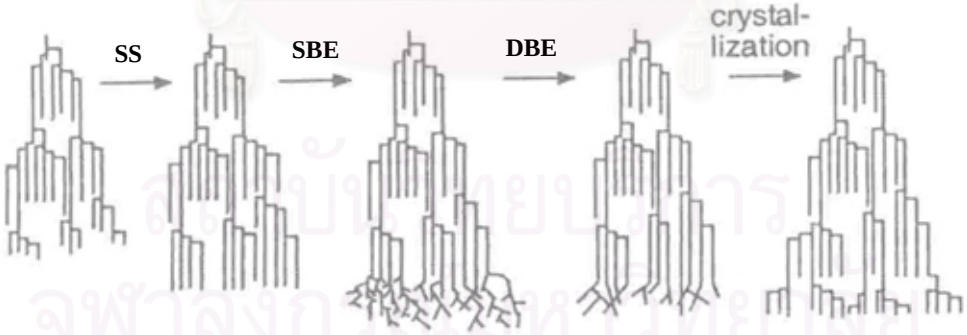


Figure 1.8 Amylopectin synthesis model (15)

1.4 Starch branching enzyme

Starch branching enzyme also called Q-enzyme (α-1,4-glucan : α-1,4-glucan-6-glucosyltransferase,EC 2.4.1.18) introduces branch points in the amylopectin molecules by hydrolysis of the α-1,4-glucan chains. It then catalyzes the formation of an α-1,6 cross linkage between the reducing end of the cleaved chain and another glucose residue (16). SBE is a member of the α-amylase family of enzyme, characterized by four highly conserved regions and a central $(\beta/\alpha)_8$ barrel domain. Apart from the barrel domain, SBEs show considerable structural variation in the length and amino acid sequences at the N- and C- terminal regions. Multiple SBE isozymes have been found individual plant species and are encoded by two gene families (families A and B) based on the primary sequence. Members of the two families display distinct enzymatic properties, presumably because of the differences in N- and C-teminal regions. Several studies have shown that the N-terminal region is important for specificity of transferred chain length and required for maximum enzyme activity, whereas the C-terminal region is involved in substrate specificity (17)

That plant almost invariably contains the multiple isoforms of starch branching enzyme (SBE) raises the possibility that different forms create chains with different length or branch points at different frequencies. Multiple forms of starch branching enzyme could thus give rise to the branching pattern and polymodal distribution of chain length that underline the cluster structure of the amylopectin. These isoforms have been classified two classes, A isoform and B isoform, based on amino acid sequence comparisons. Isoforms IIa and I of maize endosperm, III and I of rice endosperm, I and II of pea embryo (18), and II and I of potato tuber (19) fall into classes A and B, respectively. The A and B isoforms of starch branching enzyme differ both in their substrate affinities and in the length of branches they preferentially create. In vitro,

isoform A preferentially branches amylopectin, whereas isoform B preferentially branches amylose. With amylose as a substrate, isoform B preferentially transfers longer chain than isoform A (20). When expressed in a strain of *E. coli* that lacks a glycogen-branching enzyme, both isoforms can form branches in the linear product of the bacterial glycogen synthase to give a glycogen-like polymer. Consistent with their actions in vitro, the glycogen synthesized by isoform A has more shorter chains (6-9 glucose units) and fewer long chains (greater than 14 glucose units) than the glycogen synthesized by isoform B. It is likely that the difference in properties of the isoforms is general between A and B classes. The difference in properties between A and B isoforms have led to the suggesting that isoform B participates in *in vivo* synthesis of the long and intermediate length chains that will span cluster, whereas isoform A participates in the synthesis of shorter chains that lie wholly within cluster (16).

This idea is potentially testable through study of mutant and transgenic plant in which one isoform is eliminated or severely reduced in activity. Mutation at the *AMYLOSE-EXTENDER* (*AE*) loci of cereal and the RUGO-SUS(R) locus of peas lead specifically to the loss of A isoform (18). The amylopectin in *ae* mutant endosperm and *r* mutant embryo display increase in average chain length relative to that of the wild type. There is, however, no dramatic change in the distribution of the chain lengths among chains of up to 50 glucose units, and the structural periodicity of 9 nm within semicrytalline region of the granule is not affected by the mutation. No mutation affecting the B isoform has been described, but dramatic reduction of the activity of this isoform in potato tuber through expression of antisense RNA is reported to have only minor effects on the structure of amylopectin (21).

Plant branching enzyme (Q-enzyme) was first identified in potato (22), the purified SBE had monomeric molecular weight ranging from 83-103kD. The Km value

of the enzyme determined to be 0.02 mg/ml and K_{cat} was in the order of 1000 sec^{-1} using potato amylose as the substrate (23). In 1996, SBE was extracted from starch of potato tuber and separated by SDS gel electrophoresis. The specific protein bands were digested to produce peptides and then separated on the reversed phase chromatography and finally sequencing. The data showed that three isoforms of starch synthase and two isoforms of branching enzyme were present in the starch of potato tuber (24). The cDNA of potato SBE was cloned and identified as B class (major form)(25) and A class (minor form)(26). Afterward, the in vitro activities of purified starch branching enzyme I and II expressed in *E.coli* were compared using several assay method such as: substrate specificity, number of branching of linear dextrin, it was found that SBE I was more active on amylose substrate whereas SBE II was more active on amylopectin (27).

In cereal grain, SBE was found in many crops such as BE I, IIa and IIb in developing kernels of maize (28). SBE in developing rice endosperm have been purified as two isoforms QE I and QE II. After electrophoresis on a native PAGE followed by activity staining, the QE II fraction was found to be composed of two isoforms, QE IIa and QE IIb. QE IIa was detected only in the extract of endosperm, whereas QE IIb was presented in extracts of all tissues examined (29).

Cassava SBE was purified in five minutes cultivar and showed that it contained two subunits of 80 kD (30). Previously, cDNA coding for Cassava (*Manihot esculenta* Crantz.) branching enzyme was cloned from λgt11 cDNA library using a potato cDNA probe (31). Next, The cloned encoding SBE II was isolated and examined on the spatial and temporal expression of the *sbeII* gene (32).

However, molecular biology studies of SBE are needed to understand the full function of the enzyme. Although SBE's have been studied in many plants, most studies are carried out by gene cloning including cassava. Reports on starch synthesizing

enzymes in cassava are still rare. Purifications of cassava SBE isoforms have never been reported.

1.5 Objectives

Although cassava has entered the modern market economy, being used as food, feed product and other industrial products, its market price has been very low. It is, therefore, essential to add value to the crops by improving the quality of its major product i.e. cassava starch. The quality of starch is the result of interaction between starch synthase and starch branching enzyme. The characteristics of the starch, in term of texture, viscosity, stability are all strongly influenced by the relative proportion and molecular size of amylose and amylopectin fraction. It is, therefore, of our interest to understand the characteristics of this enzyme in cassava in order to be able to apply the knowledge to improve the quality of cassava. From the varieties of cassava developed in Thailand, we chose two cultivars which are different in starch content: Rarong1 the low starch cultivar and KU50 the high starch cultivars for our study on level of starch synthesizing enzymes in the tubers at different ages. Studies on the SBE isoform will be caused out by purification of the enzyme using the cultivar KU 50 which has high starch content.

The objectives of this thesis are:

1. To monitor activity of SBE in cassava roots of low and high starch cultivar (Rayong 1 and KU 5) at different ages.
2. To isolate and purify isoforms of starch branching enzyme from KU 50 cassava tuber at selected age.
3. To characterize the purified starch branching enzyme isoforms.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

Cassava tubers used were from cultivars Rayong 1 and KU 50 from Rayong Field Crops Research Center at Rayong province, Thailand.

2.2 Chemicals

Chemicals	Company
α-D-Glucose-1-phosphate hydrochloride	Sigma
α-D-[^{14}C-] Glucose-1-phosphate	Amersham Bioscience
β-Mercaptoethanol	Sigma
Acylamide	Merk
Adenosine mono phosphate	Sigma
Amylopectin, corn	Sigma
Amylose, potato	Sigma
Aquasorb	SM.lab
Benzamidine hydrochloride	Sigma
Citric acid	Carlo
Comassie blue R-250	Acros
Comassie blue G-250	Fluka
DEAE – Toyopearl	Tosho

Chemicals	Company
Dithiothreitol	Amersham Bioscience
Ethelenediaminetetraacetic acid	Carlo
Glucose	Ajax
Glutatione reduced form	Sigma
Glycine	Amersham Bioscience
Glycogen, Rabbit liver	Sigma
Hydrochloric acid	BDH
Iodoacetic acid	Sigma
Isoelectric focusing calibration kit pH 3-10	Amersham Bioscience
Maltoheptaose	Sigma
Maltohexaose	Sigma
Iodine	Fluka
Maltopentaose	Sigma
Maltose	Sigma
Maltotriose	Sigma
β-Mercaptoethanol	Scharlau
Methanol	BDH
MOPS (3-[(N-morpholino)propanesulfonic acid])	Amersham Bioscience
N,N,N',N'-Tetrametylene ethylene diamine	Fluka
N,N-methyl-bis-acrylamide	Amersham Bioscience
N-Ethylmaleimide	Sigma
Potassium iodide	Fluka

Chemicals	Company
Potassium Chloride	Merk
Potato starch soluble	Kanto chemical
Sephadex G-200	Amersham Bioscience
Sodium Chloride	BDH
Sodium citrate anhydrate	Carlo
Sodium laulyl sulphate	Sigma
Tris(hydroxymethyl) aminomethane	Amersham Bioscience
Polyethylene glycol 6000	Fluka

2.3 Equipments

Equipment/Model	Company
Blender /	Phillip
Bench top centrifuge /	Labquip
Electrophoresis unit / Hoefer Mighty small	Amersham Bioscience
Electrophoresis power supply / EPS 300	Amersham Bioscience
FPLC / Akta FPLC	Amersham Bioscience
Fraction collector	LKB
Lyophilizer	Flexi-Dry
Peristaltic pump	LKB
pH meter	Metler
Refrigerator centrifuge /Avanti J-30I	Beckman

Equipment/Model	Company
Scintillation counter	Beckman
Spectrophotometer	Beckman
Water bath shaker	Sturt

2.4 Preparation of starch branching enzyme from cassava tuber

Cassava tubers were peeled and the cortex was removed. The parenchyma was chopped and homogenized in a blender. The pH of the cassava juice was adjusted with 0.05 M Tris-HCl pH 7.5 containing 2 mM EDTA and 1 mM dithiothreitol. The cassava juice was added with Benzamidine 1 mM to prevent serine protease activity. The homogenate was centrifuged at 15000xg for 1 hour at 4 °C to remove starch and lipid. The supernatant was collected as crude enzyme and kept at 4°C for further work.

2.5 Purification of SBE from cassava tubers

2.5.1 Precipitation of crude enzyme with Polyethylene glycol 6000

Solid polyethylene glycol 6000 was added to crude enzyme to reach 10% saturation and continuously stirred for 1 hour on ice bath. Afterward, the supernatant was collected by centrifugation at 15000xg for 1 hour at 4 °C. The supernatant was collected for DEAE – Toyopearl column chromatography.

2.5.2 First DEAE-Toyopearl column chromatography

DEAE-Toyopearl was washed with 0.5 N sodium hydroxide followed with distilled water until the pH is neutral and packed into a glass column (2 x 15cm.). It was equilibrated with starting buffer (0.05 M Tris-HCl pH 7.5 contained 2 mM EDTA and 1 mM DTT). The sample in section 2.4.2 was loaded onto the column and eluted with starting buffer containing 0.15 M sodium chloride at flow rate 60 ml/h. Fractions of 18 ml were collected using

fraction collector. The eluted fractions were monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was detected by the method described in section 2.5. The fractions with enzyme activity were pooled. The enzyme was filtered and concentrated by Viva flow 50 (molecular weight cut off 30,000 Dalton) and dialyzed against starting buffer for second DEAE-Toyopearl step.

2.5.3 Second DEAE-Toyopearl column chromatography

DEAE-Toyopearl column was prepared as described in section 2.4.4 and packed in glass column (1 x 13 cm). The column was eluted stepwise with 0.04 M, 0.08 M and 0.1 M sodium chloride respectively, at flow rate 60 ml/h. Fractions of 6 ml were collected and monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was detected by the method described in section 2.5. The fractions with enzyme activity were pooled for the next step.

2.5.4 Hitrap Q-sepharose chromatography .

Hitrap Q-sepharose column was equilibrated with 0.05 Tris-HCl pH 7.5 contained 1 mM DTT, 2 mM EDTA. Each isoform of enzyme was loaded separately onto the column and unbound protein was washed until negligible A_{280} was detected followed by elution with linear gradient of 0-0.5 M NaCl at flow rate 60 ml/h. Fractions of 3 ml were collected and monitored for protein and SBE activity as described in section 2.5.3

2.5.5 Sephacryl HR S-200 chromatography

Prepacked Sephacryl HR S-200 column was run by FPLC. The column was equilibrated with 0.05 Tris-HCl pH 7.5 containing 1 mM DTT, 2 mM EDTA and 0.15 M sodium chloride. Each isoform of the enzyme was concentrated and loaded on to the column,

and eluted with the same buffer at flow rate 30 ml/h. Fractions of 2 ml were collected and detect for enzyme activity of enzyme.

2.6 Protein determination

Protein was determined by the Coomassie blue method. Using bovine serum albumin (BSA) as standard.

One hundred microliters of sample was mixed with 5 ml of Coomassie blue reagent and left for 30 minutes before recording the absorbance at 595 nm.

2.7 SBE Activity assay

SBE activity was determined by 2 methods:

2.7.1 Radioactive method

Activity of starch branching enzyme was assayed by measuring the incorporation of ^{14}C-glucose into α-D-glucan synthesized by stimulation with rabbit-muscle phosphorylase a modified from the method describe by Mizuno *et a l* (33)

The reaction was conducted in 0.2 ml. mixture containing 0.1 M MOPS-NaOH pH 7.0, 50 mM α-D-[^{14}C] glucose-1-phosphate, 1 mM AMP, 10 μg rabbit-muscle phosphorylase a and appropriate amount of enzyme. After incubation at 37°C for 1 hour, the mixture was boiled for 3 min. to terminate enzyme reaction. To the mixture, 20 μg rabbit-liver glycogen was added to co-precipitate the newly forms glucans with methanol. The amount of radioactive in methanol-insoluble portion was measured using liquid scintillation counter. One unit enzyme activity was define as 1 mmole of α-D-[^{14}C] glucose incorporation from α-D-[^{14}C] glucose-1-phosphate into the methanol-insoluble material per min under the condition used.

2.7.2 Spectrophometric method

The modified assay is based on the spectral change of the glucan-iodine complex that occurs after branching of the substrate (28)

The assayed was conducted in 0.4 ml. containing 1 mg/ml corn amylose or corn amylopectin and 0.1 M MOPS-NaOH pH 7.0 and appropriate amount of enzyme. The reaction was initiated by adding the enzyme and 0.1 ml aliquots of reaction mixture were collected at various time intervals. Each aliquot was boiled for 2 minutes mix with 0.3 ml distilled water and 2.6 ml of iodine solution I. The absorbance changes were measured at 660 and 520 nm. for amylose and amylopectin, respectively. One unit of SBE activity is defined as the decrease in absorbance of 1.0 per minute at 37°C.

2.8 Polyacrylamide gel electrophoresis (PAGE)

2.8.1 Non-denaturing starch-PAGE

Non-denaturing polyacrylamide gel was prepared as described in Appendix A, with addition of 0.6 % (w/v) soluble starch in the gel solution on slab gel (10x8x1.5) of 7.5% (v/v) separating gel and 4% (v/v) stacking gel. Cold Tris-glycine buffer pH 8.3 was used as electrode buffer. The electrophoresis was performed at a constant current of 16 mA. For SBE activity stain, gel strip was rinsed with distilled water and soaked in 50 mM MOPS-NaOH pH 7.0 for 2 hour at room temperature and incubated 15 min. in iodine solution II. The zones of cassava starch branching enzyme activity appeared sharp red-brown bands on the blue stained background.

2.8.2 SDS - PAGE

The denaturing gel was carried out with 0.1% (w/v) SDS in 10% (w/v) separating gel and 5.0% (w/v) stacking gel and Tris-glycine buffer pH 8.3 containg 0.15 (w/v) SDS was used as electrode buffer. Sample to be analyzed were treated with sample buffer and boiled for 5 min before application to the gel. The electrophoresis was performed at constant current of 20 mA per slap, at room temperature on a electrophoresis unit form cathode towards anode.

2.9 Characterization of starch branching enzyme

2.9.1 Determination of Molecular weight

2.9.1.1 Gel filtration on Sephacryl HR S-200 colum

The molecular weight of each SBE isoform was chromatographed on Sephacryl HR S-200 and molecular weight determined from calibration curve prepared from standard molecular weight marker proteins; namely catalase (232 kDa), aldolase (158 kDa),bovine serum albumin (68 kDa), ovalbumin (43 kDa). Blue dextran and riboflavin were used to determine the void volume (V_o) and total volume (V_t), respectively. The partition coefficient (K_{av}) of the standard marker protein and SBE were calculated from the formula $K_{av} = \frac{Ve - Vo}{Vt - Vo}$

2.9.1.2 SDS-PAGE

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining. The molecular weight of SBE subunits were determined form calibration curves obtained from R_f and molecular weight of standard proteins; namely Phosphorylase b (94 kDa), Bovine serum albumin (64 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa)

2.9.2 Effect of pH on starch branching enzyme activity

The purified starch branching enzyme was used to study the effect of pH on its activity. The enzyme was assayed as described in section 2.6.2; the enzyme was incubated with substrate solutions prepared in universal buffer at various pH's. After incubation for 30 minutes the reaction was stopped by heating in boiling water for 5 minute. The result was expressed as the percentage relative activity.

2.9.3 Effect of Temperature on starch branching enzyme activity

The purified enzyme was used to study effect of temperatures on its activity. The enzyme was assayed by incubation at 20, 25, 30, 37, 40, and 50 °C for 1 hour. After the reaction was stopped by heated in boil water for 5 minutes, the activity was measured as described 2.6.2.The result was expressed as the percentage relative activity.

2.9.4 Temperature stability of starch branching enzyme activity

The temperature stability of starch branching enzyme was studied. The temperatures at which more than 60% SBE activity remaining after incubation for 1 hour (experiment in section 2.8.2.1) was used for stability study (25, 30, 37, 40 and 50 °C). The enzyme was incubated at the selected temperature for 0, 6, 12, 24, 36 and 48 hours and collected for assay as described previously. After the reaction was stopped by heated in boil water for 5 minutes, the activity was measured as describe 2.6.2. The result was expressed as the percentage relative activity.

2.9.5 Effect of sulfhydryl reagents on starch branching enzyme activity

The effect of the sulfhydryl group reagents on starch branching enzyme activity were studied. Different concentrations (2 mM, 5mM, and 10 mM) of DTT, NEM, IAA and β-2 Mercaptoethanol were added to different reaction mixtures. The reactions were stopped by

heated in boiling water after incubation at 37°C for 1 hour. The activity was measured as describe 2.6.2. The result was expressed as the percentage relative activity.

2.9.6 Effect of divalent metal ions on starch branching enzyme activity

The effect of various the divalent metal ion on starch branching enzyme activity were studied. Various concentrations (1mM, 5mM, 10 mM and 100 mM) of $CuSO_4$, $ZnCl_2$, $MnCl_2$, $HgCl_2$ and $Ni(SO_4)_2$ were added to reaction mixtures and SBE activity were monitored as describe section 2.6.2

2.9.7 Isoelectric focusing polyacrylamide gel electrophoresis

2.9.7.1 Preparation of gel support film

A few drop of water was pipetted on to the plate. The hydrophobic side of the gel support film was then placed against the plate and flatly rolled the test tube to force excess water and bubbles. Subsequently, it was place down on the casting tray with the gel support film facing down so that it rest on the space bars.

2.9.7.2 Preparation of the gel

The gel solution for IEF was prepared as described appendix C. The solution was mixed and degassed about 5 min. and carefully pipitted between the glass plate and casting tray with a smooth flow rate to prevent air bubbles. The gel was left about 45 min to allow polymerization, then the gel plate was lifted from the casting tray by spatula. The gel was fixed on the gel support film and ready for used.

2.9.7.3 Sample application and electrophoresis

The sample was loaded and the gel with the absorbed samples was turned upside-down and directly placed on top of the graphite electrode. Focusing is carried out constant voltage conditions in a stepwise fashion. The gel was firstly focused at 100 V 15 min., followed by increase up to 200 V to 15 minutes and finally run at 450 V for 60 minutes. After complete electro focusing, the gel was stained. Standard protein markers with known pI in the ranges 3-10 were run in parallel. The pI of the sample protein were determined by the standard curve constructed from the pI of standard proteins and their migration distance from cathode.

2.9.8 Determination of K_m and V_{max}

The purified SBE was used to study for kinetic constant with amylose as the substrate of this enzyme. The reaction mixture consisted of various concentrations of amylose, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.25, 1.5, 1.75 and 2.0 mg/ml.

After the reaction was stopped by heated in boil water for 5 minutes, the activity was measured as described in section 2.6.2.

2.9.9 Comparison of SBE activity with amylose and amylopectin as substrates

The purified enzyme was used to study substrate specificity for amylase and amylopectin. The enzyme was assayed by incubation at 37 °C for 0, 5, 10, 20, 30, 60 and 120 minutes. After the reaction was stopped by heated in boil water for 5 minutes, the activity was measured as describe 2.7.2.The result was expressed relative to A_{660} for amylose or A_{520} for amylopectin when SBE was not added, which were assumed as 100%.

CHAPTER 3

RESULTS

3.1 SBE activities in cassava tubers at different ages and cultivars

SBE activity was monitored by radioactive method in cassava tubers harvested at 3 months intervals up to 12 months in cultivars Rayong1 and KU50. The SBE activity was expressed as specific activity. Protein content seemed to be high at 3 months old but dropped with ages while level of SBE increased (Table 3.1). Specific activities and activity per wet weight appeared to increase with growth, accelerated rapidly between 6 to 9 months in both cultivars. Maximum SBE activity per Kg wet weight was reached at 9 and 12 months for KU 50 and Rayong 1 respectively.

KU50 is the cultivar with high starch content; therefore, we used KU50 at the age of 9 months, which had highest SBE per Kg wet weight for purification of SBE for further studies.

3.2 Purification of starch branching enzyme from KU50 cassava tubers

3.2.1 Preparation of crude enzyme

Crude starch branching enzyme was prepared from 2.5 kilograms of parenchyma from KU50 cassava tubers as described in section 2.1.1. The SBE activity was measured as described in 2.6.1. The amount of protein obtained was 1920 mg with 199.7 units of starch branching enzyme (SBE) activity.

3.2.2 Precipitation with polyethylene glycol 6000

The crude enzyme was first purified by precipitation of other contaminating proteins and small starch granules with 10% polyethylene glycol as described in section 2.4.1. Significant amount of proteins was removed, leaving 1086 mg protein in the supernatant with 184 units of SBE yielding 1.6 folds of purification.

3.2.3 First DEAE-Toyopearl chromatography

The enzyme from polyethylene glycol 6000 fraction was loaded on DEAE-Toyopearl prepared as described in section 2.4. Fractions of 18 ml were collected. Unbound proteins were washed with the starting buffer and SBE was eluted with 0.15 M NaCl. The chromatographic profile is shown in Figure 3.1. The SBE activity peak was pooled and concentrated using membrane with molecular weight cut off 30,000 Dalton. This fraction contained 265 mg of protein and 200 units of SBE.

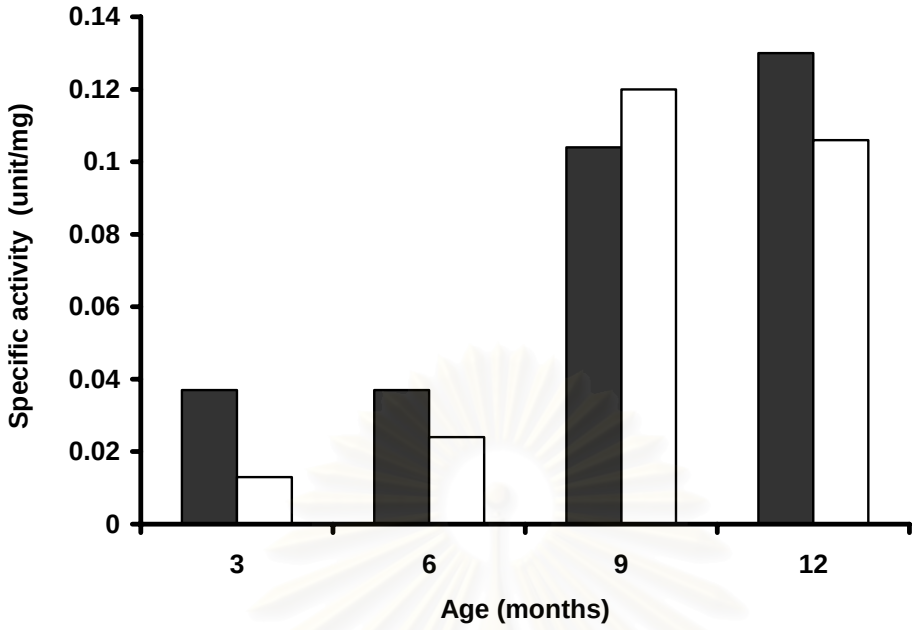


Figure 3.1 The specific activity of starch branching enzyme in tubers of KU50 and Rayaong 1 at different ages.

■ KU 50 □ Rayong 1

Table 3.1 The activity of starch branching enzyme and protein content in tuber of KU 50 and Rayong 1 at different cultivar and age

Cultivars	Age (Months)	Protein (mg/ml)	Protein/Kg. Tissue (mg/Kg. Tissue)	Activity/Kg. tissue (mmole/min/Kg. tissue)	Specific activity (Unit)
KU 50	**3**	**3.0**	**720**	**26.73**	**0.037**
	6	**4.3**	**645**	**24**	**0.037**
	9	**3.2**	**503**	**52.3**	**0.104**
	12	**2.2**	**330**	**42.6**	**0.130**
Rayong 1	**3**	**2.9**	**725**	**9.66**	**0.013**
	6	**3.3**	**495**	**11.76**	**0.024**
	9	**2.5**	**390**	**46.8**	**0.120**
	12	**3.1**	**465**	**49.62**	**0.106**

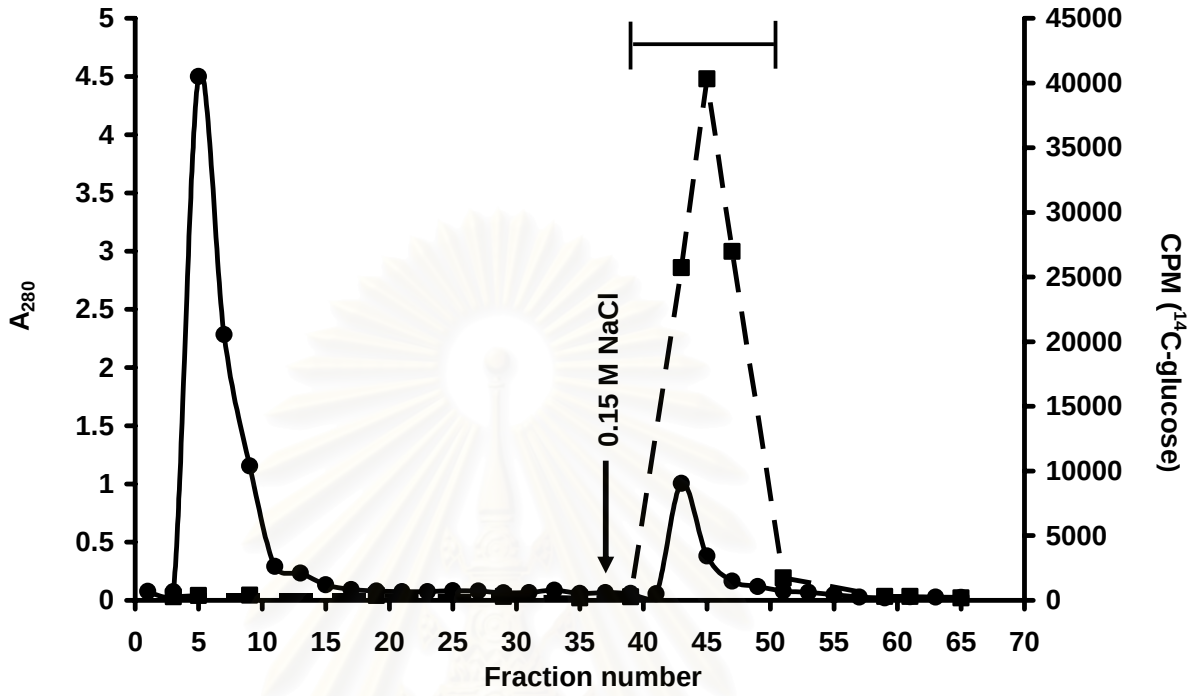


Figure 3.2 First DEAE-Toyopearl chromatographic profile of SBE from parenchyma of cassava tubers in polyethylene glycol 6000 precipitated fraction from cassava parenchyma that was eluted with 0.15 M NaCl in starting buffer. Fractions of 18 ml were collected and flow rate of 60 ml/h

● A_{280} ■ CPM

3.1.4 Second DEAE-Toyopearl chromatography

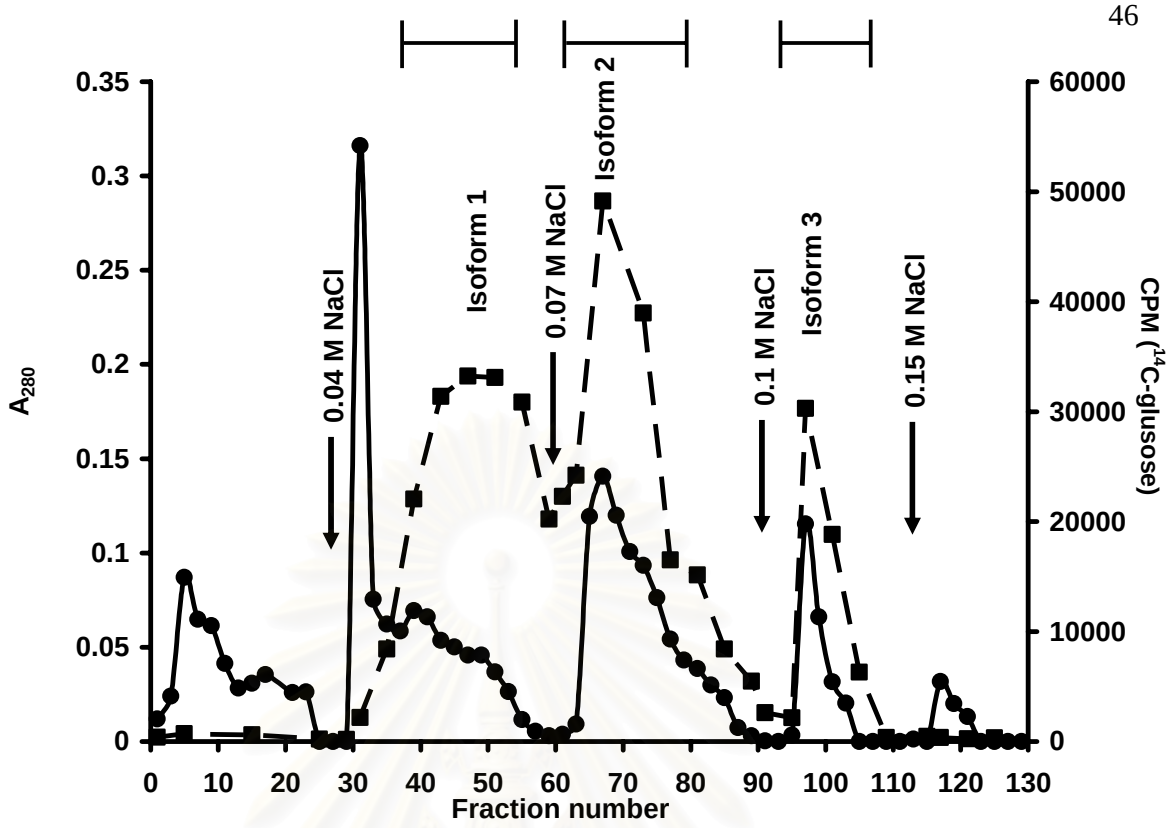
The enzyme from first DEAE-Toyopearl chromatography was loaded on a 2^{nd} DEAE-Toyopearl with different column size (1x13 cm) and eluted stepwise with 0.04, 0.07 and 0.1 M NaCl at 250, 250 and 100 ml, respectively. The chromatographic profile is shown with SBE peaks activity peaks (Figure 3.3). Each activity peak contained 1012, 321, 587 units, respectively. They were called SBE isoforms 1, 2 and 3.

3.1.5 Hitrap Q Sepharose column chromatography

Each SBE peak from the second DEAE-Toyopearl chromatography was pooled and loaded separately on Hitrap Q Sepharose column and eluted with gradient of 0-0.5 M NaCl. The first peak was eluted at 0.4 M with 39 mg protein and 503.5 SBE units. The second peak was eluted 0.3 M containing 50 mg protein and 400 SBE units and the third peak was eluted at 0.42 M NaCl with 32 mg protein and 822 SBE units.

Table 3.1 summarized the purification of SBE isoforms achieved at each step of purification. Isoforms 1, 2 and 3 were purified to 125, 77 and 250 folds respectively.

The purified isoforms and 1,2 and 3 were subjected to electrophoresis on 7% native polyacrylamide gel and stained with I_2 with the techniques described in section 2.8.1. Isoform 2 and 3 seemed to move at the same mobility while isoform 1 moved slower (Figure 3.8).



Figurer 3.3 Second DEAE-Toyopearl chromatographic profile of SBE from first DEAE-Toyopearl concentrated with Viva flow 50 (cut off 30,000 Da.) The enzyme was eluted stepwise with 250 ml of 0.04, 0.07 and 150 ml. of 0.1 M NaCl, respectively. Fractions of 6 ml were collected.

● A_{280} ■ CPM

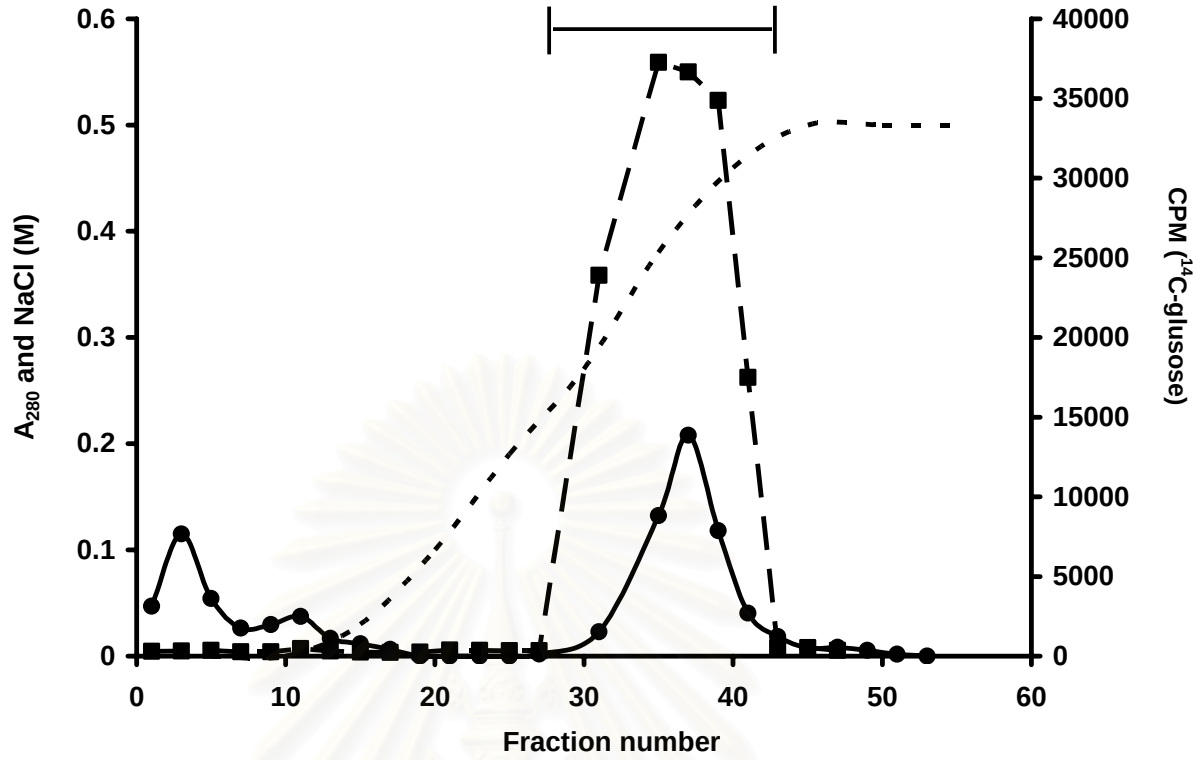


Figure 3.4 Hitrap Q-Sepharose chromatographic profile of the SBE isoform 1 from second DEAE-Toyopearl. The enzyme was eluted with linear gradient of 0-0.5 NaCl. Fractions of 3 ml were collected.

● A_{280} ■ CPM ····· [NaCl]

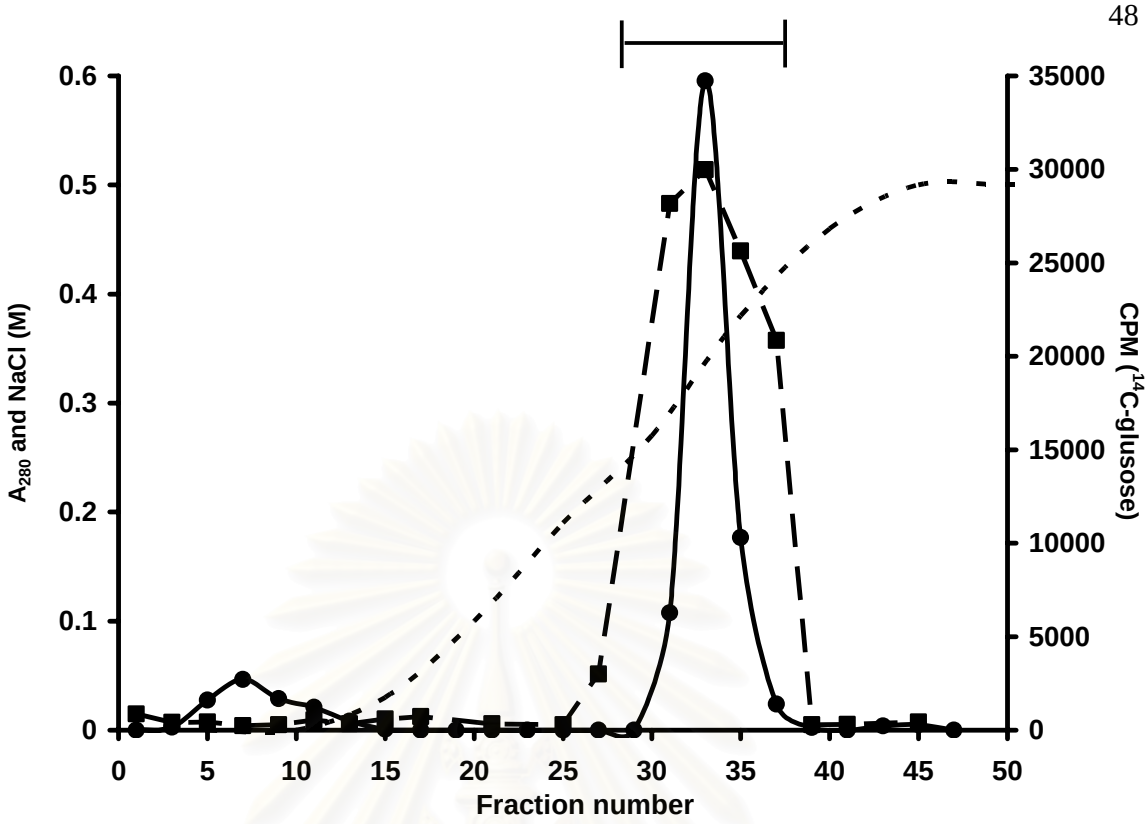


Figure 3.5 Hitrap Q-Sepharose chromatographic profile of the SBE isoform 2 from second DEAE-Toyopearl. The enzyme was eluted with linear gradient of 0-0.5 NaCl. Fractions of 3 ml were collected.

● A_{280} ■ CPM ····· [NaCl]

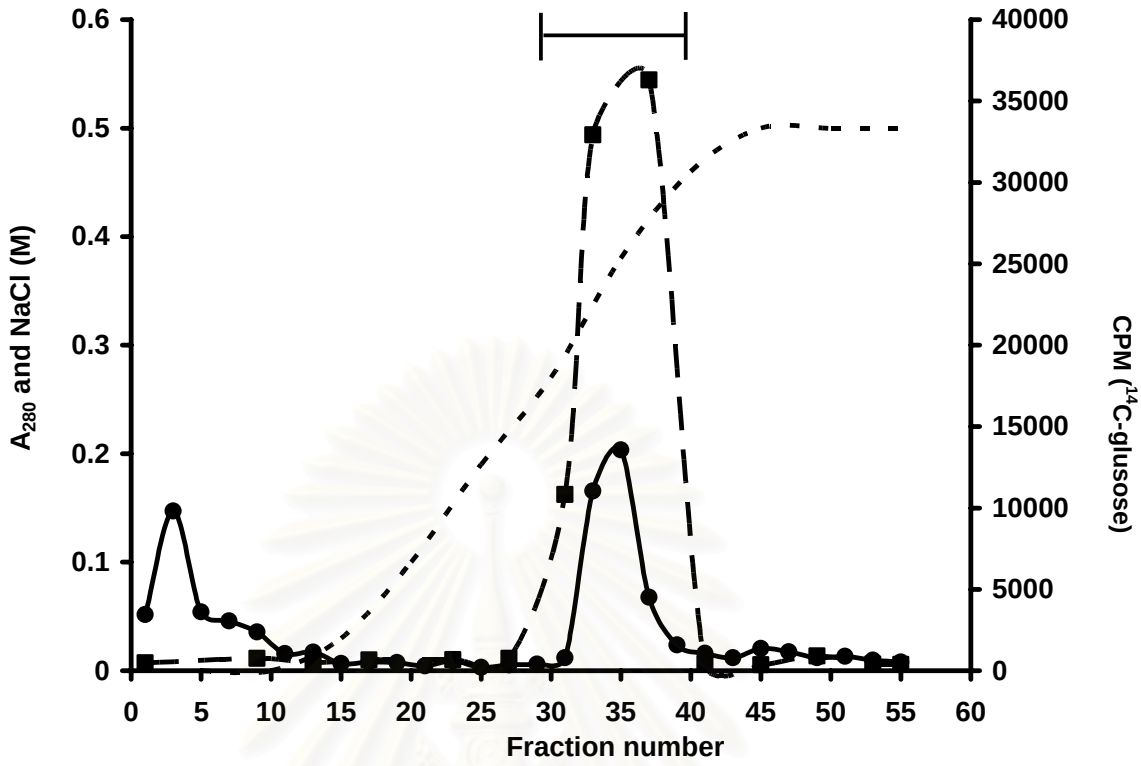


Figure 3.6 Hitrap Q-Sepharose chromatographic profile of SBE isoform 3 from second DEAE-Toyopearl. The enzyme was eluted with linear gradient of 0-0.5 NaCl. Fraction of 3 ml were collected.

● A_{280} ■ CPM ····· [NaCl]

Table 3.2 Purification table of cassava starch branching enzyme.

Fraction	Volume (ml)	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg protein)	folds
Crude	600	1920	200	0.104	1
PEG supernatant	650	1113	184	0.165	1.6
1st DEAE	195	265	200	0.75	7
Isoform 1					
2nd DEAE	325	172	1012	6	58
Hitrap Q-Sepharose	130	39	503.5	13	125
Isoform 2					
2nd DEAE	211	133	321	2.41	23
Hitrap Q Sepharose	97	50	400	8	77
Isoform 3					
2nd DEAE	227	118	587	5	48
Hitrap Q Sepharose	162	32	822	26	250

Activity unit : mmole of ^{14}C-glucose incorporated in 1 min at 37 °C

Specific activity : The activity unit per mg protein

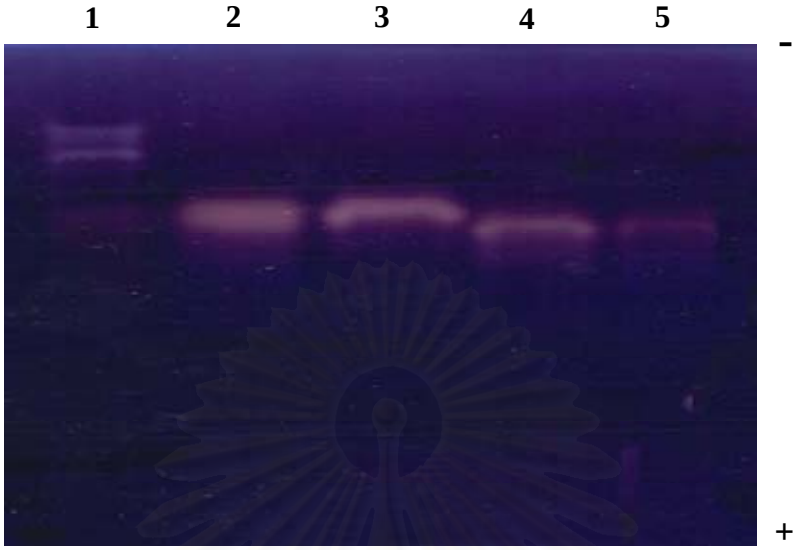


Figure 3.7 Activity staining of cassava SBE on starch polyacrylamide gel electrophoresis

Lane 1 Crude enzyme (25 units)

Lane 2 First DEAE Toyopearl (50 units)

Lane 3 Isoform 1 from Sephacryl HR S 200 (50 units)

Lane 4 Isoform 2 from Sephacryl HR S 200 (50 units)

Lane 5 Isoform 3 from Sephacryl HR S 200 (40 units)

3.3 Characterizations of starch branching enzyme

Each isoform of SBE purified from Hitrap Q-Sepharose column chromatography was used for the characterization studies.

3.3.1 Molecular weight Determination

3.3.1.1 Native molecular weight

Native molecular weight of the 3 isoforms were determined by chromatographies on Sephacryl HR S-200 column on FPLC. Standard proteins were loaded on the same column .All 3 isoforms were eluted at the same position and their molecular weight determined be 57,000 Dalton (Figures 3.8,3.9).

3.3.1.2 Molecular weight of SBE isoforms on SDS-PAGE

SBE isoforms were subjected to 10% SDS polyacrylamide gel electrophoresis as described in section 2.9.1.2. Standard proteins were run in parallel and a standard curve was constructed from their molecular weight and relative mobilities (Figure 3.10,3.11). Isoform 1 showed 2 major bands with molecular weight 108,000, 57,000 Daltons whereas isoform 2 and 3 showed one band with molecular weight 57,000 and 60,000 Daltons as determined from the standard curve.

3.3.2 Determination of pI of SBE isoforms

All 3 isoforms and standard pI marker protein were subjected to electrofocusing gel according to the method described in section 2.9.7. The result is shown in Figure 3.12. A pI standard curve was constructed from the pI's and distance migrated from cathode of the standard proteins (Figure 3.13). From the standard curve, SBE isoform 1,2 and 3 were found to have the pI's of 4.9,4.9 and 5.0, respectively.

3.3.3 Optimum pH for activity of SBE isoforms

Each isoforms was assayed at various pH's as described in section 2.8.1. The results were shown in Figure 3.14. The SBE showed highest activity at pH 8.0, 6.5 and 7.5 for isoforms 1,2 and 3 respectively. Below pH 5.5, activities of SBE isoforms 1and 2

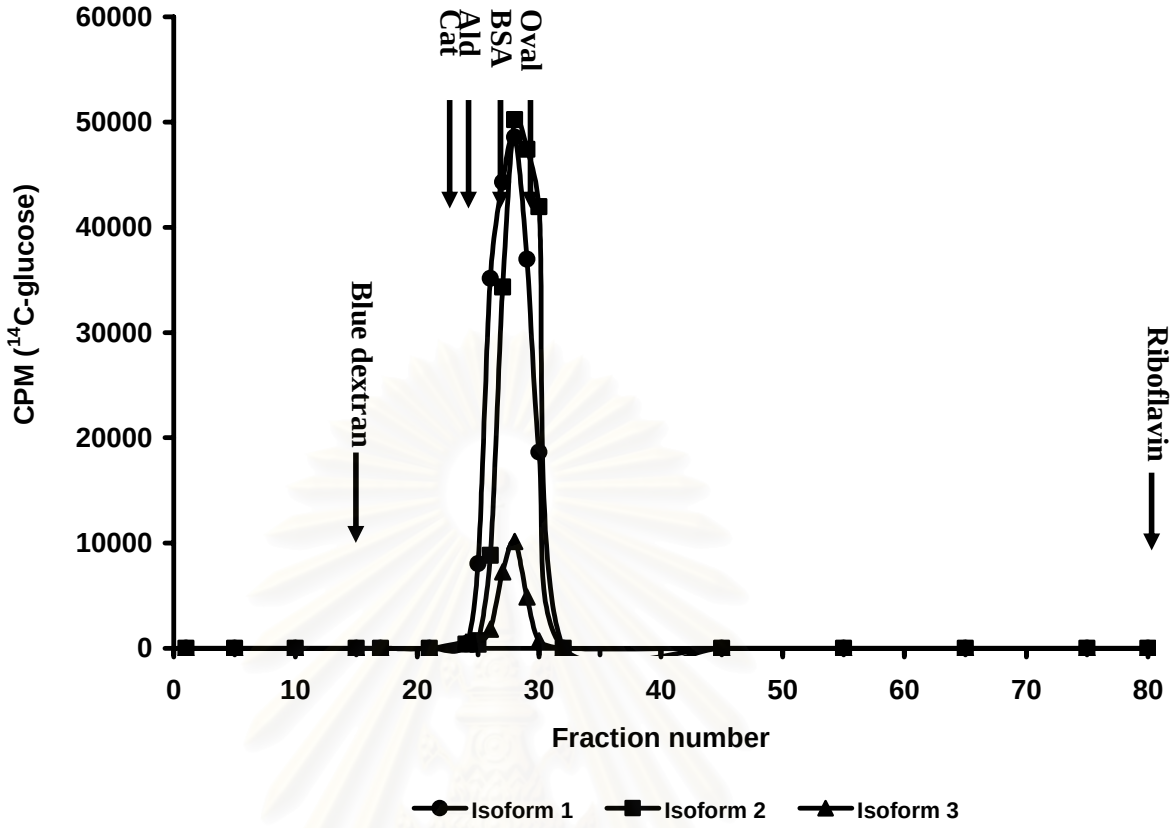


Figure 3.8 Sephacryl HR 200 chromatographic profiles of 3 SBE isoforms from Hitrap Q-Sepharose.

Cat = Catalase Mw = 232,000 Da

Aldo = Aldolase Mw = 158,000 Da

BSA = Bovine serum albumin Mw = 66,000 Da

Oval = Ovalbumin Mw = 43,000 Da

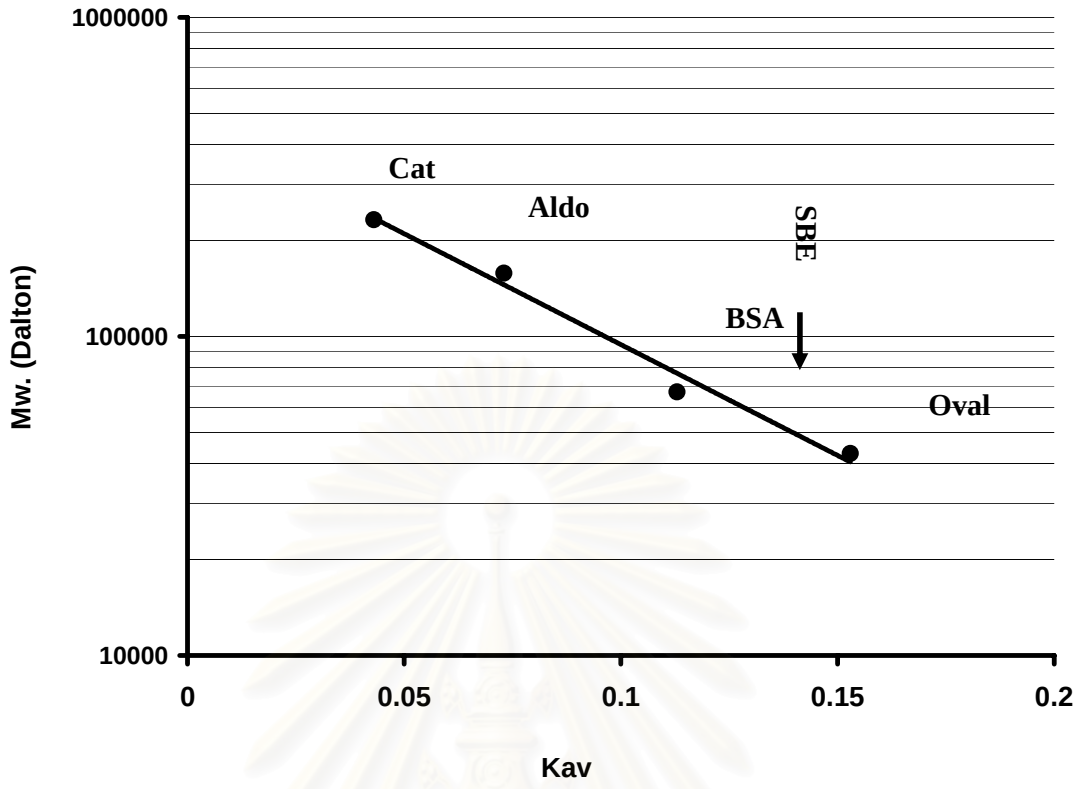


Figure 3.9 Calibration curve for native molecular weight determined by chromatography on Sephacryl HR 200 column.

Cat = Catalase Mw = 232,000 Da

Aldo = Aldolase Mw = 158,000 Da

BSA = Bovine serum albumin Mw = 66,000 Da

Oval = Ovalbumin Mw = 43,000 Da

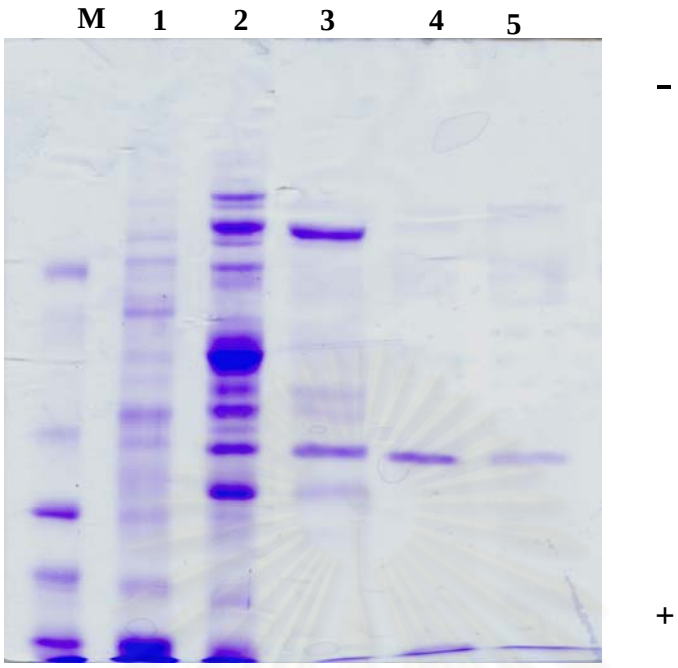


Figure 3.10 SDS-PAGE pattern of cassava SBE

Lane M Standard molecular weight proteins

Phosphorylase b (94 kDa)

BSA (67 kDa)

Ovalbumin (43 kDa)

Carbonic anhydrase (30 kDa)

Soybean trypsin inhibitor (20.1 kDa)

Lane 1	Crude enzyme	20	µg
Lane 2	First DEAE-Toyopearl	10	µg
Lane 3	Sephacryl HR S 200 isoform 1	5	µg
Lane 4	Sephacryl HR S 200 isoform 2	5	µg
Lane 5	Sephacryl HR S 200 isoform 3	5	µg

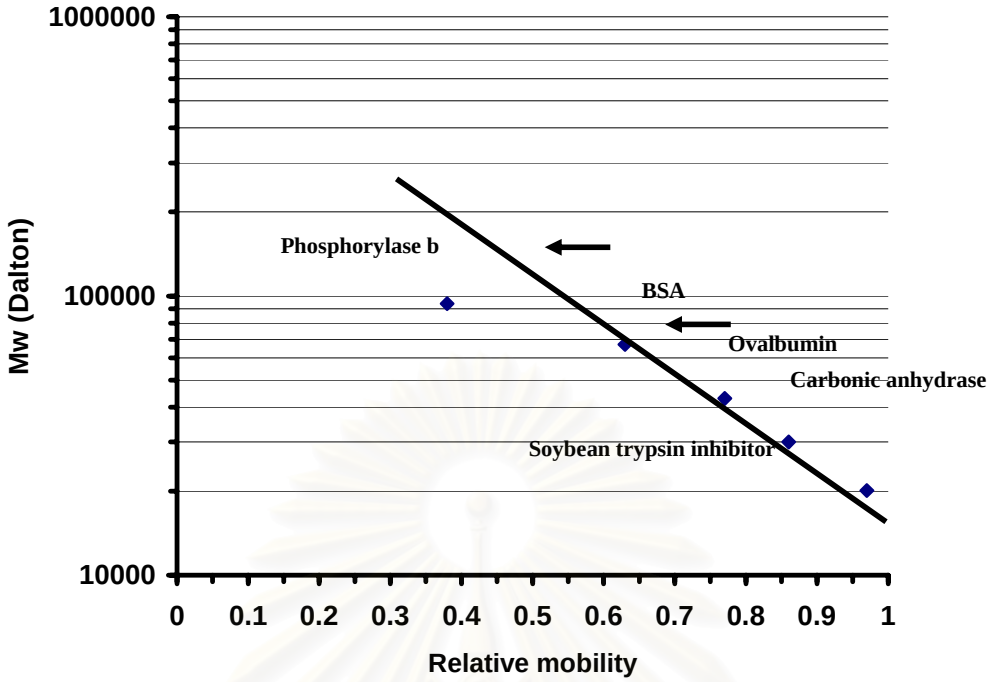


Figure 3.11 Calibration curve of molecular weight markers on SDS-PAGE.

The molecular weight of the standard markers were plotted against their relative mobility on 10% SDS-PAGE

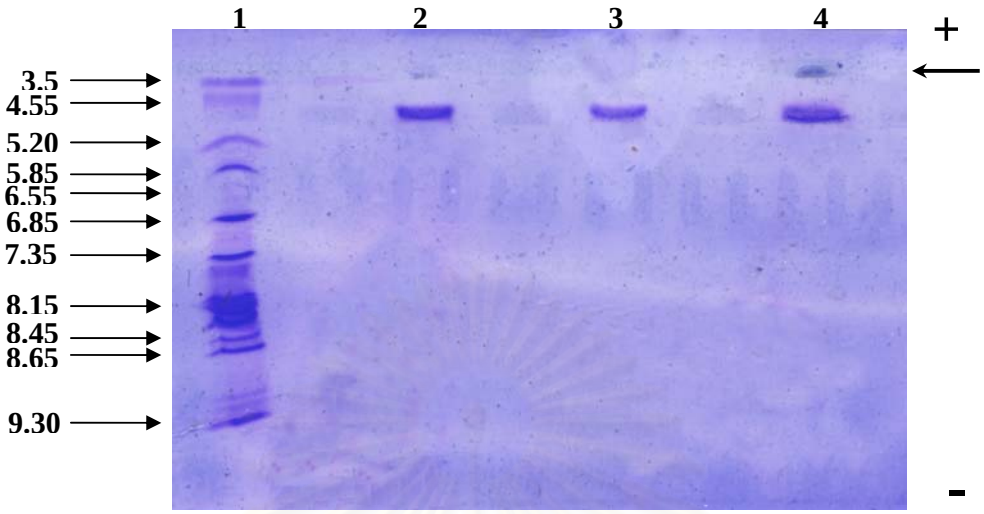


Figure 3.12 Isofocusing polyacrylamide gel electrophoresis of SBE isoform.

Lane 1 standard pI marker :

Amyloglucosidase	pI 3.5
Soybean trypsin inhibitor	pI 4.55
β-lactoglobulin	pI 5.20
Bovine carbonic anhydrase B	pI 5.85
Human carbonic anhydrae 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 purified cassava SBE isoform 1 5 μg

Lane 3 purified cassava SBE isoform 2 5 μg

Lane 4 purified cassava SBE isoform 3 5 μg

← Sample application

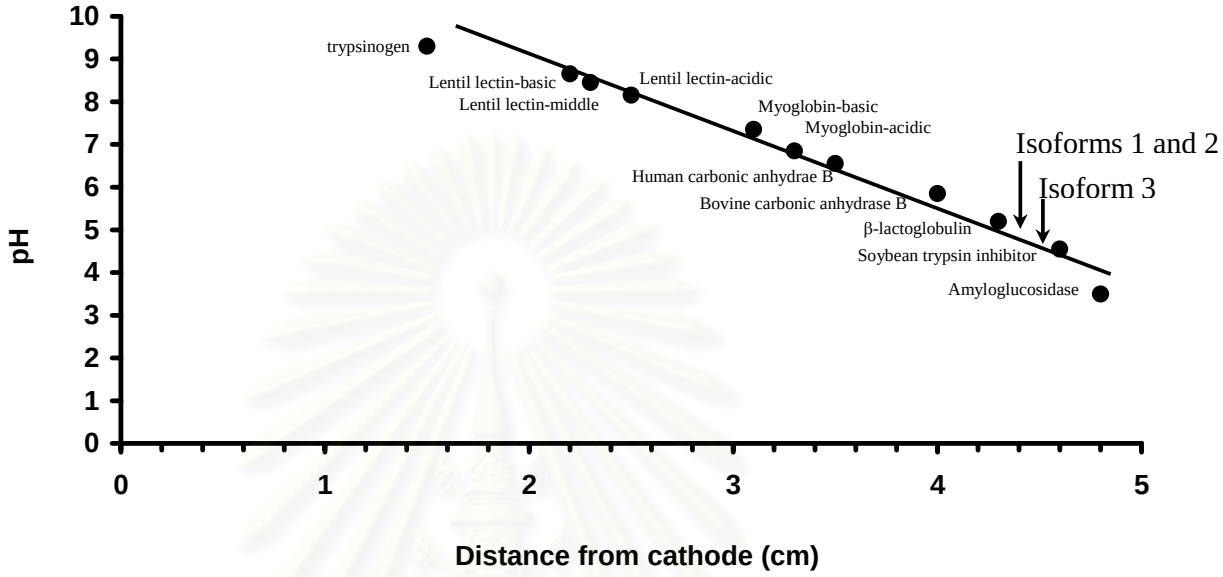


Figure 3.13 Calibration curve of standard pI markers.

The migration distance from cathode of the pI standard markers were measured and plotted against pI.

decreased to less than 50% of optimum pH, while activity of isoform 3 decreased to about 70% , isoforms 2 and 3 retained activity over 50% at pH 8.5

3.3.4 Optimum temperature for activities of SBE isoform

The isoforms of SBE were assayed at different temperatures as described in section 2.8.2. The result is shown in Figure 3.15. Isoform 3 showed highest activity at 30 °C while isoforms 2 and 3 at 37 °C. At 40°C, all isoforms retained more than 60% of then activities. At 5 and 45 °C, the SBE activities of all isoforms decreased to less then 50% and absolute loss of activities occurred at 50 °C.

3.3.5 Temperature stability of activities of SBE isoform

Each SBE isoform was tested for its stability at various temperatures according to the method described in section 2.8.3. It was found that all 3 isoforms showed similar stability profiles and were quite stable at 25, 30 and 37 °C at which more than 80% of the activities were retained after incubation for 48 hours. At 40 °C, more than 50% of activities of all isoform were still retained. At 50 °C, all isoforms completely lost their activities after 15 hours (Figure 3.16).

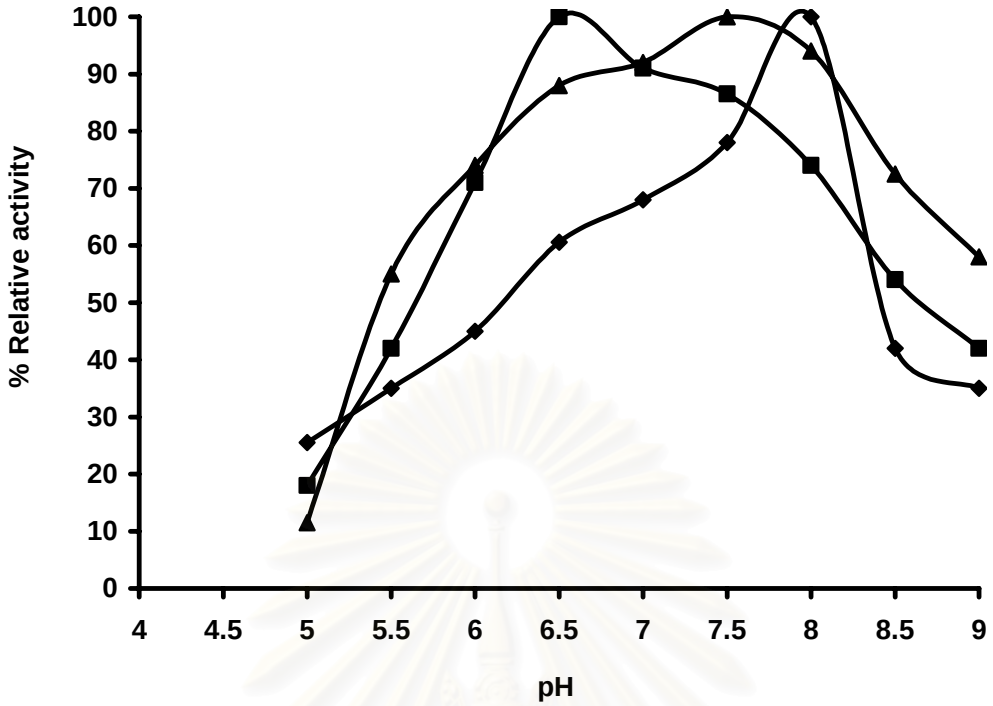


Figure 3.14 Effect of pH on activity of SBE isoforms.

The experiment was performed at various pH in universal buffer, calculations were performed using activities at pH 8, 6.5 and 7.5 as 100% of isoform 1, 2 and 3, respectively.

◆ = isoform 1 ■ = isoform 2 ▲ = isoform 3

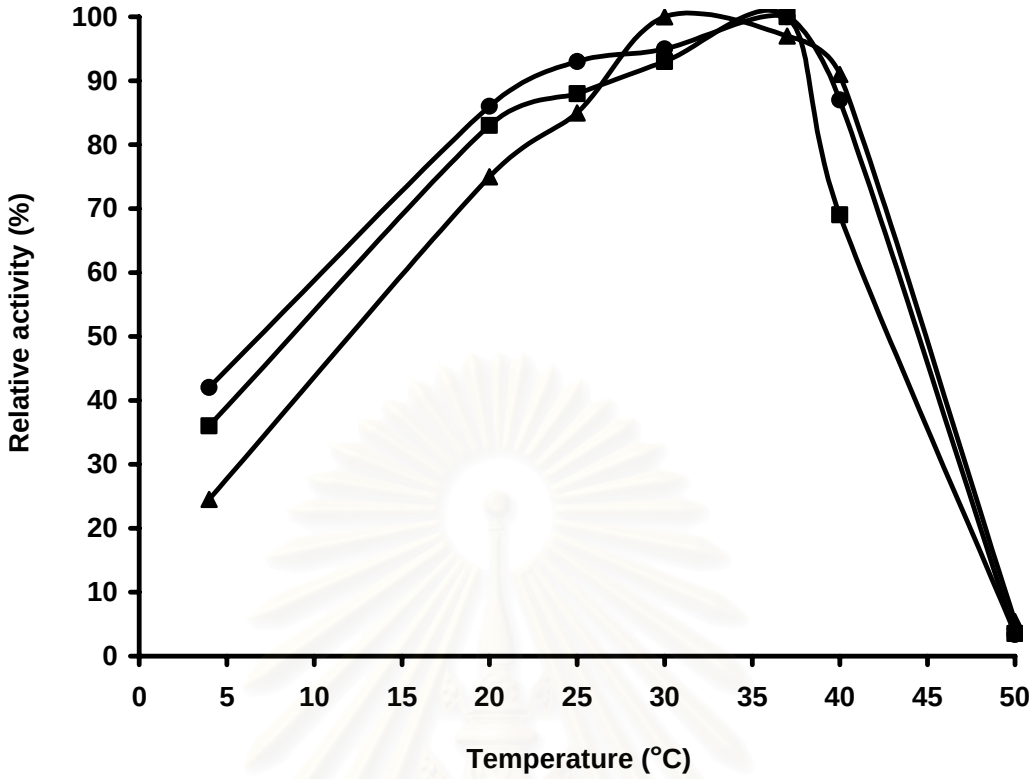


Figure 3.15 Effect of temperature on activity of SBE isoform.

The experiment was performed at various temperatures calculations were performed optimal temperature with activities at 100%

● = isoform 1 ■ = isoform 2 ▲ = isoform 3

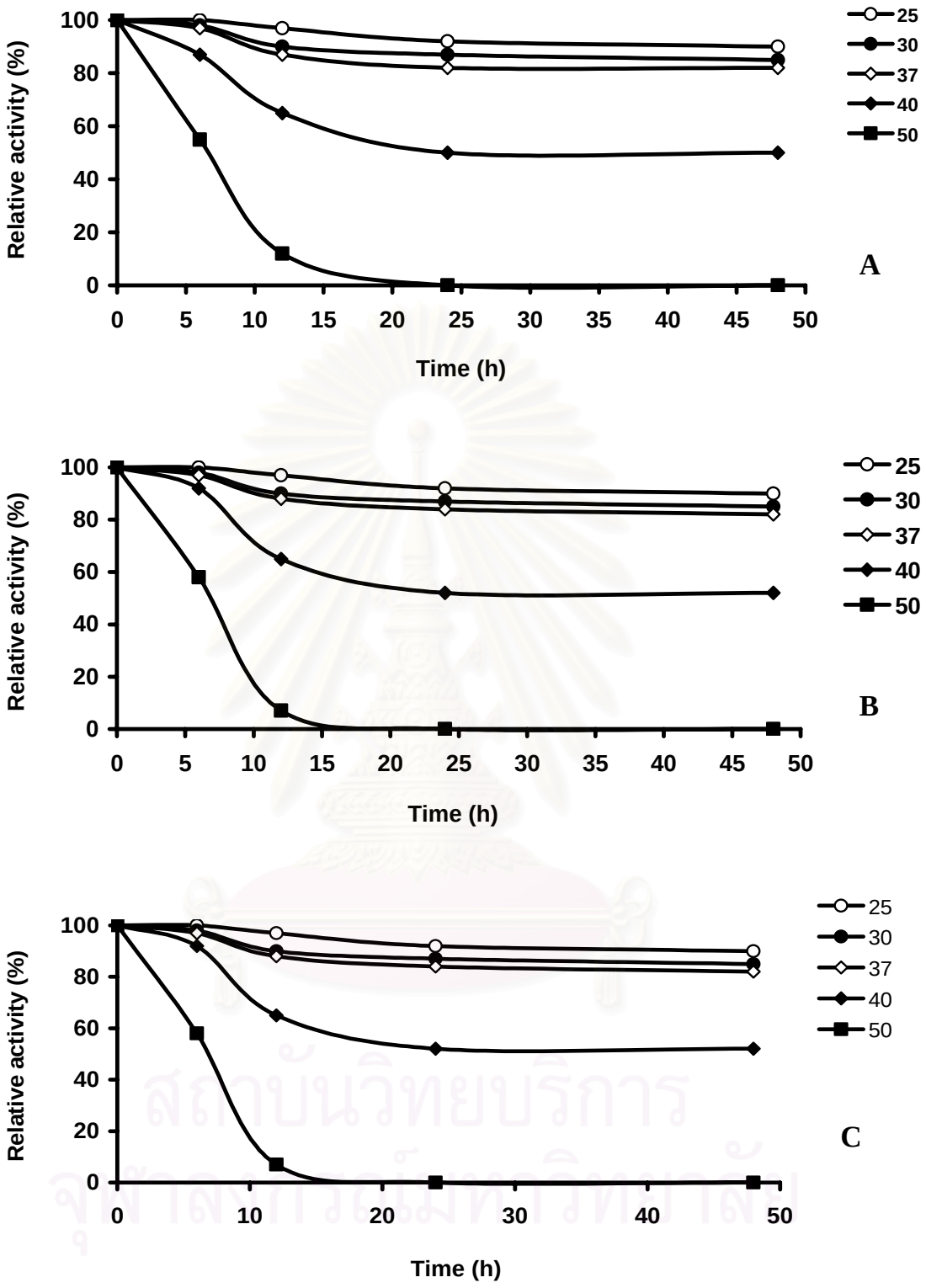


Figure 3.16 Temperature stability of SBE activities.

Each isoform was assayed for its remaining activity after incubation at various temperatures for 0-48 hours as described in section 3.34

A = isoform 1 B = isoform 2 C = isoform3

3.3.6 Effect of sulfhydryl reagent on starch branching enzyme activity

Cassava SBE was assayed with added sulfhydryl reagents to determine the effect SH-group on its activity as described in 2.8.5. The results are shown in Figure 3.17. All sulfhydryl reagents test at 2, 5 and 10 mM shown no observable effect on enzyme activity.

3.3.7 Effect of divalent metals ion on starch branching enzyme activity

Cassava SBE was assayed with added divalent metal ion at 1, 5 and 10 mM to determine the effect of divalent ions on its activity as described in 2.8.6. The divalent ion at 1 mM were absolutely inhibitory on activity of all isoforms (Table 3.3)

3.3.8 K_m and V_{max} of SBE isoforms with amylose as substrate

Cassava SBE was assayed with amylose as substrate at 37 °C for 30 minutes as described in 2.8.5. Figure 3.18, 3.19 and 3.20 showed the saturation curves and Liweaver-Burk plot of isoform 1, 2 and 3, respectively. At very high substrate concentrations, the reaction rate of all isoform did not showed characteristic saturation curve but dropped gradually with in creasing amylose concentration after saturation was briefly attended. From Lineweaver-Burk plot, K_m for amylose were calculated at 1.12, 1.37 and 2.17 mg/ml and the V_{max} were 1.03, 0.83 and 0.57 ΔA_{660}/30 min. for isoforms 1,2 and 3, respectively

3.3.9 Comparison of SBE isoform activity with the amylose and amylopectin substrates

To compare the substrate specificity, corn amylose and amylopectin were used as substrates and assayed as described in 2.8.9. The results were shown in Figure 3.21. Isoforms 1 and 2 utilized amylose better than isoform 3 (Figure 3.21 A) while isoform 3 was more active on amylopectin (Figure 3.21 B).

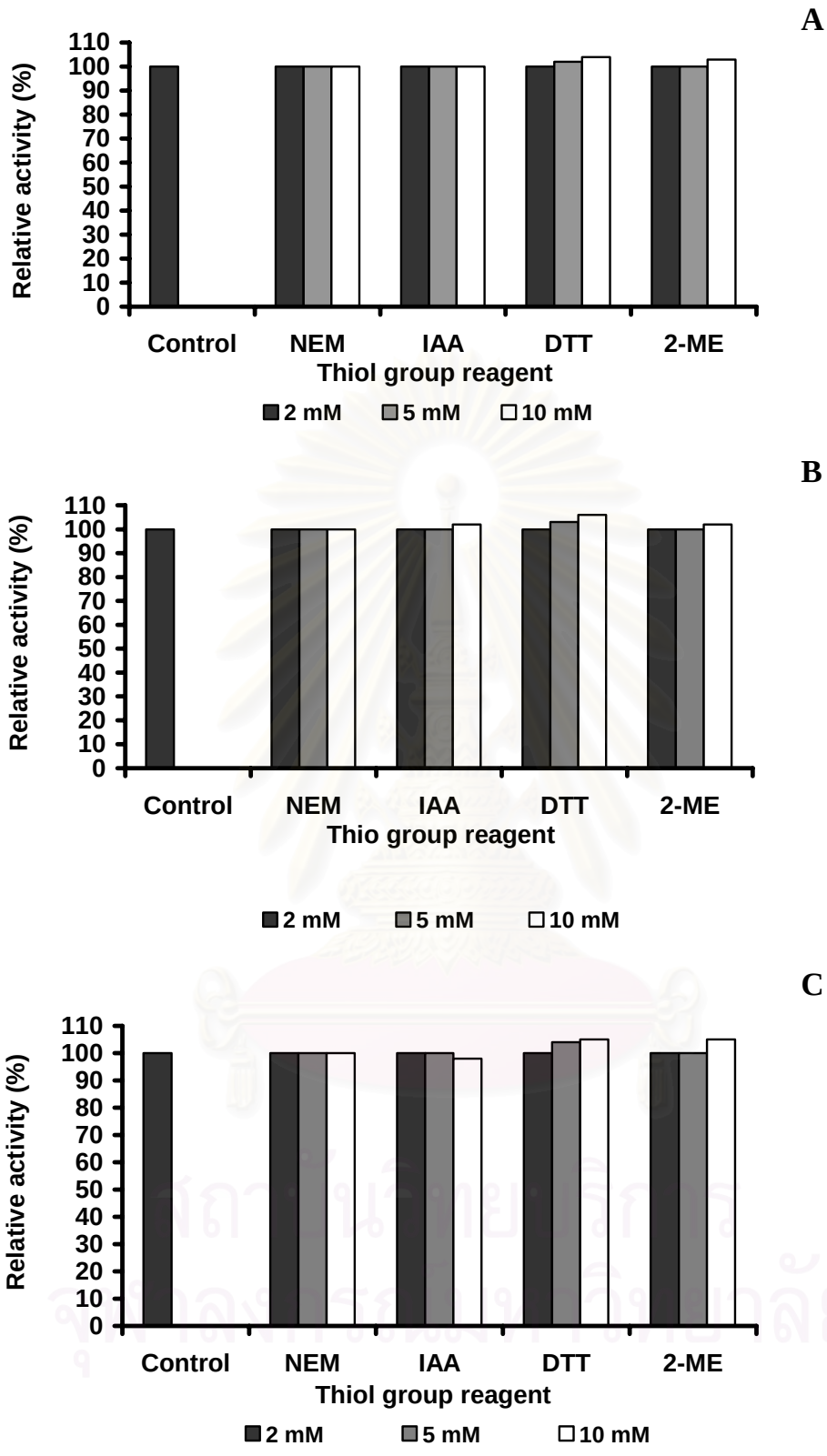


Figure 3.17 Effect of Sulfhydryl group reagent

A) Isoform 1 B) Isoform 2 C) Isoform 3

Table 3.3 Effect of divalent metal ions on SBE

Metal ion	**Relative activity (%)**								
	Isoform 1			**Isoform 2**			**Isoform 3**		
	1 mM	**5 mM**	**10 mM**	**1 mM**	**5 mM**	**10 mM**	**1 mM**	**5 mM**	**10 mM**
Co^{2+}	0	0	0	0	0	0	0	0	0
Cu^{2+}	0	0	0	0	0	0	0	0	0
Hg^{2+}	0	0	0	0	0	0	0	0	0
Mn^{2+}	0	0	0	0	0	0	0	0	0
Ni^{2+}	0	0	0	0	0	0	0	0	0
Zn^{2+}	0	0	0	0	0	0	0	0	0

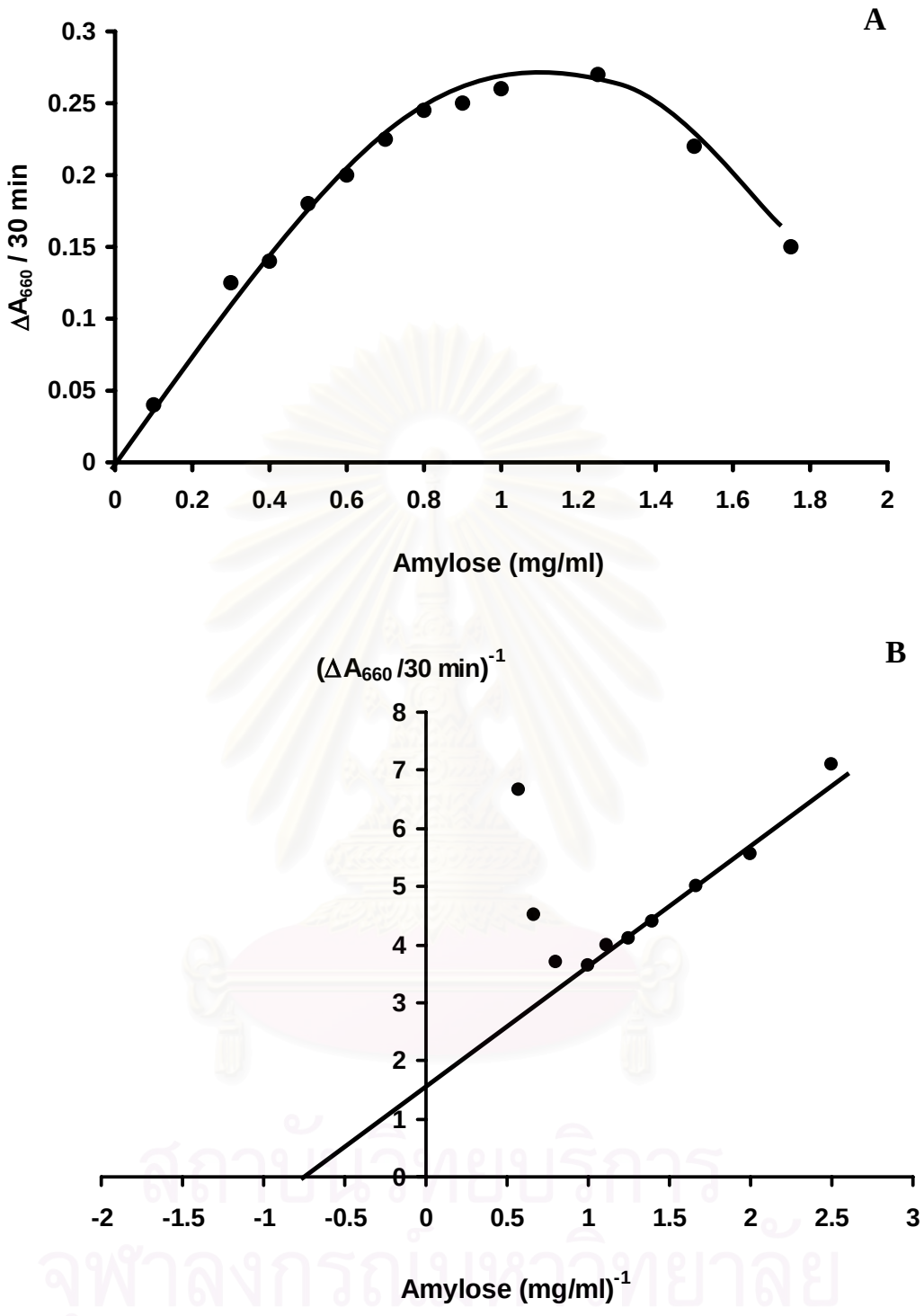
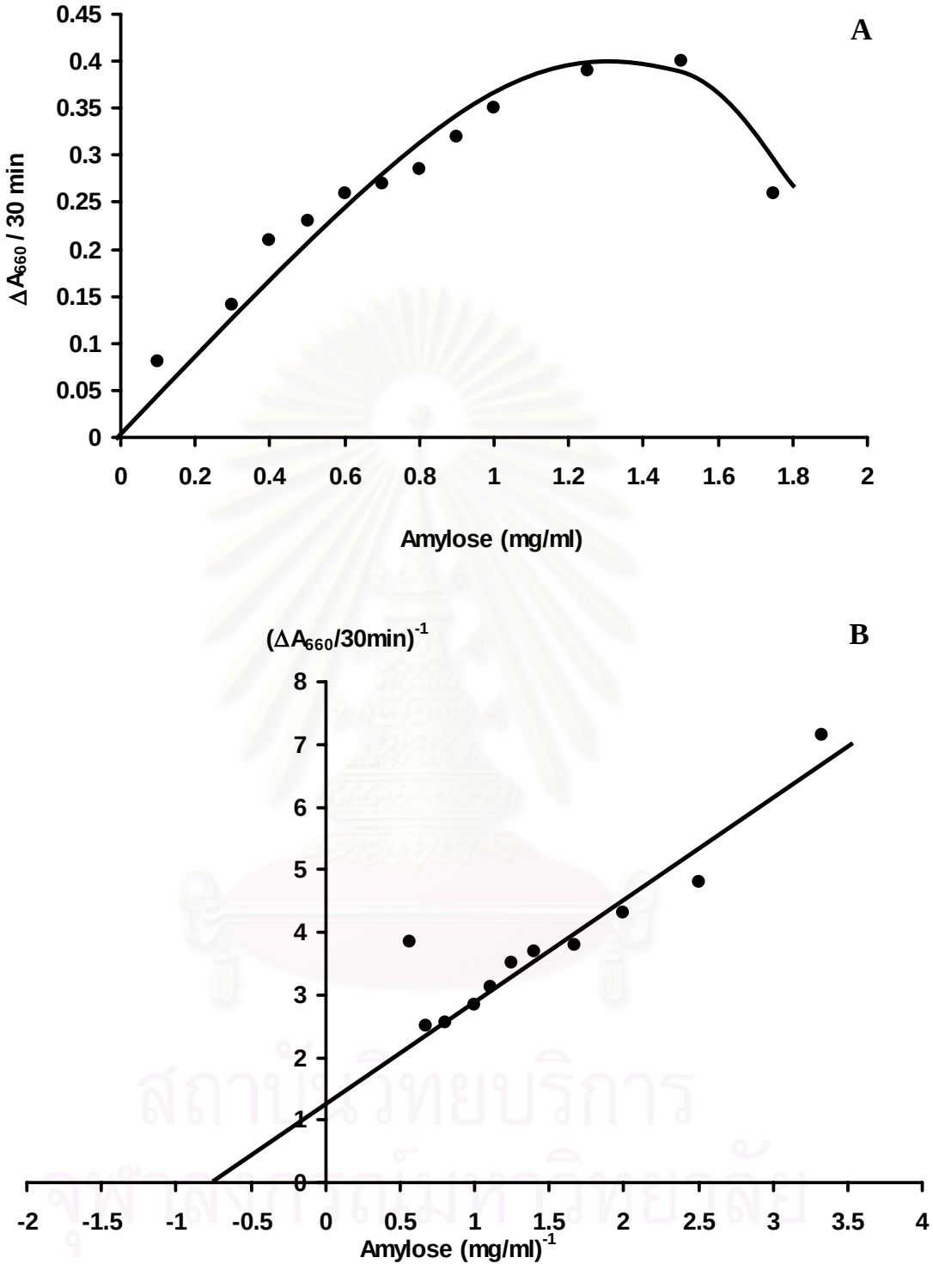


Figure 3.18 Kinetic studies of SBE isoform 1 activity with amylose as substrate

A) Saturation curve

B) Lineweaver-Burk plot of amylose for SBE activity in isoform 1



igure 3.19 Kinetic studies of SBE isoform 2 activity with amylose as substrate

A) Saturation curve

B) Lineweaver-Burk plot of amylose for SBE activity in isoform 2

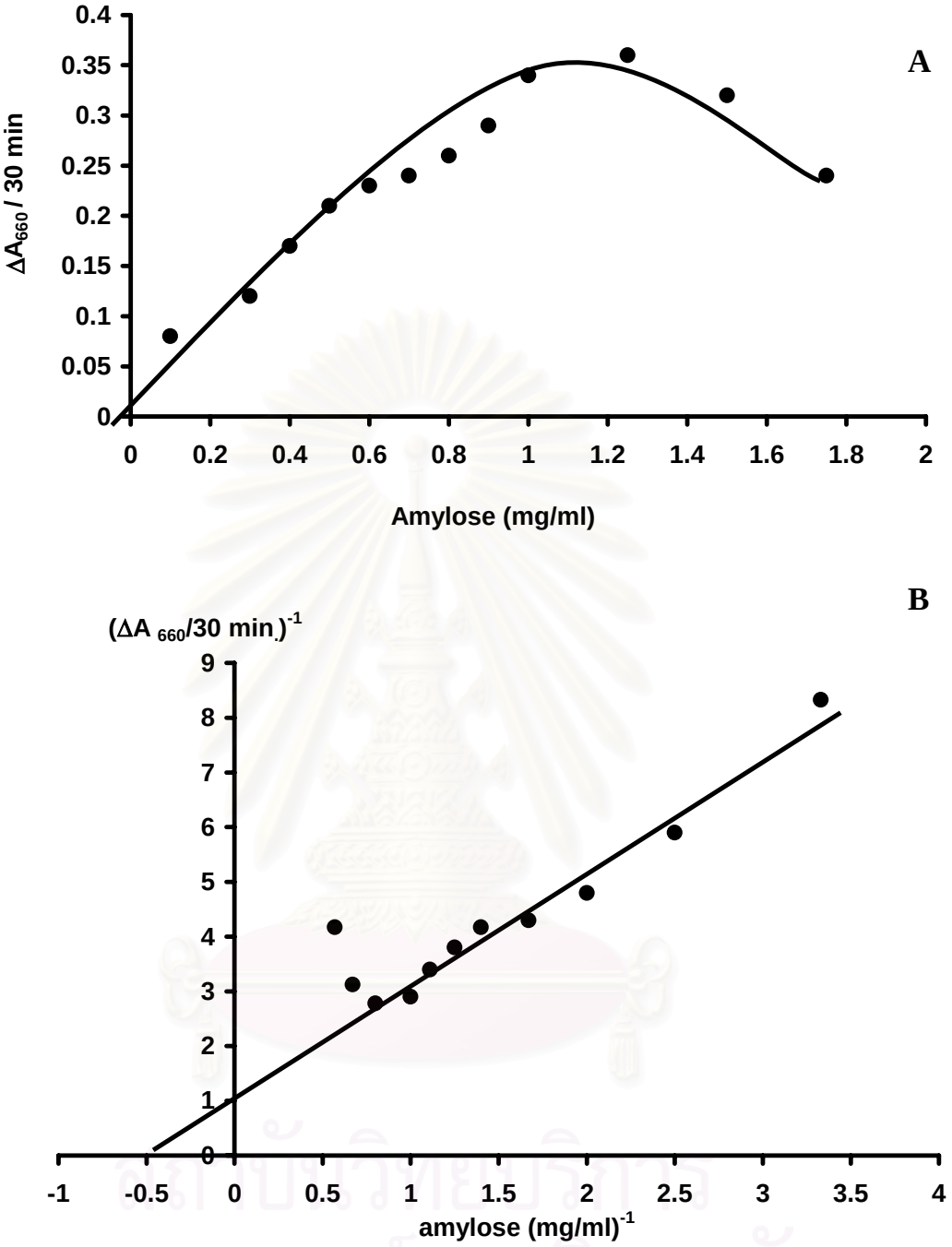


Figure 3.20 Kinetic studies of SBE isoform 3 activity with amylose as substrate

A) Saturation curve

B) Lineweaver-Burk plot of amylose for SBE activity in isoform 3

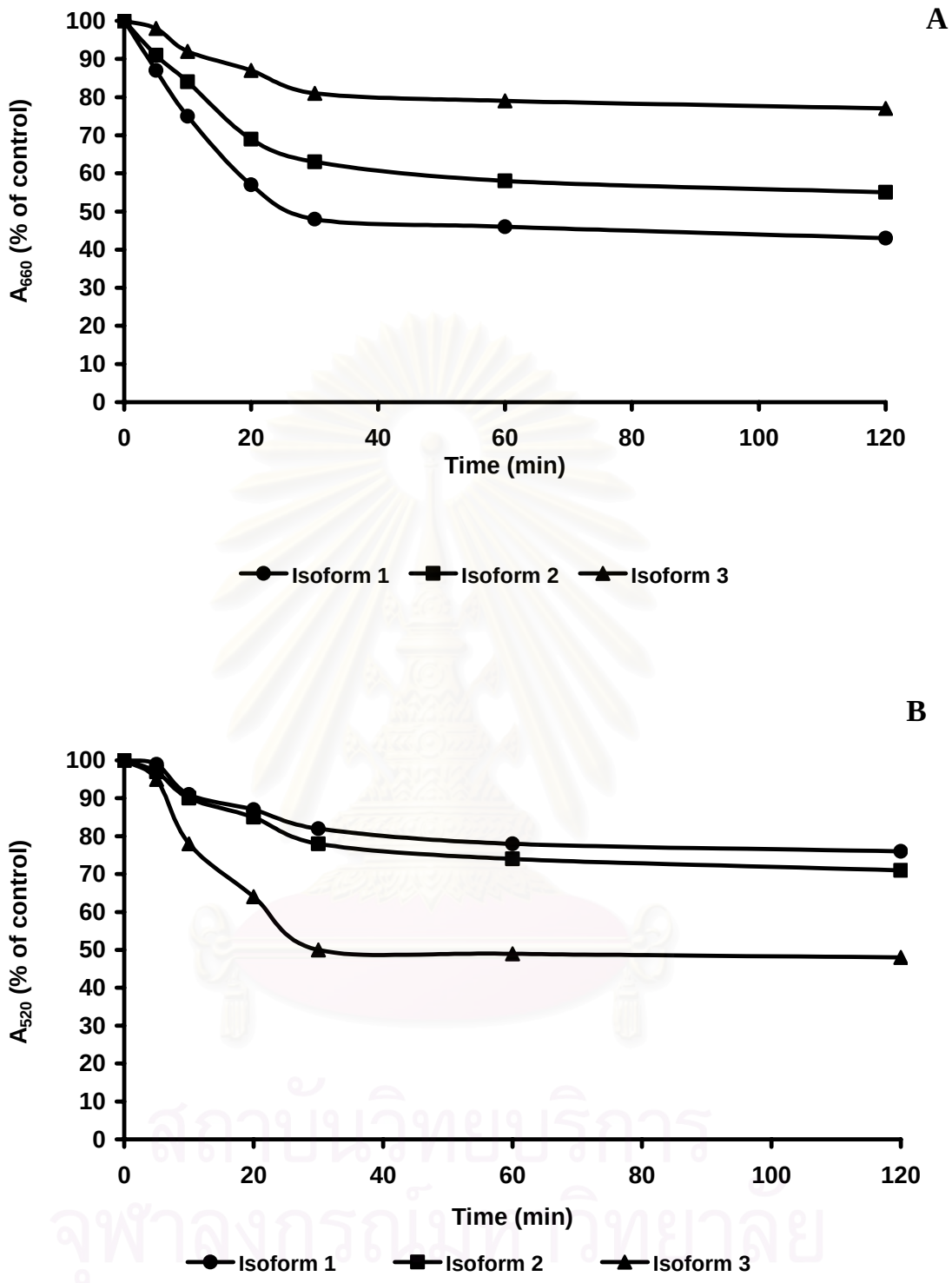


Figure 3.21 Activity of SBE isoforms on amylose (A) and amylopectin (B)

100 units of each isoforms were used in each experiment.

CHAPTER 4

DISCUSSION

Cassava is one of the most economically important crops in Thailand that are produced in large scale and most of it is exported. However, cassava needs to be increase in qualities and quantities of starch to add more value to its products. One way of approach is by way of manipulation in breeding to give rise to novel cultivars. In Thailand, there have been many cultivars being developed for examples Rayong 1, Rayong, 3 Rayong 60 and KU 50 that yield different content of starch. Biochemically, starch synthesis is one of the important key to improve starch quality. Several studies on the enzymes involving in starch synthesis such as starch synthase and starch branching enzyme have been performed in maize (28) but only a few reports in cassava. This thesis concentrated on the understanding of isoforms of starch branching enzyme in cassava tuber which is one of the enzymes involved in amylopectin synthesis and starch quality.

4.1 Assay methods for SBE

In the study of starch branching enzyme, it was necessary to select a method that would allow monitoring of enzyme activity. There are at least 2 methods of assaying SBE activity.

4.1.1 Radioactive method

Activity of starch branching enzyme can be assayed by measuring the incorporation of ^{14}C-glucose into α-D-glucan synthesized by stimulation with rabbit-muscle phosphorylase a. This method was modified from the method described by Mizuno *et a l.*(33)

Rabbit muscle phosphorylase a functioned in transferring ^{14}C-glucose from ^{14}C-glucose-1-phosphate into α-D-glucan. In this procedure, the ^{14}C-glucose was incorporated in α-D-glucan by 1,4 linkage. SBE synthesized α-1,6-glucosyl-α-1,4-glucan

from α-D-glucan. Finally, α-1,6-glucosyl-α-1,4-glucan could be primed with glycogen which enhanced precipitation of the radioactive glucans by methanol (Figure 4.1). The amount of ^{14}C-glucose in the precipitate can be measured and converted to SBE activity. This method was sensitive and can detect low level of SBE. However, it was expensive using radioactive and the experimentation was tedious. In addition it involved more than one enzyme. This rendered the method unsuitable for kinetic studies.

4.12 Spectrophotometric method

The assay was based on the spectral difference of the glucan-iodine complex that occured with branched and unbranched glucan substrate. The amylose interacted with iodine to form amylose-iodine complex which have λ_{max} at 640. SBE changed amylose to amylopectin by creating branches in the molecule, resulting in decrease of A_{660}. If amylopectin was used, amylopectin-iodine complex has λ_{max} at 550 nm (Figure 4.2).

From preliminary test comparing the above methods, it was found that radioactive assay was suitable for monitoring activity of SBE in purification steps because of its sensitivity. However, it was not suitable for some characterizations and kinetic studies because the method also involved another enzyme (Phosphorylase a). Effects of pH, temperature, chemicals may resulted from the effects on phosphorylase a which would lead to false interpretation of results.

Therefore, spectrophotometric assay was the assay method used for characterizations of SBE on account that it directly measured purified SBE activity. However, this method may

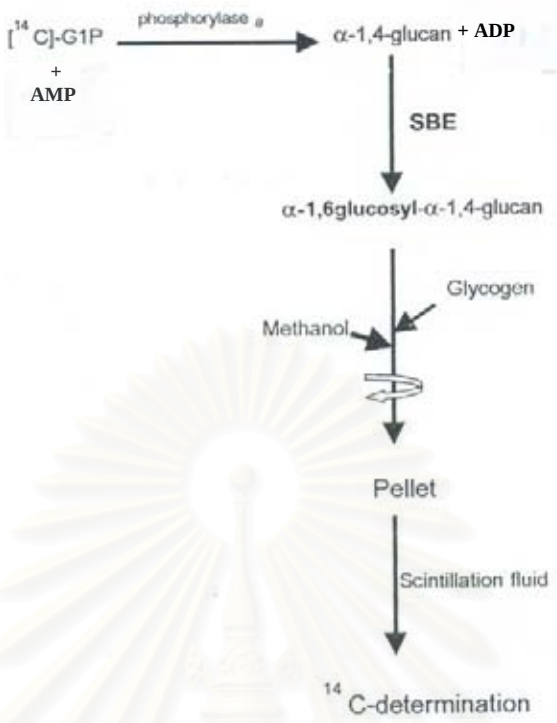


Figure 4.1 Assay of SBE activity by radioactive assay.

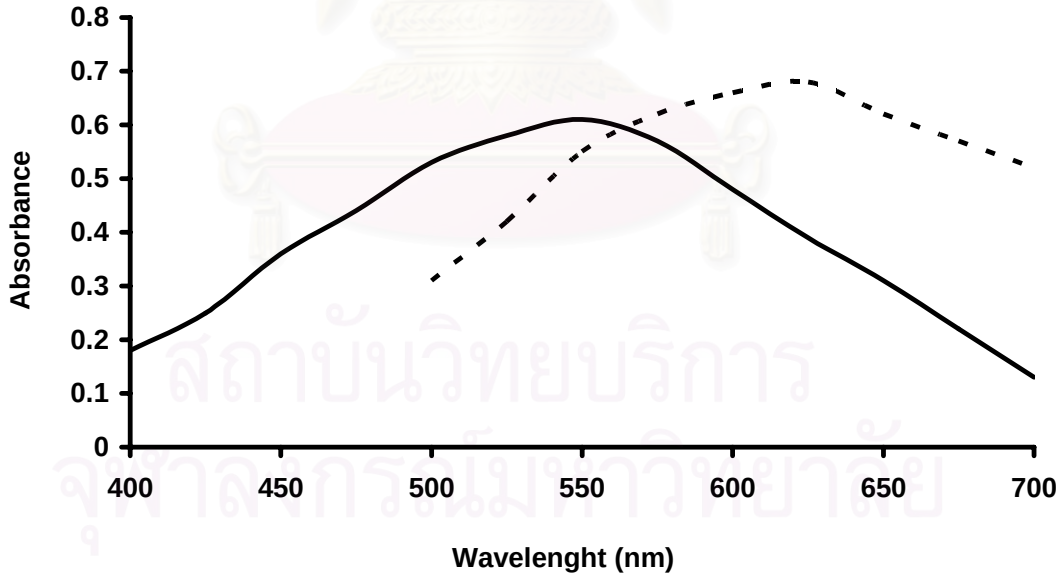


Figure 4.2 Spectrum of amylose-iodine complex and amylopectin-iodine complex.

Amylopectin ····· Amylose

give false results in presence of other carbohydrate hydrolyzing enzymes; Therefore, it is suitable only for assay purified SBE.

4.2 Starch branching enzyme activity during growth of cassava tubers in cultivars Rayong 1 and KU 50

Two cultivars of cassava bred in Thailand, Rayong 1 and KU 50 were selected for monitoring SBE activity in their tubers during growth at ages up to 12 month. Tuber were harvested at 3 months intervals. Rayong 1 has low starch content but is tolerant to extreme environmental of conditions: seasons, soil and pesticide. The KU 50 is a recently bred cultivar with high starch content. SBE activity was found to increase with age whereas the amount of protein per tuber mass decreased. SBE when expressed as specific activity and activity per tuber mass increased rapidly between 6 to 9 months in both cultivars. The decrease in protein per tuber mass and increase in SBE activity per tuber mass or specific activity during growth may reflect that the increase in tuber mass during growth was the contribution of increasing amount of starch being produced by the increasing starch synthesizing enzymes.

4.3 Isolation and purification of isoforms in SBE

Several steps were employed to purify SBE from cassava tuber. The first step of purification involved the use of polyethylene glycol 6000 to precipitate other contaminating proteins especially amylose and starch synthase (Figure 4.3)(23). In our experiment, 10% PEG can remove 834 mg proteins.

In our experiment, it was found that 10% PEG removed about 40% of total protein while 90% of SBE was retained. Specific activity of SBE activity was shown to increase by 1.6% after this step (Table 3.1). Although, this step did not give high purification but is was an essential step because of the removal of other starch metabolizing enzymes.

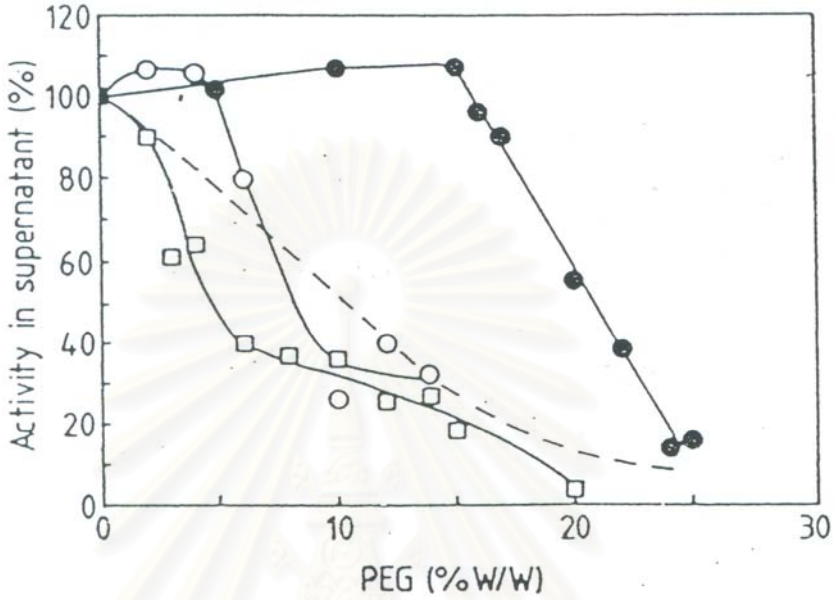


Figure 4.3 Precipitation curves of protein in potato juice with polyethylene glycol (22)

····· Protein; ● Starch branching enzyme

□ Starch synthase; o α-amylase

Two step of ion exchange column chromatography on DEAE-Toyopearl were performed.

In preliminary experiment which SBE was chromatographed on DEAE-Toyopearl and eluted with NaCl gradient, the activity peak showed appearance of possible existence of 3 isoforms. Several adjustment of NaCl gradient did not successfully separate the isoforms. It was decided to run the first column as a step to get rid of contaminating protein and separated the pooled SBE peak on a second DEAE-Toyopearl column using step wise elutions with different NaCl concentrations. The second DEAE-Toyopearl column yield good separation of 3 SBE isoforms. The poor separation of SBE isoforms on the 1st DEAE-Toyopearl column may be due to the effect of contaminating PEG in the sample. DEAE-Toyopearl was found to have more resolving power than other DEAE resin like DEAE-Cellulose (fibrous form). Previous experiments using DEAE-Cellulose (Fibrous form) to separate SBE isoforms were not successful (data not shown). This kind of resin were recently becoming popular as a purification tool (40,41,42)

Hitrap Q-sepharose, a strong ion exchange column with gel filtration matrix further enhanced purity of each isoforms. The strong anionic resin removed some of the protein bound to DEAE-Toyopearl and co-eluted with SBE as unbound proteins.

From each column chromatography used in purification of SBE isoform, there were some interesting observation on the total activities obtained (Table 3.2). Firstly, the pooled fraction which should consist all three isoforms contained 200 units of SBE activity. When all isoforms were separated and purified through Hitrap Q-sepharose column, the sum of total activities of all isoforms amounted to 1326 units which was six folds of the crude enzyme. Secondly, each isoforms showed similar increase in total activity after Hitrap Q-Sepharose step: 2.5 folds, 2 folds and 4 folds for isoforms 1,2,3 respectively. The possible explanation was existence of some inhibitory compounds in crude enzyme which were removed through column chromatographies led to the enhanced SBE activities observed. Isoform 1, however, showed a peculiar 5 folds increase in second DEAE-Toyopearl and lost

half of this later through the Hitrap Q-Sepharose column. Profiles of second DEAE-Toyopearl (Figure 3.3) showed that there was overlapping of the peaks of isoform 1 and isoform 2. The pooled isoform 1,therefore, also contained some portion of isoform 2 which was possibly removed in Hitrap Q-sepharose step. Thus, the drop of isoform1 activity may be caused by the removed contaminated isoform 2 removed. Ion exchange chromatographies on DEAE-Toyopearl and Hitrap Q-Sepharose were performed in Tris-HCl buffer pH 7.5 and SBE activities were all in bound fractions, indicating their pI's were lower than 7.5. This was confirmed by the electrofocusing experiment of which the pI's of all isoform were 4.9-5.0. From 4 step of purification, there isoforms were purified to 125, 77 and 250 folds, respectively. All of the 3 purified isoforms showed characteristics reddish bands which is specific for SBE stained with I_2 (38)

4.3 Characterization of SBE isoforms

4.3.1 Molecular weight and pI determination

Native molecular weight of all isoforms appeared to be the same at 57 kDa by gel filtration on sepharose HR S 200 column run by FPLC. On SDS-PAGE, isoform 1 showed 2 bands at 108 kDa and 57 kDa. Although the band at 108 kDa was approximately 2 times at 108 kDa and 57 kDa it was unlikely an aggregate of 2 molecules of the 57 kDa unit since no activity peak was detected in Sephacryl S-200 column. Since 57 kDa was shown by Sephacryl HR S-200 column to be the native molecular weight. Isoforms 2 and 3 showed single band at 57 kDa and 60 kDa. Table 4.1 summarized the reported molecular weights and other properties of SBE in other plants. The only report on purified cassava SBE was by Aroonrungsawadi in 2000 (30) in 5 minutes cultivars which reported one SBE with molecular weight of 160 kDa with 2 identical subunit of 80 kDa and pI 5.4. Several plants were also reported to have isoforms of almost the same molecular weight. Most SBE seemed to have

molecular weight determined by SDS-PAGE in the range 70-90 kDa with one report on the smaller molecular weight of 40 kDa in rice.

Of all the characterized SBE shown in Table 4.1, pI's were not reported. Electrofocusing of the SBE isoforms purified in our study show that isoform1 and 2 were focused at the same pI of 4.9 while isoform 3 showed slightly different pI of 5.0

Variation in size of SBE in different plants were observed (Table 4.1) but size of isoforms in each plant seemed to be quite close. Therefore, our observation that cassava SBE isoforms we have isolated had the same molecular weight complied with previous observation. These isoforms may differ only in some amino acid sequence or if there is any size difference, it was too small to detect.

4.3.2 Effect of pH and temperature on SBE activity

Each isoform was incubated in various pH's and its activity measured. It was found that isoforms 1,2 and 3 were most active at pH 8.0,6.5 and 7.5, respectively, and the activity decrease about 50% at pH lower than 6.0. When the enzyme was incubated at various temperatures, the activity showed the highest temperature at 30°C for isoform 3 and 37°C for isoforms 1 and 2, after which the enzyme activity dropped steadily to lower than 50% in all isoforms. When stability of all isoforms were studied by incubation up to 48 hours at 25-50°C, it was found that all isoform still maintained more than 80% of their activities in the temperature range 25-37°C. At 40°C, activities of all isoform steadily declined during the first 20 hour then maintained at 60% activity up to 48 hours. At 50°C, all isoforms completely lost their activity after 15 hours. Therefore, SBE should be kept around 25-37 °C when carry out experiments. The assay should be performed at pH 7-8 and 30-37°C. In other plant, the optimum pH and temperature of SBE differ for different isoform (Table 4.1).

Table 4.1 Comparative properties of starch branching enzymes from various plants.

	Isoform	Mw* (kDa)	Native molecular weight (kDa)	pI	pH optimum	Temperature optimum	Reference
Rice	QE-1 QE-II	40 85	-	-	-	-	34
Wheat	WBE-I WBE-II	88 80	-	-	-	-	35
Maize	BE-I BE-IIa BE-IIb	70-90	-	-	-	33 °C 25 °C 12-20 °C	36
Kidney bean	KBE1 KBE2	80 72	-	-	7.0	25-30 °C	37
Potato	SBE-I SBE-II	95	-	-	7.5	-	23
Cassava (Five minute)	SBE	80	160	5.4	-	37 °C	30
Cassava (KU 50)	SBE 1 SBE 2 SBE 3	108,57 57 60	57 57 57	4.9 4.9 5.0	8.0 6.5 7.5	37 °C 37 °C 30 °C	-

* The value determined by SDS-PAGE

4.3.3 Effect of sulfhydryl reagents and divalent ions on activity of starch branching isoforms

The purified enzymes was incubated with thiol reagents, IAA, NEM, DTT and β-2 Mecaptoethanol, it was found that non significant effected on SBE as a result it has not SH- group related active site but the sulfhydryl reagent such as DTT is stabilizing agent which protect SH- group from oxidizing agent of conformation of enzyme (22).

Metal ion have been reported to affect enzyme activities either activate or inhibit. Of all the divalent ions tested, all SBE isoforms were inhibited even at 1 mM.

4.3.4 Kinetic constants of SBE

Starch branching enzyme employed amylose as main substrate. Experiments were carried out to investigate the K_m and V_{max} for amylose of each isoform (Figures 3.18, 3.19 and 3.20). The K_m constant was 1.12, 1.37 and 2.17 mg/ml and V_{max} were 1.03,0.83 and 0.57 ΔA_{660} / 30 min for isoforms 1,2 and 3 respectively. This indicated that isoforms 1 and 2 more active toward amylose than isoform 3.

High concentration of substrate can have inhibitory effect on enzyme activity. Normally, an increase in substrate concentration increases the velocity of the enzyme reaction. Some enzymes, however, display the phenomenon of *excess substrate inhibition*. This means that large amounts of substrate can have the opposite effect and actually slow the reaction down. A familiar example of this is the enzyme *invertase*, or beta-fructofuranosidase (EC3.2.1.26), which is responsible for hydrolyzing the disaccharide sucrose to its component monosaccharide, glucose and fructose. It's thought that substrate inhibition happens with this enzyme when two substrate molecules bind to the active site at the same time. They can only do this by approaching the active site in an end on fashion which prevents either of them from positioning itself in such a way that the enzyme can attack it. As long as both substrate molecules are attached to the active site the enzyme is effectively inactive, and therefore

inhibited. For this process to occur the second substrate must approach the active site very rapidly after the first, otherwise the first substrate would quickly attain the correct catalytic placement. As collisions between enzyme and substrate are completely random this is only likely to occur at high substrate concentrations when the frequency of random collisions is greatly increased, so inhibition is only seen in the presence of excess substrate.

4.3.5 Comparison of SBE isoform activity with amylose and amylopectin as substrate

There have been reports that some SBE isoforms, although catalyzed branching of amylose, preferentially used amylopectin as substrate (section 1.4). To investigate if any of 3 isoforms isolated displayed such isoforms activities, experiments were performed using both corn amylose and amylopectin as substrates (Figure 3.21). Isoforms 1 and 2 showed higher activity towards amylose than isoform 3 whereas isoform 3 was more active in utilizing amylopectin as substrate. Isoform 1 was most active in utilizing amylose, followed by isoforms 2 and 3 (Figure 3.21 A). When amylopectin was used as substrate, isoform 1 and 2 were less active. Isoform 3 can utilize amylopectin 30% better than isoforms 1 and 2 (Figure 3.22 B). This result agreed with the Km for amylose of the isoform.

SBE has been classified to two classes by amino acid sequences, class A preferentially branches amylopectin whereas class B preferentially branches amylose. It was reported that C-terminal domain of SBE is involved in substrate specificity whereas N-terminal domain is important for specificity of transferred chain length and require or maximum enzyme activity. From the above information, isoforms 1 and 2 should be grouped in class B. whereas isoform 3 which is specific for amylopectin should be grouped in class A.

Monitoring of SBE development with ages in cassava tubers has never been reported before. Further more, this is also the first report on purification and characterization of SBE isoforms form cassava tubers. The finding that cassava SBE existed in the both class

A and class B isoforms was also new. Although this research was still in its early stage, further detailed study of these isoforms together with similar studies on starch synthase and debranching enzymes could render useful informations which could lead to enzyme manipulations to yield starch of desired quality and quantity.

Table 4.2 Comparative properties of starch branching enzymes from KU 50 of cassava

Isoform	Mw* (kDa)	Native molecular weight (kDa)	pI	pH optimum	Temperature optimum	Kinetic Constant	
						K_m	V_{max} (Δ A_{660} / 30 min)
SBE 1	57	57	4.9	8.0	37 °C	1.12	1.03
SBE 2	57	57	4.9	6.5	37 °C	1.37	0.83
SBE 3	60	57	5.0	7.5	30 °C	2.17	0.57

* The value determined by SDS-PAGE

CHAPTER 5

CONCLUSIONS

1. SBE activity in cassava tuber both KU 50 and Rayong 1 cultivars with was increased growth between 6 to 9 months. Maximum activity per wet weight was at 9 month in KU 50 and 12 months in Rayong 1
2. Crude SBE from 9 month old tubers of KU 50 was purified by 10% polyethylene glycol precipitation and first DEAE-Toyopearl.
3. Three isoforms of SBE were isolated by second DEAE-Toyopearl column by stepwise elution with NaCl solution at concentrations of 0.04, 0.07 and 0.1 M
4. Each isoforms were further purified by Hitrap Q-Sepharose with linear gradient and were eluted at 0.4, 0.3 and 0.4 M NaCl with perform folds of 503.5, 400 and 822 respectively.
5. The native molecular weight of each isoform was estimated to be 57 kDa by gel filtration to be and estimated by SDS –PAGE to be 57 kDa for isoform 1 and 57 kDa. for isoform 2 and 3.
6. Activity stain of all isoforms on native gel electrophoresis showed characteristics reddish bands of SBE on iodine stain. Isoform 1 moved slower than isoforms 2 and 3 which had same mobilities
7. The optimum pH for isoform 1, 2 and 3 were 8.0, 6.5 and 8.5. Optimum temperature was 37 °C for isoforms 1 and 2 and 30°C for isoform 3. All isoform were at 40 °C
8. The isoelectric point (pI) estimated by isoelectricfocusing gel were 4.9 for isoform 1, 2 and 5.0 for isoform 3
10. The sulfhydryl group reagent showed no effect on activities of all isoforms indicating no involvement of SH- group in SBE activity

11. Divalent ions strongly inhibited SBE activities
12. The K_m for amylose were 1.12, 1.37 and 2.17 mg/ml and V_{max} were 1.03, 0.83 and 0.57 ΔA_{660} / 30 min for isoforms 1, 2 and 3 respectively.
13. Isoforms 1 and 2 were more specific to amylose and grouped as class B SBE while isoform 3 was more specific for amylopectin grouped as class A SBE.

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APPENDICES

APPENDIX A

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

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

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

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

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

Solution B (SDS PAGE)

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

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8	50	ml
10% SDS	4	ml
Distilled water	46	ml

2. Non- denaturing PAGE

7.0 % Seperating gel

30 % Acrylamideml solution	1.75	ml
1.5 M Tris-HCl pH 8.8	2.5	ml
Soluble starch	1.0	ml
Distilled water	2.14	ml
10% $(NH_4)_2S_2O_8$	100	µl
TEMED	10	µl

4.0 % Stacking gel

30 % Acrylamideml solution	0.67	ml
0.5 M Tris-HCl pH 6.8	1.0	ml
Distilled water	3.27	ml
10 % $(NH_4)_2S_2O_8$	50	µl
TEMED	10	µl

5X Sample buffer

1 M Tris-HCl pH 6.8	3.1	ml
Glycerol	5.0	ml
1 % Bromophenol blue	0.5	ml
Distilled water	1.4	ml

One part of sample buffer was added to four parts of sample.

Electrophoresis buffer, 1 liter (25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

Dissolve in distilled water to 1 liter. Do not adjust pH (final pH should be 8.3).

3. SDS-PAGE

10 % Separating gel

30 % Acrylamideml solution	2.5	ml
Solution B	2.5	ml
Distilled water	2.39	ml
10% $(NH_4)_2S_2O_8$	100	µl
TEMED	10	µl

4.0 % Stacking gel

30 % Acrylamide solution	0.67	ml
Solution C	1.0	ml
Distilled water	3.27	ml
10 % $(NH_4)_2S_2O_8$	30	µl
TEMED	5.0	µl

5X Sample buffer

1 M Tris-HCl pH 6.8	0.6	ml
50% Glycerol	5.0	ml
10% SDS	2.0	ml
2-Mercaptoethanol	0.5	ml
1 % Bromophenol blue	1.0	ml
Distilled water	0.9	ml

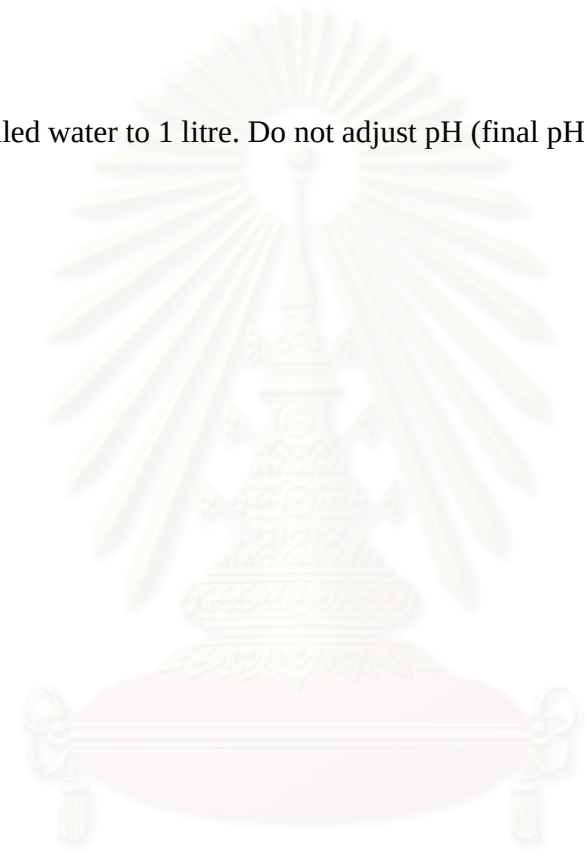
One part of sample buffer was added to four parts of sample. The mixture was heated 5 min. in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g
SDS	1.0 g

Dissolve in distilled water to 1 litre. Do not adjust pH (final pH should be 8.3).



APPENDIX B

Preparation for isoelectric focusing gel electrophoresis

Stock solution for isoelectric focusing gel electrophoresis

24.25% Acrylamide; 0.75% bis-acrylamide

Acrylamide	24.25 g
N,N-methylene-bis-acrylamide	0.75 g

Adjusted volume to 100 ml with distilled water

0.1% Riboflavin

Riboflavin	50 mg
Distilled water	40 ml

Heat until solubility and adjusted volume to 50 ml with distilled water

Monomer-ampholyte solution

25% polyacrylamide	1 ml
Distilled water	2.75 ml
25% Glycerol	1 ml
Ampholyte	0.25 ml

This solution was degassed

0.1% Riboflavin	50 μl
10% Ammonium persulfate	15 μl
TEMED	5 μl

Fixative Solution, 100 ml

Sulfosalicylic acid	4 gm.
Trichloroacetic acid	12.5 gm
Methanol	30 ml

Immerse gels in this solution for 30 minutes

Staining Solution, 100 ml

Ethanol	27 ml
Acetic acid	10 ml
Coomassie brilliant blue R-250	0.04 gm
$CuSO_4$	0.5 gm
Distilled water	63 ml

Dissolve the $CuSO_4$ in water before adding the methanol. Either dissolve the dye in alcohol and filtrate or add it to the solution at the end.

Immerse the gel into stain for approximately 1-2 hours.

Destaining Solution

First destaining solution

Ethanol	12 ml
Acetic acid	7 ml
$CuSO_4$	0.5 gm
Distilled water	81 gm

Dissolve the $CuSO_4$ in water before adding the ethanol. Immerse the gel in two or three change of this solutiomunit the background nearly clear.

Second destaining solution

Ethanol	25	ml
Acetic acid	7	ml
Distilled water	68	gm

Immerse the gel in this solution removethe last traces of the $CUSO_4$

APPENDIX C

Iodine's Solution

Iodine solution I

0.05% Potassium iodide; 0.005% Iodine

Potassium iodide	0.05 g
Iodine	0.005 g
Adjust to 100 ml distilled water	

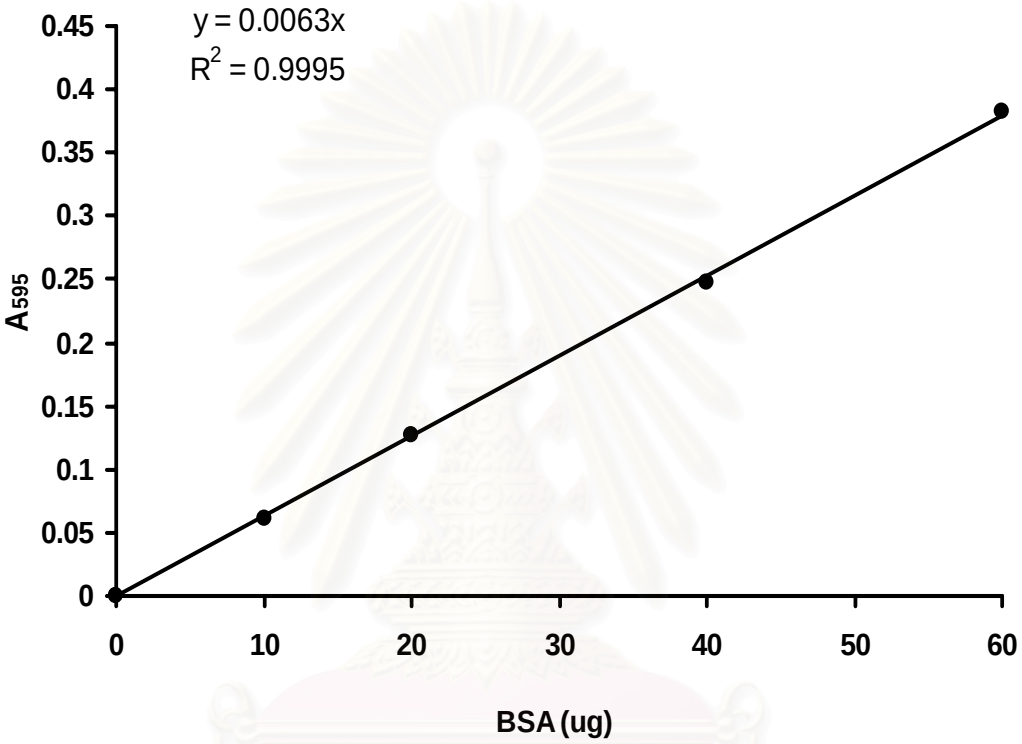
Iodine solution II

1% Potassium iodide; 0.1% Iodine

Potassium iodide	1 g
Iodine	0.1 g
Adjust to 100 ml distilled water	

APPENDIX D

Calibration curve of protein concentration



APPENDIX E

Calculation of SBE activity

Blk = CPM of reaction mixture with out SBE

X = CPM of SBE products

Y = CPM of ^{14}C in 50 mmol

Incubation time = 60 minutes

$$\text{SBE activity} = \frac{\text{X-Blk}}{\text{Y}} \times 50 \times 10^3 \times \frac{1}{60} \text{ umol/min}$$

BIOGRAPHY

Mr. Surachai Yaiyen was born in June 16th, 1977 in Bangkok. He finished Mattayom 6 at Wat Borvorniwet school, Bangkok and enrolled in the Faculty of Science Ramkhamhaeng University in 1994 and graduated with B.Sc. in Biotechnology in 1999. He continued for M.Sc. in Biochemistry later that year.