การแสดงออกของยีนสตาร์ชบรานชิ่งเอนไซม์ที่โคลนได้จากมันสำปะหลัง *Manihot esculenta* Crantz และ การติดตามเอนไซม์ทางอิมมูน

นาย สุรชัย ใหญ่เย็น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Expression of Starch Branching Enzyme Genes Cloned from Cassava *Manihot esculenta* Crantz. and Immunological Monitoring of the Enzymes

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctoral Philosophy of Program in Biotechnology
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Thesis Title	Expression of Starch Branching Enzyme Genes Cloned from Cassava *Manihot esculenta* Crantz and Immunological Monitoring of the Enzymes
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สุรชัย ใหญ่เย็น : การแสดงออกของยีนสตาร์ชบรานชิงเอนไซม์ที่โคลนจากมันสำปะหลัง *Manihot esculenta* CRANTZ และการติดตามทาง เอนไซม์ทางอิมมูน (Expression of starch branching enzyme genes cloned from cassava *Manihot esculenta* CRANTZ and immunological monitoring of the enzyme) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. ทิพาพร ลิมปเสนีย์ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. สุพัชรี เนตรพันธุ์, 120 หน้า

เอนไซม์สร้างโซ่กิ่ง (starch branching enzyme) หรือ Q เอนไซม์ (alpha-1,4-glucan : alpha-1,4-glucan-6-glucosyltransferase, EC 2.4.1.18) มีหน้าที่เร่งปฏิกิริยาการสังเคราะห์อะไมโลเพคตินโดยการสลาย แอลฟา 1,4-กลูแคน แล้วนำไปเชื่อมกับกลูโคสด้วยพันธะ แอลฟา-1,6 ไกลโคซิดิกที่ด้านปลายรีดิวซ์ของกลูโคส ในการศึกษานี้ได้นำ cDNA ของเอนไซม์สร้างโซ่กิ่งจากมันสำปะหลังสายพันธุ์ KU50 ซึ่งมีสองยีน คือ *sbeI* และ *sbeII* มาทำพีซีอาร์ได้ชิ้นส่วนยีนขนาด 2.8 และ 2.7 กิโลเบสซึ่งถูกทำการโคลนเข้ากับ พลาสมิด pET28c และ pET28b โดยใช้ *E. coli* rosetta gami (DE) เป็นเซลล์เจ้าบ้าน ซึ่งแสดงออกได้ดีเมื่อถูกกระตุ้นด้วย 0 .4 mM IPTG เป็นเวลา 4 ชั่วโมง ได้รีคอมบิแนนท์เอนไซม์คือ SBERI และ SBERII หลังจากนั้นทำให้เอนไซม์บริสุทธิ์โดยการผ่านโครมาโตกราฟีแบบจำเพาะคือ hitrap His-tag ได้ค่าความบริสุทธิ์ของรีคอมบิแนนท์เอนไซม์ SBERI และ SBERII เพิ่มขึ้น 5 เท่า และมีน้ำหนักโมเลกุลเท่ากับ 90 กิโลดาลตันเมื่อวิเคราะห์ด้วยอีเลกโทรฟอเรซิสแบบเสียสภาพ จากนั้นนำรีคอมบิแนนท์เอนไซม์ทั้งสองไปศึกษาลักษณะสมบัติพบว่า เอนไซม์สามารถทำปฏิกิริยาได้ดีที่ pH 7.0 และอุณหภูมิ 37 องศาเซลเซียส เอนไซม์มีค่าทางจลนพลศาสตร์ต่ออะไมโลส คือ K_m เท่ากับ 2.13 และ 3.46 มิลลิกรัมต่อมิลลิลิตร และ ค่า V_{max} เท่ากับ 1.7 และ 3.4 ΔA_{660}/min ตามลำดับ SBERI มีความจำเพาะต่ออะไมโลสมากกว่า SBERII ซึ่งมีความจำเพาะต่ออะไมโลเพคตินมากกว่า เมื่อศึกษาบทบาทของกรดอะมิโนต่อแอกทิวิตี้โดยการใช้สารเคมีดัดแปลงกรดอะมิโน พบว่า ไทโรซีน, และอาร์จินีน มีบทบาทกับแอคติวิตี้ของเอนไซม์ทั้งสอง นอกจากนี้ ในSBERI กรดอะมิโนฮีสติดีนมีบทบาทเกี่ยวข้องกับการทำปฏิกิริยาของเอนไซม์ด้วย จากการวิเคราะห์องค์ประกอบของกรดอะมิโนของเอนไซม์สร้างโซ่กิ่งในหัวมันสำปะหลังสายพันธุ์ KU50 (SBE1,SBE2,SBE3) พบว่า มีอัตราส่วนของกรดอะมิโนที่คล้ายกับกรดอะมิโนที่มาจากรีคอมบิแนนท์เอนไซม์ โดย SBE1 และ SBE2 มีความคล้ายกับ SBERI และ SBE 3 มีความคล้ายกับ SBERII เมื่อนำไอโซฟอร์มบริสุทธิ์ทั้งสามจากหัวมันสำปะหลังและรีคอมบิแนนท์เอนไซม์มาทำพอลิโคลนอลแอนติบอดี พบว่า แอนติบอดีของเอนไซม์แต่ละชุด สามารถจับกับเอนไซม์อีกชุดหนึ่งได้ เมื่อนำเอนไซม์สกัดหยาบที่มาจากมันสำปะหลังสายพันธุ์ KU50 อายุ 6, 9 และ 12 เดือน มาแยกด้วยอีเลกโทรฟอเรซิสแบบเสียสภาพและ ให้จับกับ Anti-SBERI และ anti-SBERII ด้วยวิธี western blot พบว่า สามารถทำปฏิกิริยากับเอนไซม์ในหัวมันสำปะหลังสายพันธุ์ KU50 ได้ โดยพบแถบเอนไซม์ที่ 108 และ 60 กิโลดาลตัน และ ความเข้มของแถบสีที่เกิดปฏิกิริยาเพิ่มมากขึ้นตามอายุของหัวมันสำปะหลัง

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต .....

ปีการศึกษา.....2552..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4873862523 : MAJOR BIOTECHNOLOGY
KEYWORDS: ST ARCH BRANCHING ENZYME/ CASSAVA / POLYCLONAL ANTIBODY

SURACHAI YAIYEN : EXPRESSION OF STARCH BRANCHING ENZYME GENES CLONED FROM CASSAVA *Manihot esculenta* Crantz AND IMMUNOLOGICAL MONITORING OF THE ENZYME THESIS ADVISOR : ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D. THESIS CO-ADVISOR SUPATCHAREE NETRPHAN, Ph.D., 120 pp.

Starch branching enzyme (SBE) also called Q-enzyme (alpha-1,4-glucan : alpha-1,4-glucan-6-glucosyltransferase, EC 2.4.1.18 introduces branch points in the amylopectin molecules by hydrolysis of the alpha-1,4-glucan chains. It catalyzes the formation of an alpha-1,6 cross linkage between the reducing end of the cleaved chain and glucose residue. In this study the cDNA of two SBE genes, *sbe*I and *sbe*II gene were cloned and expressed. Their respective PCR products were 2.8 and 2.7 kb were cloned into pET 28c for *sbe*I and pET 28b for *sbe*II. They were expressed in *E. coli* Rosetta gami (DE). The optimum expression condition was induction with 0.4 mM IPTG for 4 hours for both transformants. Purification by Hitrap His-Tag chromatography resulted in 5 folds purification with specific activities of 8.4 and 7.6 units/mg protein, respectively. Both SBERI and SBERII showed similar characteristics: molecular weight of 90 kDa by SDS-PAGE, optimum pH at 7.0, optimum temperature of 37°C. However, their kinetic characteristics were different, K_m and V_{max} for amylose were 2.13 and 3.46 mg/ml and 1.7 และ 3.4 ΔA_{660}/min for SBERI and SBERII respectively. SBERI was more active than SBERII towards amylose as substrate whereas SBERII was more active with amylopectin as substrate. Modification of tyrosine, argine and tryptophan altered the activities of both SBERI and SBERII whereas modification of histidine only affected SBERI. Comparison of amino acid compositions and kinetic parameters indicated SBE1 and SBE2 were similar to SBERI and were probably the product of *sbe*I gene while SBE3 was related to SBERII. Polyclonal antibody of each native isoforms could react with both recombinant enzymes and similar interactions were observed with polyclonal antibodies of recombinant enzyme and native SBE isoforms. Polyclonal antibodies of the recombinant enzyme were used to monitoring enzyme extract of tubers at different ages of 6, 9 and 12 months by western blots. Anti-SBERI stained more intensely with both bands of 108 kDa and 60 kDa. whereas anti-SBERII showed less intensity on both bands. The band intensities also increased with ages.

Field of study.....Biotechnology..... Student's signature.....

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LIST OF ABBREVIATION

A	Absorbance
AMP	Adenosine-5’-monophosphate
BSA	Bovine serum albumin
CPM	Count per minute
DBE	Debranching enzyme
DTT	Dithiothreitol
EDTA	Ethelenediaminetetraacetic acid
FPLC	Fast performance liquid chromatography
xg	gravitational acceleration
GBSS	Granule-bound starch synthase
HCl	Hydrochloric acid
kDa	kilo Dalton
M	Molar
mM	Millimolar
MW	molecular weight
NEM	N-ethylmaleimide
NaCl	Sodiumchloride
NaOH	sodium hydroxide
SDS	Sodium dodecyl sulfate
SSs	Soluble starch synthase
TEMED	N,N,N’,N’-tetramethyl ethylene diamine

CHAPTER 1

INTRODUCTION

1.1 Cassava

Cassava *Manihot esculenta* Crantz. is a tropical, dicotyledonous plant (Figure 1.1A) in the botanical family Euphorbiaceae. Cassava is a perennial woody shrub, an annual growth. Cassava is a major source of low cost carbohydrates for populations in the humid tropics. The largest producer of cassava is Brazil, followed by Thailand, Nigeria, Zaire and Indonesia. Production in Africa and Asia continues to increase, while that in Latin America has remained at relatively constant level over the past 30 years. Thailand is the main exporter of cassava with the major portion going to Europe. It is a staple food in many parts for western and central Africa and is found throughout the humid tropics. The world market for cassava starch and meal is limited, due to the abundance of substitutes.

Cassava is a tropical root crop, requiring at least 8 months of warm weather to produce a crop. It can tolerate drought and can be grown on soils with low nutrient capacity. It also responds well to irrigation or higher rainfall conditions. Cassava has high yield and is highly resistant to the damage from serious pests and diseases (Osuntokun, 1973).

Cassava is grown for its enlarged starch-filled tubes, which contains nearly the maximum theoretical concentration of starch on a dry weight basis among food

crops. Fresh tubes contain about 30% starch and very little protein.Tubers are prepared much like potato. They can be peeled and boiled, baked, or fried. It is not recommended to eat cassava uncooked, because of potentially toxic concentrations of cyanogenic glucosides that are reduced to innocuous levels through cooking. In traditional settings of the Americas, tubers are grated and the sap is extracted through squeezing or pressing. The cassava is then further dried over a fire to make a meal or fermented and cooked. The meal can then be rehydrated with water or added to soups or stews. In Africa, tubers are processed in several different ways. They may be first fermented in water. Then they are either sun-dried for storage or grated and made into a dough that is cooked. Alcoholic beverages can be made from the tubers. In addition to being used for human consumption, dried cassava is used as animal feed and cassava starch , it 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.

Global cassava production in 2008 is forecasted as 238.5 million tonnes, 5% above the record of the previous year. Cassava production is anticipated to record strong growth in Asia, much on account of Thailand, where, according to the annual planting survey, a 15percent rise in production is forecast in 2008 to a record 29.15million tones (Table 1.1). Global trade in cassava products in the current year is likely to fall to an eight year low of 7.5 million tonnes (pellet equivalent). The forecast is based on a significant decline in the competitiveness

of cassava feedstuffs and starch relative to grain based products, combined with lower international demand for cassava as a feedstock for ethanol production. This expectation is in line with a weaker pace of cassava shipments by Thailand to date, by far the world's largest international supplier. Overall, the country is anticipated to ship just over 7 million tonnes (pellet equivalent) of cassava chips, pellets and starch in 2008. Countries in Asia are once again expected to be the major destination of internationally traded cassava products in aggregate. The implementation of the free-trade zone between China and Thailand, which resulted in the abolishment of a 6 percent tariff levied on Thai cassava products, has provided a boost to cassava trade between the two countries in recent years, and in doing so, firmly established China as the world's leading importer of cassava products. However, 2008 marks a shift in China's status, especially in the context of chips and pellets imports. While Thailand is foreseen to export 40 percent less than what it did in 2007, China's share in that market is expected to fall to 35 percent in 2008 from a high of 90 percent in 2006. Ample supplies of cheaper domestic grain based feedstuffs and home grown cassava for China's ethanol industry are likely to depress cassava inflows into the country. A permanent retreat from the import market of the European Union, once the major destination of international cassava shipments (mainly for animal feed), appears to have come to an end. Thailand is preparing to ship as much as 1.4 million tonnes of pellets to the community, similar to the level last year but four times the volume delivered in 2006. The European Union has emerged as the main destination for pellets in the current year. However, the momentum in European Union purchases has slowed

in the past few months, coinciding with the increased availability of feedstuffs among member states following the recent grain harvest. As for cassava starch and flour, global trade is again expected to contract, but not to the same degree foreseen in the chips and pellets market. The fall in trade would similarly reflect the price advantage that grain based starch is forecast to maintain over cassava. Japan appears likely to overtake China as the principal starch buyer, with Indonesia, the Chinese Province of Taiwan and Malaysia all engaging in significant international purchases during the course of the year.

Cassava accumulates food in its tubers. After growing leaves and other green parts, it starts to produce carbohydrate. The ability to produce and accumulate starch depends on the variety, the age at which it is harvested, the amount of rainfall and other factors. For tapioca with the age of 12 months and sufficient amount of rainfall, the composition is as follows Table 1.3. The composition of cassava tuber is, apart from water, mainly starch. Therefore, cassava is a source of carbohydrate, so important to man and animals. Usually, cassava tuber with low starch content will have a high density. For a rapid test of starch content, if the cassava tuber placed in the water is light, the starch content is low. On the other hand, if the weight of the tuber in the water is heavier, then the starch content will be high.

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

	2005	2006	2007	2008
	Thousand tonnes			
World	207,437	222 559	228 138	238 450
Africa	114 602	118 078	117 888	124 000
Nigeria	41 565	45 721	45750	49 000
Congo	14 974	14 989	15 000	15 300
Ghana	9 567	9 638	9 650	10 300
Angola	8 606	8 810	8 800	9 000
Mozambique	6 500	7 500	7 350	7 750
Tanzania	7 000	6 500	6 600	7 000
Uganda	5 576	4 926	4 456	4 000
Latin America				
Brazil	25 872	26 639	27 313	26 300
Paraguay	4 785	4 800	5 100	5 300
Colombia	2 050	2 000	2 100	2 200
Asia	55 917	67 190	70 745	76 650
Thailand	16 938	22 584	25 348	29 150
Indonesia	19 321	19 928	19 610	20 000
Viet Nam	6 646	7 714	8 900	10 000
India	5 855	7 620	7 600	7 700
China, Mainland	4 000	4 300	4 350	4 500
Cambodia	536	2 182	2 000	2 100
Philippines	1 678	1 757	1 829	2 000

* Forecast

Table 1.2 Thai Trade in Cassava [1]

	2005	2006	2007	2008
	Thousand tonnes			
Total	**6 240**	**8 964**	**9 240**	**7 026**
Flour and starch				
Total	**3 212**	**4 616**	**4 416**	**4 132**
Japan	622	694	729	921
China	525	723	694	586
Chinese Prov	502	676	548	482
Indonesia	348	968	667	450
Malaysia	229	312	256	353
Others	986	1 244	1 523	1 341
Chips and pellets				
Total	**3 028**	**4 348**	**4 824**	**2 894**
China	2 766	3 963	3 127	1 032
EU	246	341	1 436	1 392
Others	16	44	261	470

Source: TTTA, FAO

[1] In product weight of chips and pellets

Table 1.3 A typical cassava root compositions.

Composition of tapioca root	Amount per 100 gram
Water	60.21-75.32
Peel	4.08-14.08
Flesh (Starch)	25.87-41.88
Cyanide (ppm)	2.85-39.27

Composition of tapioca flesh	Amount per 100 gram of dried weight
Starch	71.9-85.0
Protein	1.57-5.78
Fiber	1.77-3.98
Residue	1.20-2.80
Fat	0.06-0.43
Non-Starch carbohydrate	3.59-8.66

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)

(www.cassava.org). 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) (Sriroth *et al.*, 2000).

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 1.4 Cassavas varieties in Thailand (*www.cassava.org*)

	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 1.5 Comparison of cassava varieties in Thailand

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

1.2 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 tubers. 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, that very in size and shape among cereal, legume and tuber (Banks and Muir, 1980; French, 1984; Hoseney, 1994)

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) (Bank and Muir, 1980). Amylase and amylopectin has to constitute 20-30% and 70-80% of the starch, respectively (shanon and Gerwood, 1994). In addition, small amounts of lipids (0.1-1.0%), proteins (0.05%-0.5%) and trace element (such as phosphate).

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 1.6 Percent of amylose and amylopectin in reserve plant starch

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

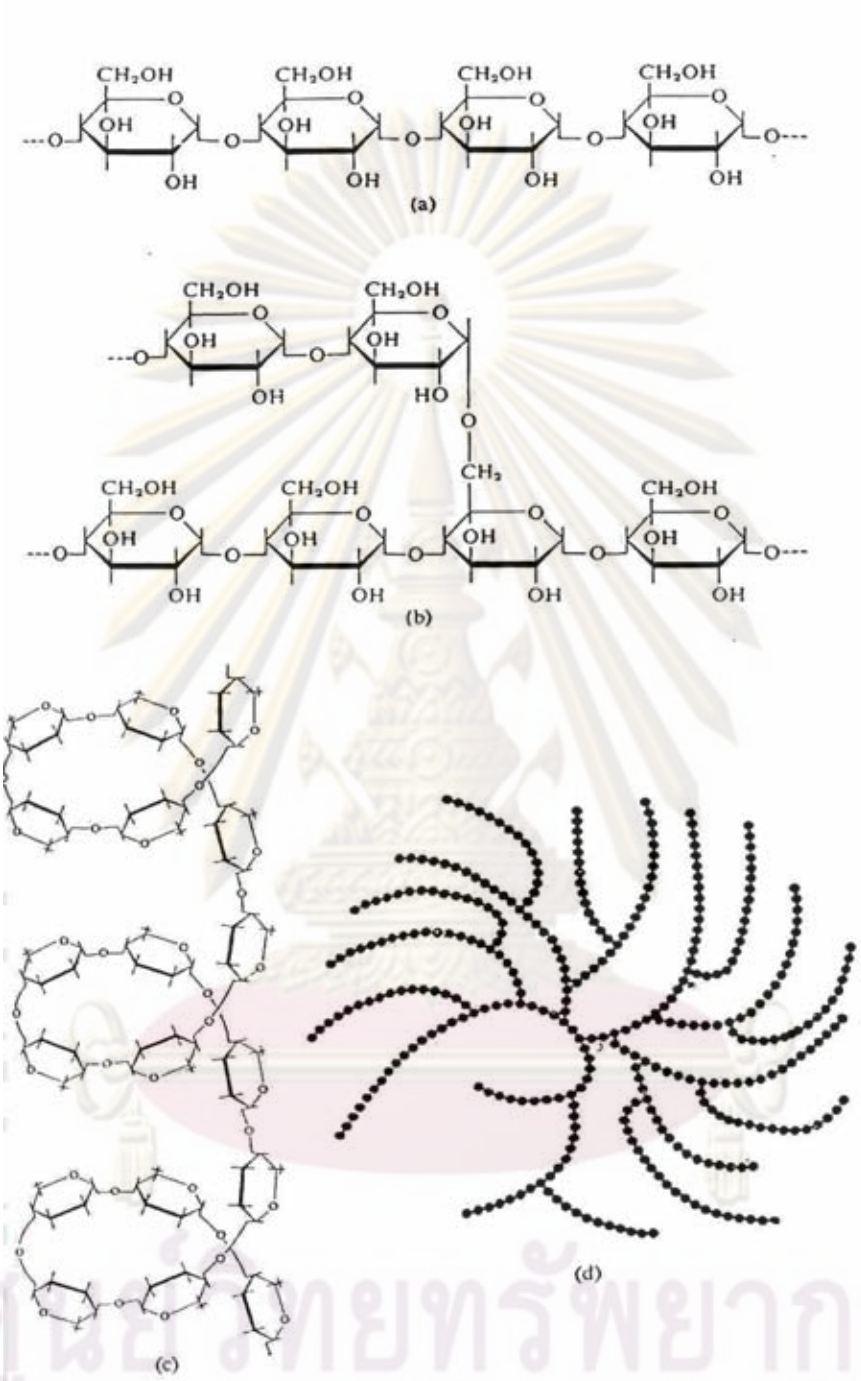


Figure 1.2 Structures of amylose and amylopectin

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 (Bank and Muir, 1980). 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).

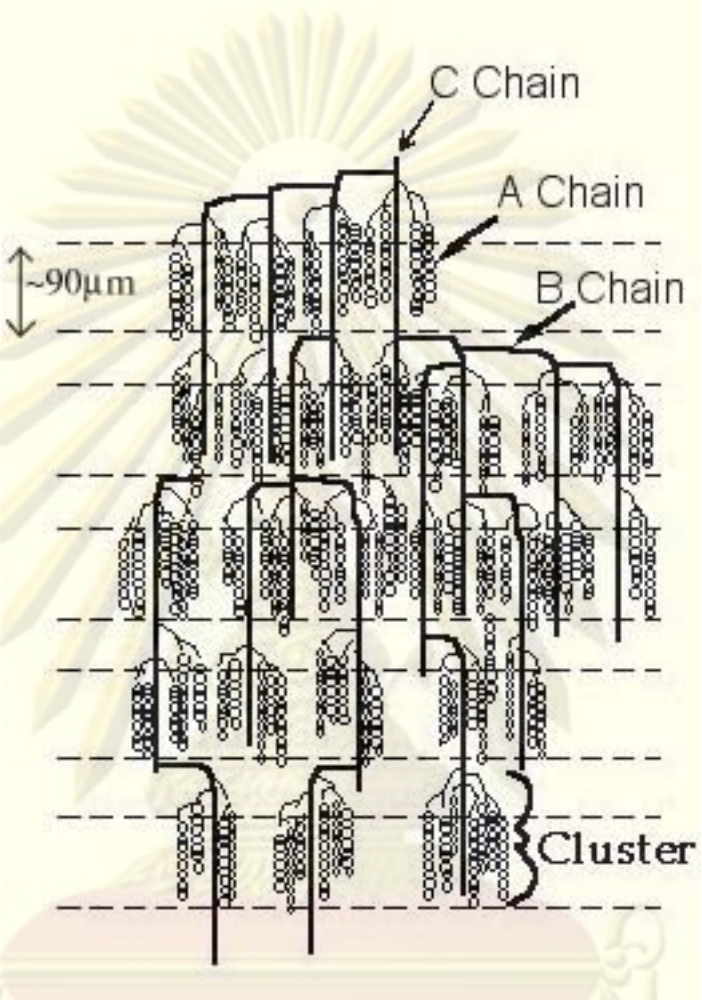
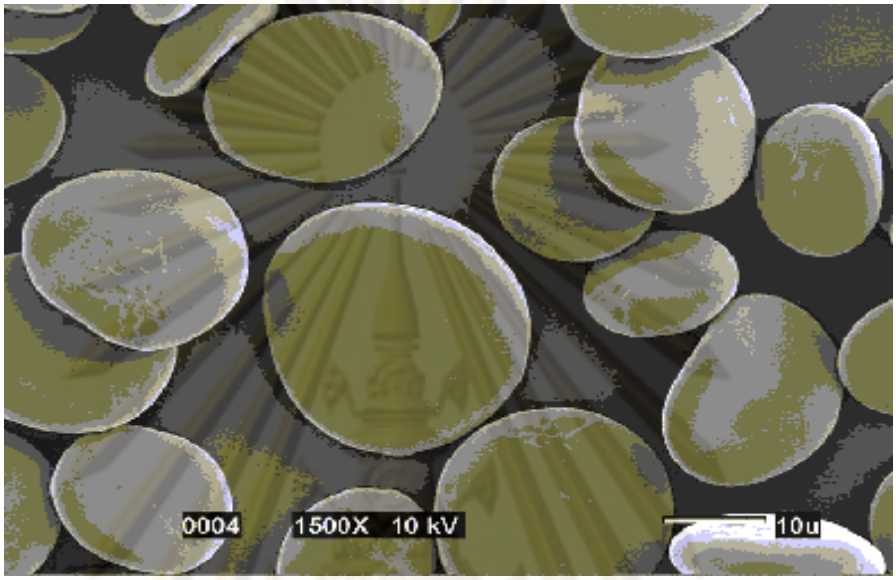


Figure 1.3 The branch structure of amylopectin

Table 1.7 Properties of the amylose and amylopectin components of starch

Property	Amylose	Amylopectin
General structure	Essential linear	Branched
Color with iodine	Dark blue	Purple
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.

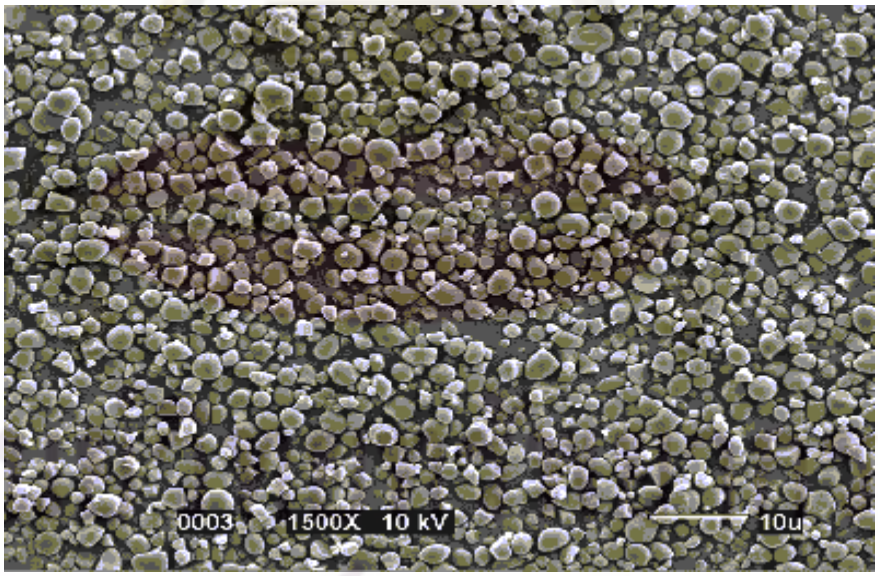


Figure 1.4 The starch granule in Wheat.

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 (Smith *et al.*, 1997).

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. Other components within all starch granules are proteins (0.5% in cereal endosperm and 0.05% in potato tuber), which 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

The starch biosynthesis is well established in the chloroplast of leaves while transported to amyloplasts of storage organs. There are consensus that starch synthesis takes place in plastids from many enzymes (Figure 1.6) (Myer *et al.*, 2000). ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27), Starch synthase (SS, EC 2.4.1.21), Starch branching enzyme (SBE, EC 2.4.1.18), Debranching enzyme (DBE, EC 2.4.1.25). On the other hand, some evidence indicate dispropotionation enzyme (D-enzyme, EC 2.4. 1.25) is also involved in starch biosynthesis.

In chloroplast, the starch is transistory and synthesized from ADP-glucose by the combined action of SS and SBE. ADP-glucose is synthesized in leaves from fructose-6-phosphate, from calvin cycle. Three enzymes; phosphoglucoisomerase, phosphoglucomutase and ADP-phosphorylase are involved in ADP-glucose synthesis (Figure 1.3). In previously, the mutant in *Clakia xantiana*, *Arabidopsis thaliana* and *Nicotiana sp.* are effect to these enzymes in starch-less or low level of starch phenotypes in leaves (Casper *et al.*, 1985; Jones *et al.*, 1986; Hanson and McHale, 1988; Neuhass and Stit, 1990).

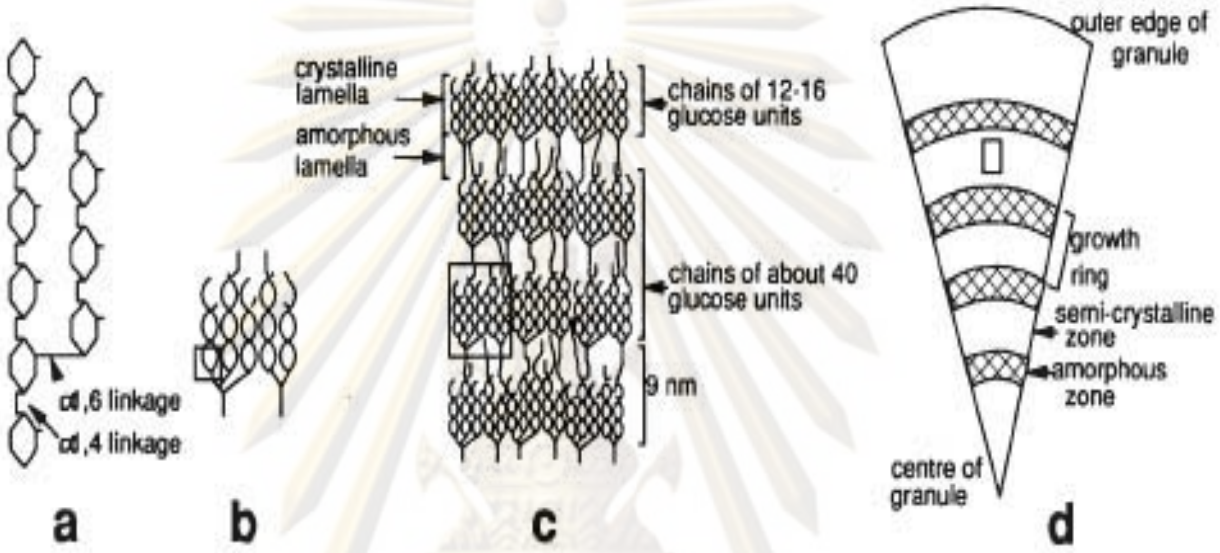


Figure 1.5 Schematic representation of levels of organization within the starch granule. The boxes within the diagrams in panel b, c and d represent the area occupied by the structure in the preceding panel (Smith *et al*., 1997).

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 semi crystalline material, consisting of crystalline and amorphous lamellae, and amorphous material.

In amyloplast, a stored organ and synthesized storage starch is synthesize, The synthesis pathway is same as chloroplast but the precursor for ADP-glucose synthesis comes from the catabolism of sucrose, a primary carbon source in the cytosol. The amyloplast has two pathways for starch synthesis. Then, ADP-glucose pyrophosphorylase pathways, sucrose is catabolized into triose / hexose phosphate that is transported into amyloplast with phosphate translocator after triose/hexose phosphate takes place by the combined action of AGPase, SS, SBE and DBE (Figure 1.6) (Okita, 1992; James *et al*., 1995; Martin and Smith, 1995).

However, the enzymes are role in amylose and amylopectin synthesis, which it depends on quality, physical and biochemical properties in plant starch.

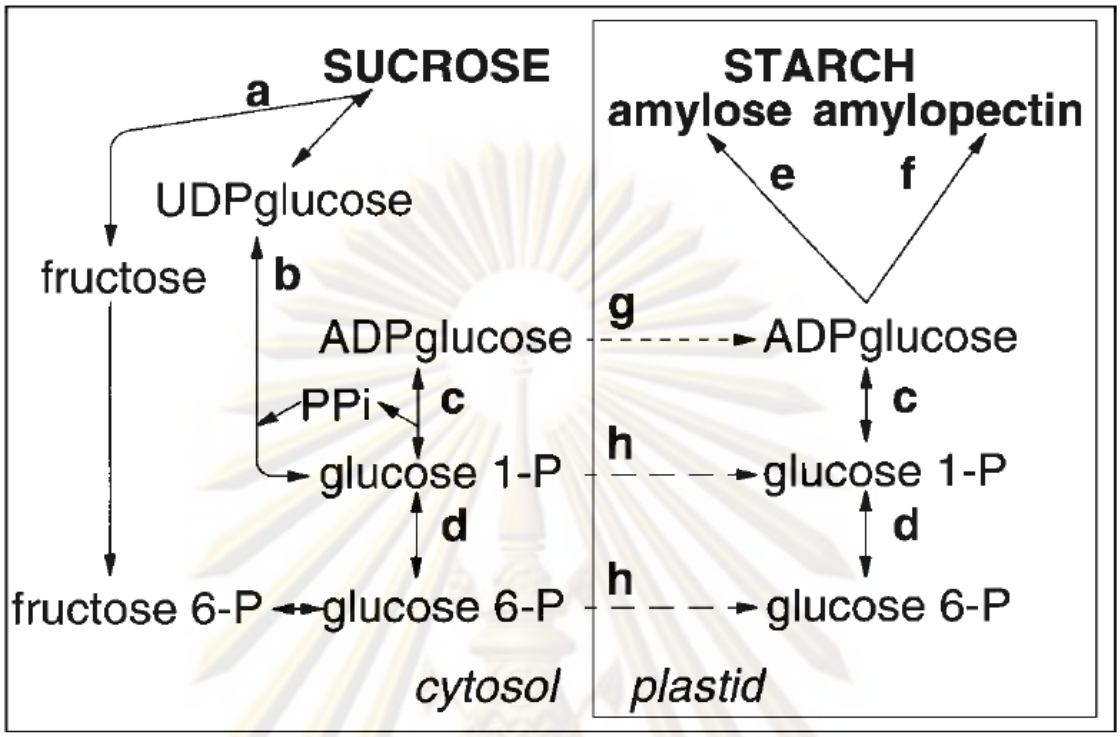


Figure 1.6 The major metabolites and enzyme involved in the conversion of sucrose to starch in storage organs. Carbons is shown entering the plastid either as a hexose phosphate (Smith *et al*.1997). or as ADP-glucose: a. sucrose synthase; b. UDP-glucose pyrophosphorylase; c. ADP-pyrophosphorylase; d. 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 (Denyer *et al*., 1999). 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 (Smith *et al.,* 1997).

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) (Myer *et al*., 2000). 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 (Myer *et al*., 2000). D-enzyme may play a direct role in amylopectin formation, or it could be involved in indirect recycling of glucosyl unit from water-soluble polysaccharide or pre-amylopectin back into amylopectin biosynthesis. Thus, D-enzyme should be considered in addition to SS and SBE as a potential direct determinant of chain length distribution. However, it is not clear how D-enzyme acts on amylopectin biosynthesis (Figure 1.8) (Muyakikawa *et al.*, 1997).

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. The SBE is multiple isoforms , it have been found in Spinach leaf (Hawker *et al.,* 1974), maize endosperm and leaf (Boyer and Preiss, 1978; Dang and Boyer, 1988), Rice endosperm (Smyth, 1988), pea embryo (Matters and Boyer, 1981) and potato (Borovsky *et al*., 1975). 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. The eight regions of parallel β-sheets form central cylinder which acts as a scaffold for substrate binding and catalysis. The members of this family possess four highly conserved amino acid sequence that catalytic and some substrate binding residues. Apart from the barrel domain, SBEs show considerable structural variation in the length and amino acid sequences at the N- and C-terminal regions. Comparison of the deduced amino acid sequence indicated that SBEI and SBEII are structurally related very similar to glycogen branching enzyme from bacteria (Burton *et al*., 1995). The length and composition of the amino acid loops that connect the seventh β-sheet and α-helix may be related to difference in the branch length introduced by *E.coli* glycogen BE and maize SBEI (Jesperson *et a*l., 1993). Comparison of the deduce amino acid sequences of SBEI and SBEII indicated a difference loop size between 8 β-sheet and 8 α-helix involving anextra 11 amino acid domain that is well conserved in SBEII members (Burton *et al.,* 1995). This difference in the length of the loop might be significant since SBEI and SBEII catalyse different branching reactions. 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 (Hamada *et al*., 2002). Between two SBE isoforms, they is 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 (Denyer *et al*., 1995), and II and I of potato tuber (Drummond *et al*., 1972) 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 (Guan and Preiss, 1993). 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 (Munyikawa *et al*.,1997).

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 (Denyer *et al.*, 1995). 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 (Flipse *et al.*,1996).

In pea, three isoforms of SBE that differ in kinetic and physical properties were separated by ion exchange chromatography. Two of the three proteins with the estimated molecular weight of 114 and 108 kDa eluted at low salt concentration while a higher salt concentration. Immunologically, the 114 and 108 kDa proteins were in distinguishable while the 100 kDa protein was only weekly antigenic related to 114 and 108 kDa (Smith, 1988).

In rice endosperm, purification of four isoforms of SBE but it differ immunological and proteinase digestion patterns in two forms. Further studies revealed that the SBEII isoform was composed of two proteins (SBEIIa and SBEIIb) whish SBEIIa was found only in the endosperm (Mizuno *et al.*,1992; Nakamura *et al*., 1992).

Plant branching enzyme (Q-enzyme) was first identified in potato (Drummond *et al*., 1975), the purified SBE had monomeric molecular weight ranging from 83-103kD. The K_m 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 (Blennow and Johanssons, 1991). 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 (Larsson et al.,1996). The cDNA of potato SBE was cloned and identified as B class (major form) (Kobmann *et al*., 1991) and A class (minor form) (Safford *et al*.,1999). 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 (Rydberg *et al.*, 2001).

In cereal grain, SBE was found in many crops such as BE I, IIa and IIb in developing kernels of maize (Boyer and Preiss,1978). 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 (Yamonchi and Nakamura, 1992).

The three isoforms of SBE were purified from tubers of cassava cultivar KU 50 (Yaiyen *et al.*, 2004). Form 1 and 2 showed the molecular weight of 105 and 57 kDa and

preferred amylopectin as substrate. Form 3 showed the molecular weight of 57 and preferred amylose as substrate.

In molecular characterization, hereafter the cDNA or gene encodes the SBEI and SBEII isoforms will be referred to as *sbe*1 and *sbe*2 as proposed by the commission on plant gene nomenclature (Smith and Preiss, 1944). The cDNA encoding the SBEI isoform has been isolated from maize (Baba *et al.*, 1991), potato (Poulsen and Kreiberg, 1993), rice (Mizuno *et al.*, 1992), cassava (Salehuzzamen *et al.*,1992) and pea (Burton et al., 1995). The encode the SBEII has been isolated from maize (Fisher *et al.*, 1993), rice (Mizuno *et al.*, 1992), cassava (Johnsons *et al.*, 2003) and pea (Burton *et al.*, 1995). The cDNA coding for Cassava (*Manihot esculenta* Crantz.) branching enzyme was cloned from λgt11 cDNA library using a potato cDNA probe (Salehuzzaman *et al.*, 1992). Next, The cloned encoding SBE II was isolated and examined on the spatial and temporal expression of the *sbeII* gene (Baguma *et al.*, 2003).

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.

Aims of the Dissertation

1. To clone genes of SBE from cDNA of the enzymes prepared for cassava tuber KU50.
2. To Express the recombinant enzymes and characterize them in compare with nature SBE.
3. To prepare antibodies against all SBE isoforms from native and recombinant enzyme and detect the immunological activity by western blot.
4. To use the antibodies to follow developmental pattern of SBE in the tubes at different ages.

CHAPTER II

MATERIALS AND METHODS

2.1 Material

2.1.1 Full length cDNA and Native enzyme

Full length cDNA was cloned from Dr. Supatcharee Netrphan

- full-length *sbeI* cDNA in pCR-Blunt vector

- full-length *sbeII* cDNA in pTriplEx2 vector

Native enzyme from cassava tuber (*Manihot esculenta* Crantz. cv. KU50)

2.1.2 Enzyme and Restriction enzymes

Go taq DNApolymerase I (Promega U.S.A.)

Restriction enzyme: *Sal*I (New England BioLabs, Inc., U.S.A.)

*Xho*I (New England BioLabs, Inc., U.S.A.)

*BamH*I (New England BioLabs, Inc., U.S.A.)

RNase: Sigma, U.S.A

T_4DNA ligase: New England BioLabs, Inc., U.S.A

2.1.3 Vectors

pET 28 is bacterial expression vector with T7 and *Lac* promoters, adds T7 and 6xHis tags, includes *Lac*I gene; kanamycin restistance; *Cpo*I-based in-frame, single-cut directional cloning.

2.1.4 Host cells

E. coli rosetta gami with genotype : Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA PvuII phoR araD139 ahpC galE galK rpsL* (DE3) F′[*lac+ lacIq pro*] *gor522*::Tn*10 trxB* pLysSRARE2 (CamR, StrR, TetR), Novagen

2.2 Methodology

2.2.1 Cloning for starch branching enzyme

2.2.1.1 Plasmid extraction

The cDNA of *sbeI* and *sbeII* was cloned in to pCR-Blunt vector (Invitrogens) and pTriplEx2 vector (Clontech), respectively. They were extracted by alkaline lysis (Sambrook *et al*., 1989).

2.2.1.2 PCR amplification of *sbeI* and *sbeII* gene

Each starch branching enzyme (*sbe*) genes were amplified using PCR method. Primers for *sbe* genes were designed , the forward primer of *sbe* genes were designed to cover open reading frame (*ORF*) and restriction site, while the reverse primer were designed for stop codon usage and restriction site(Table2.1). The reaction mixture contained 2.5 units of Taq polymerase, 10 mM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer and 0.5 µg of template. The condition followed Table 2.2. The PCR product was electrophoresed on agarose gel. Finally, the selected PCR fragments were harvested from agarose gel by gel extraction kit (Geneaid).

2.2.1.3 Vector and PCR product preparation

Different type of pET28 plasmid was used with each *sbe* gene, *sbeI* gene was expressed in pET28c where as *sbeII* was expressed in pET28b (Figure 2.1). They were linearized with *Sal*I and *Xho*I in pET28c and *Sal*I and *BamH*I in pET28b. The

reaction mixture contained 2 units of each restriction enzymes, and incubated at 37°C for 20 hours. The linear form of pET28 was recovered from agarose gel by gel extraction kit (Geneaid). After that, the PCR product was ligated in pGEM-T Easy vector for further used.

The *sbe* fragments from pGEM-T Easy vector were digested with restriction enzymes, *sbeI* with *Sal*I and *Xho*I and *sbeII Sal*I and *BamH*I. The reaction mixture contained 2 units of each restriction enzymes, and incubated at 37°C for 20 hours. They were recovered from agarose gel by gel extraction kit (Geneaid).

2.2.1.4 Ligation of vector and *sbe* gene fragments

The digested *sbe* fragments were ligated to the selected vectors, *sbeI* to pET28c and *sbeII* to pET28b. The ligation mixture contained 30 nmoles of vector, 100 nmoles of fragment and 4 units of T_4 ligase and incubated overnight at 16°C. The recombinant plasmid from this reaction was further transformed into host cell by electroporation

2.2.1.5 Transformation

A fresh overnight culture *E. coli* rosetta gami (DE) was inoculated into LB broth. Cells were grown to log phase (A_{600} about 0.4-0.5). The culture was chilled on ice for 15 minutes and then centrifuged at 2500xg for 15 minute at 4°C. The cells were washed with cold distilled water and centrifuged at 2,500xg for 15 minute at 4°C. The procedure was repeated twice. The cells were thenwashed with 10% glycerol and centrifuged at 2,500xg for 15 minutes at 4°C. Finally, cell pellet was resuspended in 2 ml of 10% glycerol. This suspension was stored at -80°C in 50µl aliquot until used.

2.2.1.6 Electroporation

The recombinant plasmids from 2.2.1.4 were transformed into competent cells of *E. coli* rosetta gami by eletroporation. In each electroporation mixture, one microliter of recombinant plasmid was mixed with 50µl of competent cells and placed on ice for 1 minute. The mixture was transferred to cold electroporation cuvette and applied one pulse with Gene pulser (Biorad). Subsequently, LB medium was immediately added to the cuvette, quickly resuspended and transferred to new sterile tube. This was incubated at 37°C for 1 hour and spreaded on LB agar plate containing 50 µg/ml Kanamycin, 34 µg/ml Chloramphenicol,12.5 µg/ml Streptomycin and incubated at 37°C for 20 hours. The cells containing the recombinant plasmids which could grow on the selective plates were picked and the plasmids were isolated.

Table 2.1 Primers for *sbe* genes

gene	Upstream primer	Downstream primer
sbeI	ACGCGTCGACGATGTTAGGTTCTTTGGGTCTGT	CCGCTCGAGTCATTTGTCATCCGATGTCTCT
sbeII	CGGGATCCTCTCAGCGAAATGGGACACT	ACGCGTCGACTATATCTTAACCGGCGACAGG

Table 2.2 Procedure's PCR of *sbe* gene

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 minute	1 cycle
Denaturation	95°C	1 minute	30 cycles
Annealing	60°C	1 minute	
Extension	72°C	2 minute	
Final extension	72°C	5 minute	1 cycle
Soak	4°C		

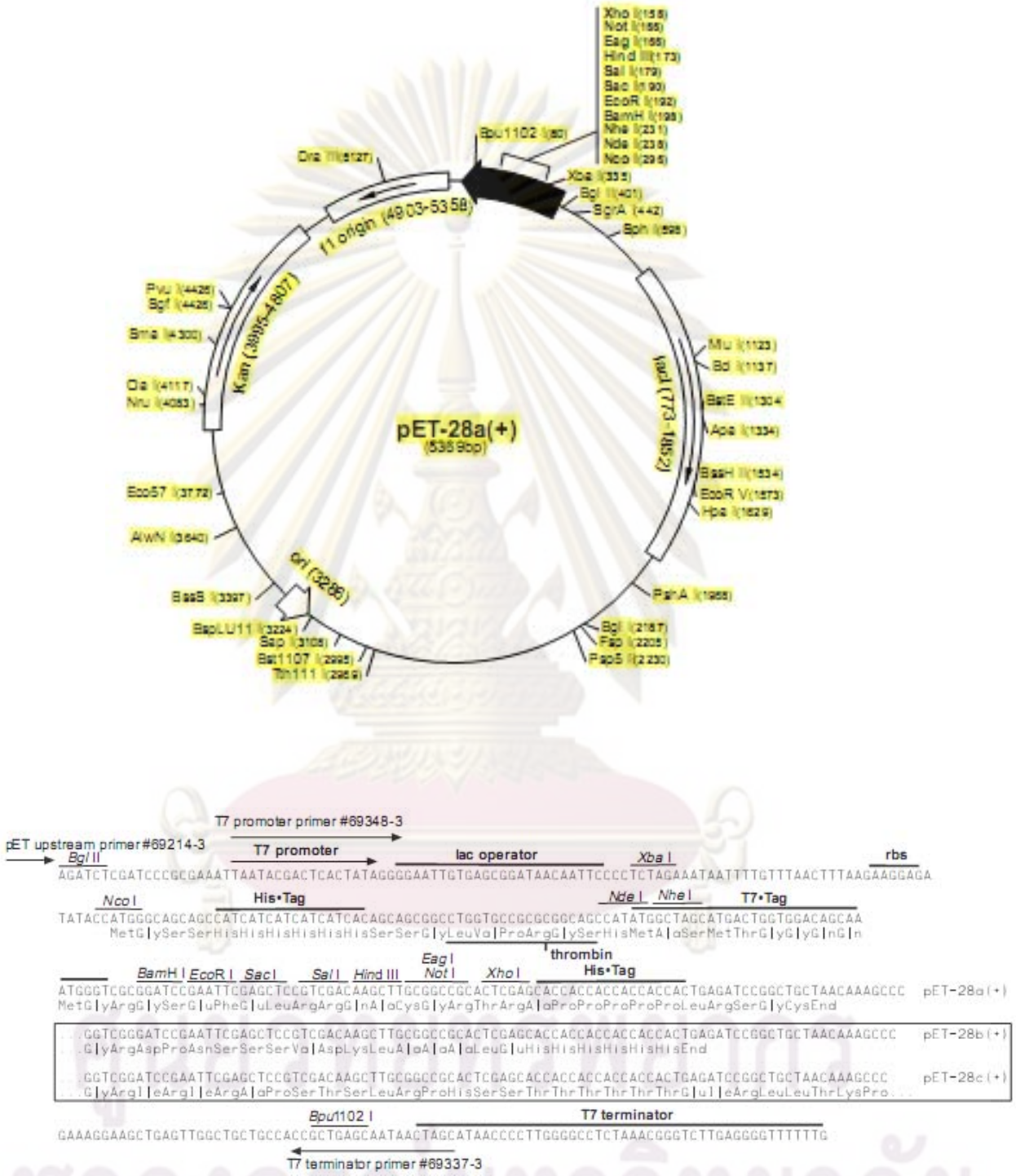


Figure 2.1 Expression vector of pET28 a-c(+)

2.2.2 Expression of *sbe* genes

2.2.2.1 Characterization of recombinant plasmids

The recombinant cells were grown in 50 µg/ml Kanamycin. The growing condition was 37°C for 20 hours with shaking. The cell cultures were collected in microcentrifuge tubes. Then, it was extracted by alkaline lysis. After that, the recombinant plasmids of *sbeI* and *sbeII* were completely digested with *Sal*I and *Xho*I, *Sal*I and *BamH*I, respectively. Finally, the digestion mixture was estimated by agarose gel electrophoresis.

2.2.2.2 Optimization of condition for *sbe* gene expression

The transformant of *E. coli* rosetta gami were grown overnight at 37°C in 5 ml LB broth containing 50 µg/ml Kanamycin, 34 µg/ml Chloramphenicol and 12.5 µg/ml Streptomycin. After, that , 1.0% of each cell culture was inoculated in 50 ml of same medium at 37°C with 250 rpm shaking. When, turbidity at A_{600} had reached 0.4, the transformant cuture was induced by IPTG at final concentration of 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM. The cells were harvest at various times 0, 2, 4, 6, 8, 12 and 16 hours and collected by centrifugation. For crude extraction preparation, the cell pellet was resuspended in 1 ml of cold extraction buffer and cells disrupted by sonication on ice. Unbroken cell and cell debris was removed by centrifugation at 12,000xg for 30 min at 4°C. The supernatant was assayed for SBE activity and protein concentration.

A portion of the cell harvest at various time points by centrifugation were resuspended in 100 µl of 5x SDS sample buffer and analyzed by SDS-PAGE.

2.3 Protein determination

Protein content 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.

2.4 Assay of SBE Activity assay

SBE activity was determined by 2 methods:

2.4.1 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 , method modified from that described by Mizuno *et a l.*, 2003

The reaction mixture of 0.2 ml contained 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 the methanol-insoluble portion was measured using liquid scintillation counter. One unit enzyme activity was defined 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.

2.4.2 Spectrophometric method

The modified assay was based on the spectral change of the glucan-iodine complex that occured after branching of the substrate (Boyer and Preiss, 1978).

The assayed was conducted in 0.4 ml reaction mixture containing 1 mg/ml potato amylose or potato amylopectin and 0.1 M MOPS-NaOH pH 7.0 with 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. mixed 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 was defined as the decrease in absorbance of 1.0 per minute at 37°C.

2.5 Polyacrylamide gel electrophoresis (PAGE)

2.5.1 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.

2.5.2 SDS - PAGE

The denaturing gel was carried out on slab gelwith 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 an electrophoresis unit form cathode towards anode.

2.6 Preparation of recombinant SBE

2.6.1 Crude enzyme

E. coli rosetta gami transformants were grown overnight at 37°C in 5 ml LB broth medium containing 50 µg/ml Kanamycin, 34 µg/ml Chloramphenical and 12.5 µg/ml Streptomycin. After that, 1.0% cell culture was inoculated in 50 ml of same medium at 37°C with 250 rpm shaking. When the tubidity at A_{600} had reached 0.4, IPTG was added to final concentration 0.4 mM to induce *sbeI* and *sbeII* gene expression and continue cultivation for 4 hours. The cell was harvested at 10,000xg for 15 min, and then washed with cold 0.85% NaCl. After that, the cell pellet was washed once in cold extraction buffer (0.05 M Tris-HCl pH 7.4 containing 0.1 mM PMSF, 1 mM DTT and 2 mM EDTA) and centrifuged again. The cell pellet was stored at -80°C until used.

Preparation of crude extract was performed by resuspending the cell pellet in 5 ml of cold extraction buffer, then the cells were disruptedl by sonication on ice. Unbroken cells and cell debris were removed together by centrifugation at 12,000xg for 30 min at 4°C. The supernatant was collected for further step.

2.6.2 Enzyme purification

The recombinant enzyme was purified by His-tag column chromatography. Firstly, the column was charged by 0.1 M $NiSO_4$, and equilibrated with starting buffer (0.05 M Tris-HCl pH 7.5) containing 0.2 mM Imidazole and 1 mM DTT. Recombinant enzyme was applied to the column at flow rate 0.75 ml/min followed by washing with starting buffer. Finally, the recombinant SBE was eluted by starting buffer containing 0.5 M Imidazole, 0.3 M NaCl and 1 mM DTT.

2.7 Preparation of native starch branching enzyme from cassava tubers

2.7.1 Crude starch branching enzymes

Cassava tubers at different ages 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.

2.7.2 Purification of SBE isoforms

The native crude enzyme from 9 month old tubers was purified according to Yaiyen (2008). Crude enzyme was precipitated with PEG 6000 to remove contaminating enzymes. Contaminating proteins in the supernatant was further removed by first DEAE-Toyopearl column eluted with 0.15 M NaCl in 0.05 M Tris-HCl pH 7.5 contained 2 mM EDTA and 1 mM DTT. at flow rate 60 ml/h. The three isoforms were

separated with second DEAE-Toyopearl column by stepwise elution with 0.04 M, 0.08 M and 0.1 M sodium chloride respectively, at flow rate 60 ml/h. followed by Q-Sepharose column chromatography of each isoform. They were collected as purified isoforms and kept at 4°C for further work.

2.8 Western blot analysis

2.8.1 Preparation of crude enzyme at different ages

Cassava tubers in different ages 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

2.8.2 Preparation of SBE antibodies

The purified forms of three SBE isoforms from cassava tuber and recombinant enzymes were sent for rasing their polyclonal antibodies in rabbit at Department of Medical technology, Chiang Mai University. The polyclonal antibodies were conjugated with horse radish peroxidase. N,N'-diclohexyl carbodiimide was used as cross linking reagent by the method adapted from that described by Wehiky *et al.* (1969).

2.8.3 Western blot

Following electrophoresis by SDS-PAGE using method described in section 2.5.2, the gel as well as the nitrocellulose membrane were incubated for 15 min in tank blotting transfer prior to blotting. Sheets of material were placed up against one another

in this order while avoiding air bubble: filter paper on fiber pad, the gel, the membrane, filter paper and finally fiber pad (Figure in Appendix C). The installed electroblot unit was filled with about 2 liters of tank blotting transfer buffer. The separated protein on the gel were then electrically transferred onto the nitrocellulose membrane from the cathode towards anode at 50 volts for 2.5 hours. During the transfer, the system was cooled down to 4°C on ice bath. After the transfer, the orientation of the gel was marked on the membrane. The membrane was washed twice ,10 minute each time, with PBS buffer (0.1 M Phosphate buffer pH 7.4 containing 0.15 M NaCl) at room temperature. The membrane was incubated in the blocking solution (3% BSA in PBS buffer) at 37°C for 1 hour. After that, the membrane was washed twice for 10 minute in PBS buffer containing 0.025% (v/v) Tween 20 and rinsed 10 min with PBS buffer at room temperature. Subsequently, the membrane was incubated 1 hour in polyclonal antibody (at 1:100 dilution). The membrane was washed twice for 10 min of PBS buffer at room temperature. After washing immunoactivity staining was developed while keeping the membrane stationary with 3,3'-Diaminobezidine (DAB) and 30% hydrogen peroxide until bands were visible. The chromogenic reaction was stopped by rinsing membrane with distilled water.

2.9 Characterization of recombinant SBE

2.9.1 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 Chemical modification of starch branching enzymes

The effects of the modified reagent at 10 mM of IAA (iodoacetic acid), NEM (N-ethyl-maleimide), EDC (1-Ethly-3-(3-diaminopropyl) carbodiimide), TNBS (Trinitrobenezene sulphonic acid), PGO (Phynylglyoxal), DEPC (Diethypyrocarbonate), NAM (N-Acetylimidazol) and NBS (N-Bromo-succinimide) were added separately to reaction mixtures containing appropriate amount of SBE. The reactions were stopped by heating in boiling water after incubation at 37°C for 1 hour. The SBE activity in each reaction was measured as described in section 2.4.1. The result was expressed as the percentage relative activity.

2.9.3 Effect of pH on starch branching enzyme activity

The purified recombinant SBEs were used to study the effect of pH on its activity. The enzyme was assayed as described in section 2.4.2.The enzyme was incubated with substrate solutions prepared at various pH's in appropriate buffer. 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.

2.9.4 Effect of Temperature on starch branching enzyme activity

The recombinant SBEs were 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 temperature of the reaction was brought to 37 °C , the activity was measured as described in section 2.4.2.The result was expressed as the percentage relative activity.

2.9.5 Determination of K_m and V_{max}

The recombinant SBEs were used to study for kinetic constant with potato amylose as the substrate of this enzyme. The reaction mixture consisted of various concentrations of amylose, 0.05, 0.075, 0.1, 0.2, 0.3, 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.

The reaction was incubated for 30 min and stopped by heating in boiling water for 5 minutes, the enzyme activity was measured as described in section 2.4.2.

2.9.6 Comparison of SBE activity using amylose and amylopectin as substrates

The recombinant SBEs were used to study substrate specificity for amylase and amylopectin. The enzyme was assayed by incubation with each substrate at 37 °C for 0, 5, 10, 20, 30, 60 and 120 minutes. After the reaction was stopped by heating in boiling water for 5 minutes, the activity was measured as describe in section 2.4.2.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%.

2.9.7 Analysis of amino acid compositions

To determined amino acid compositions, each native enzyme were deproteinized with C18 cartridge. The amino acid in the each native enzyme were deriavatized before injection. The derivatization was mixed with ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC). Derivatized samples were analyzed by reversed phase HPLC equipped with diode array detector. The separation was performed using amino acid column (200x2.1 mm). The oven temperature was 40°C. The injection program was shown in table 2.3.

The gradient was established with two solvents: A (20 mM sodium acetate pH7.2 containing 0.018% triethylamine) and B (20% of 100 mM sodium acetate pH 7.2 containing 40% acetonitrile and 40% methanol. The gradient program was shown in Table 2.3. The detection wavelength was 338 nm.

Table 2.3 Gradient used for analysis of amino acid

Elution Time (min)	%Solvent A	%Solvent B	Flow rate (ml/min)
17	100	Auto	0.45
18	40	60	0.45
18.1	0	100	0.45
18.5			0.8
23.9			0.8
24		100	0.45
25	100		0.45

CHAPTER 3

RESULTS

In the following chapters, both native and recombinant starch branching enzymes (SBEs) were studied. To avoid confusion, the following abbreviations would be used to represent each form of SBEs:

SBE1 = isoform 1 of native SBE from cassava tuber.

SBE2 = isoform 2 of native SBE from cassava tuber.

SBE3 = isoform 3 of native SBE from cassava tuber.

SBERI = recombinant SBEI encoded by *sbe* I gene.

SBERII = recombinant SBEII encoded by *sbe* II gene.

3.1 Cloning of cassava *sbe*I and *sbe*II cDNAs into *E. coli* expression vector

Two cDNA clones encoding full-length SBEI, in pCR-Blunt vector, and full-length SBEII, in pTriplEx2 vector, were kindly obtained from Dr. Supatcharee Netrphan, BIOTEC, and used for the production of recombinant proteins in an *E. coli* expression vector.

3.1.1 Characterization of the polypeptides encoded by *sbe*I and *sbe*II cDNAs from cassava

The nucleotide and deduced amino acid sequences of *sbe*I and *sbe*II cDNAs were shown in Figure 3.1 and 3.2, respectively. The open reading frame of *sbe*I cDNA is comprised of 2,559 nucleotides, thus coding for polypeptides of 852 amino acids. The

open reading frame of *sbe*II cDNA is 2,514 nucleotides long and codes for a polypeptide of 837 amino acids. The polypeptides encoded by *sbe*I and *sbe*II cDNAs were calculated to have molecular weight of approximately 95 kDa.

The deduced amino acid sequences of *sbe*I and *sbe*II cDNAs showed 49% similarity by CLUSTALW2 program (Figure 3.3) and 72.2% when using EMBOSS align (Figure 3.4). When compared with SBEs from other plants, the polypeptide encoded by *sbe*I cDNA showed 62, 68 and 67% similarity to SBEI from wheat (*Triticum aestivum*), maize (*Zea may*) and potato (*Solanum tuberosum*), respectively (Figure 3.5), while the polypeptide encoded by *sbe*II cDNA showed 70 and 72% similarity to SBEII from wheat (*Triticum aestivum*) and potato (*Solanum tuberosum*), respectively (Figure 3.6).

3.1.2 Amplification of *sbe* cDNA fragments for cloning into pET expression vectors

PCR was utilized to obtain the fragments that span to cover the coding regions of sbeI and sbeII cDNAs. The forward primers were designed to locate upstream of the start codon, while the reverse primers were located downstream of the stop codon. To facilitate directional cloning of the amplified product into pET expression vectors, suitable restriction sites were added to the 5'-region of both primers. The PCR was performed using the conditions described in section 2.3 and the amplified products of approximately 2.8 and 2.7 kb were obtained from using *sbe*I and *sbe*II cDNA templates, respectively (Figure 3.7). These amplified products were purified from the gel and digested with *Sal*I and *Xho*I (for *sbe*I) and *Sal*I and *BamH*I (for *sbe*II).

(A)

GGACACTGACATGGACTGAAGGAGTAGAAACGACTGGAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAACGACTGGAGCACGAGGACA
CTGACATGGACTGAAGGAGTAGAAACGACTGGAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAACGACTGGAGCACGAGGACACTGAC
ATGGACTGAAGGAGTAGAAACGACTGGAGCACGAGGACACTGATGGATTGAATCGCTGAGGAAACACTGGAGCACGAGGACACTGACATGGAC
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GGCTGTGGAAAGAAACTGTCAAATTGTCAAACAAAAACAGATTGAACTGACTGGATGTCGAAAATTGCCTGGTTGTTCTAGATTCCTTTTTCT
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CTATCGTGAGTGGGCCCCTGCAGCTCAGGAAGCACAAGTTATTGGGGACTTTAATGGTTGGGATGGTTCCAACCATAGGATGGAAAAGAATGA
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TGGAGTTTGGGTTGATCGAATTCCAGCTTGGATTAGATATGCCACTGTGGACCCCACAAAATTTGGAGCACCATATGATGGTGTCTACTGGGA
TCCTCCACCTCCAGAAAGGTACCAATTCAAGTATCCCCGGCCTCCAAAAGCCCAGGCCCCTCGCATATATGAGGCTCATGTGGGAATGAGTAG
CTCAGAACCTCGCATTAATACATACAGAGAGTTTGCTGATGATGTTCTGCCTCGTATACGGGCAAACAACTATAATACGGTTCAGTTAATGGC
TGTTATGGAGCATTCATATTATGGGTCGTTTGGGTACCATGTTACAAACTTTTTTGCTGTAAGCAGTAGATCTGGAACTCCTGAGGATCTTAA
ATATTTAATTGATAAAGCTCATAGCTTGGGTTTAAGTGTTCTGATGGATGTTGTTCACAGTCACGCAAGTAACAATATCACTGATGGACTTAA
TGGCTTTGATGTTGGCCAAAGCACTCAAGATTCCTACTTTCACACTGGAGATCGAGGCTACCATAAGCTATGGGATAGCAGACTCTTTAACTA
TGCTAATTGGGAAGTTATTCGCTTCCTTCTGTCCAACTTAAGATGGTGGCTTGAGGAGTACAAATTTGATGGATTCCGATTTGACGGAGTAAC
ATCAATGTTGTACCATCATCATGGGATCAACATGGCATTTACAGGGGATTATAATGAGTATTTCAGTGAGGCAACTGATATTGATGCCGTTGT
TTATCTGATGCTGGCCAATTCTCTGATTCACAACATCTTGCCTGATGCTACTGTGATTGCTGAAGATGTTTCTGGCATGCCTGGGCTTGGCCG
TTCTGTCTCTGAGGGGGGAATAGGTTTTGACTATCGCCTGCAATGGCCATCCCTGACAAATGGATCGATTACTTGAAAAACAAGAGTGATGAA
GAGTGGTCAATGAAGGAAATCTCATGGAGCTTAACTAATAGGAGATACACTGAGAAATGTGTTGCTTATGCTGAGAGTCATGACCAAGCCATT
GTAGGTGACAAGACGGTTGCCTTTTTATTAATGGATAAAGAAATGTATTATGGAATGTCTTGTTTGACAGATGCTTCACCTATGGTTGATCGA
GGGGTAGCGCTTCATAAGATGGTTCATCTTTTAACTATGGCTTTAGGAGGTGAGGGCTACCTTAATTTTATGGGAAATGAGTTTGGCCATCCT
GAGTGGATTGACTTCCCAAGAGAAGGCAATGGGTGGAGTTATGACAAGTGCAGACGCCAATGGAACCTAGTTGACACTGAACACTTGAGATAC
AGGTTCATGAATGCATTTGACAAGGCTATGAACTTGCTTGATGAAAAGTATTCATTTCTAGCATCAACAAAGCAGATTGTGAGCAGCACAAAT
GAAGAGGATAAGGTTATCGTCTTTGAGCGTGGGGACCTGGTTTTTGTATTCAATTTTCATCCAGAGAATACATATGATGGGTACAAGGTTGGT
TGCGACTTGCCCGGAAAGTATCGAGTTGCATTGGATAGTGATGCTTGGGAGTTTGGTGGACGTGGAAGAGTGGGCCATGATGTGGACCATTTT
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GTTTACTATAGAGTTGAAGAAAAAGAAGGAAATCACAACAGTAGTGATATTGGTGCTGCAAATGAGACATTGACAGACATTGCAAAGCTGGGA
GATTTTGAAGGTATCAACGAGACATCACCAGCAGATGCTGTGGCAAAGCAGGAGGATCTTAAGGCAGCACAACCTTCTTTGATTGCCGATGAT
ATTGCAACAAAGGCAAACACAGAAACAGAAGAGATTGAGGAAGAGACATCGGATGACAAATGAAAGTGACAATCCATCATCCAATTCCTGAGT
TCAGGTTTAGACATGAGGCTTTATCACTGGGAAGTTCTTTTTCAGGTTTTAAATTGCAAGGAGATTTTGTATGGCCCTCATCAGTAGACATCA
GTAGGTATGCTGCTGTAAATAAGTCAGCTTGCTGTTAAGTTATGCTTTGCTTAATTGCTGGGGAGTTGTCTTGTAAATAAAAAATAAATAAAT
CCTTATCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

(B)

MLGSLGLFPAPDFGSLSPSLAKNSKRAVERNCQIVKQKQIELTGCRKLPGCSRFLFLPRISIDKRVKQGLAISAAVADEKKMITSFEEDMEIT
GLLSIDPGLESFKDHFRYRMQRFTNQKQLIEKYEGGLEEFSNGYLKFGFNREAGGIVYREWAPAAQEAQVIGDFNGWDGSNHRMEKNEFGVWS
INIPDSGGNPAIHHNSRVKFRFKHGDGVWVDRIPAWIRYATVDPTKFGAPYDGVYWDPPPPERYQFKYPRPPKAQAPRIYEAHVGMSSSEPRI
NTYREFADDVLPRIRANNYNTVQLMAVMEHSYYGSFGYHVTNFFAVSSRSGTPEDLKYLIDKAHSLGLSVLMDVVHSHASNNITDGLNGFDVG
QSTQDSYFHTGDRGYHKLWDSRLFNYANWEVIRFLLSNLRWWLEEYKFDGFRFDGVTSMLYHHHGINMAFTGDYNEYFSEATDIDAVVYLMLA
NSLIHNILPDATVIAEDVSGMPGLGRSVSEGGIGFDYRLAMAIPDKWIDYLKNKSDEEWSMKEISWSLTNRRYTEKCVAYAESHDQAIVGDKT
VAFLLMDKEMYYGMSCLTDASPMVDRGVALHKMVHLLTMALGGEGYLNFMGNEFGHPEWIDFPREGNGWSYDKCRRQWNLVDTEHLRYRFMNA
FDKAMNLLDEKYSFLASTKQIVSSTNEEDKVIVFERGDLVFVFNFHPENTYDGYKVGCDLPGKYRVALDSDAWEFGGRGRVGHDVDHFTSPEG
IPGVPETNFNNRPNSFKILSAARTCVVYYRVEEKEGNHNSSDIGAANETLTDIAKLGDFEGINETSPADAVAKQEDLKAAQPSLIADDIATKA
NTETEEIEEETSDDK*

Figure 3.1 Full-length *sbe*I cDNA from cassava

A) The coding region of *sbe*I cDNA was highlighted in grey.

B) The deduced amino acid sequences of *sbe*I cDNA were shown.

(A)

GACACTCTCTCTAATTTCTCAGCGAAATGGGACACTACACCATATCAGGAATACGTTTTCCTTGTGCTCCACTATGCAAATCTCAATCTACCG
GCTTCCATGGCGATCGGAGGACCTCCTCTTGCCTTTCCTTCAACTTCAAGAAGGAGGCGTTTTCTAGGAGGGTCTTCTCTCTGGAAAGTCATCTC
ATGAATCTGACTCCTCAAATGTAATGGTCACTGCGTCTAAAAGAGTCCTTCCTGATGGTCGGATTGAATGCTATTCTTCTTCAACAGATCAAT
TGGAAGCCCCTGGCACAGTTTCAGAAGAATCCCAGGTGCTTACTGATGTTGAGAGTCTCATTATGGATGATAAGATTGTTGAAGATGAAGTAA
ATAAAGAATCTGTTCCAATGCGGGAGACAGTTAGCATCAGAAAAATTGGATCTAAACCAAGGTCCATTCCTCCACCCGGCAGAGGGCAAAGAA
TATATGACATAGATCCAAGCTTGACAGGCTTTCGTCAACACCTAGATTACCGGTATTCACAGTACAAAAGACTCCGAGAAGAAATTGACAAGT
ATGAAGGTGGTCTGGATGCATTTTCTCGTGGCTATGAAAAGTTTGGTTTCTCACGCAGTGAAACAGGAATAACTTATAGAGAGTGGGCACCAG
GAGCTACGTGGGCTGCATTGATTGGAGATTTCAATAACTGGAATCCTAATGCAGATGTCATGACTCAGAATGAGTGTGGCGTCTGGGAGATCT
TTTTGCCGAATAATGCAGATGGTTCACCACCAATTCCCCATGGTTCTCGAGTAAAGATACGCATGGATACTCCATCTGGCAACAAAGATTCTA
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TCAAAAATCCTCAGCCAAAGAGACCAAAATCACTTCGGATTTATGAGTCGCACGTTGGAATGAGTAGTACGGAGCCAGTAATTAACACATATG
CCAACTTTAGAGATGATGTGCTTCCTCGCATCAAAAAGCTTGGCTACAATGCTGTTCAGCTCATGGCTATTCAAGAGCATTCATATTATGCTA
GTTTTGGGTATCACGTCACAAACTTTTATGCAGCTAGCAGCCGATTTGGAACTCCTGATGATTTAAAGTCTCTAATAGATAAAGCTCACGAGT
TAGGTCTTCTTGTTCTCATGGATATTGTTCATAGCCATGCATCAACTAATACGTTGGATGGGCTGAATATGTTTGATGGTACGGATGGTCACT
ACTTTCACTCTGGACCACGGGGTCATCATTGGATGTGGGACTCTCGCCTTTTCAACTATGGGAGCTGGGAGGTTCTAAGGTTTCTTCTTTCAA
ATGCAAGGTGGTGGTTGGATGAGTACAAGTTTGATGGGTTCAGATTTGATGGGGTGACTTCAATGATGTACACCCATCATGGATTGCAGGTAG
ATTTTACCGGCAACTACAATGAATACTTTGGATATGCAACTGATGTAGATGCTGTGGTTTATTTGATGCTGTTGAATGATATGATTCATGGTC
TCTTCCCAGAGGCTGTCACCATTGGTGAAGATGTTAGTGGAATGCCAACAGTTTGCATTCCGGTTGAAGATGGTGGTGTTGGCTTTGATTATC
GTCTCCACATGGCTGTTGCTGATAAATGGGTTGAGATTATTCAGAAGAGAGATGAAGATTGGAAAATGGGTGACATTGTACACATGCTGACCA
ACAGGCGGTGGTTGGAAAAGTGTGTTTCTTATGCTGAAAGTCATGACCAGGCCCTTGTTGGTGACAAAACTATTGCATTTTGGCTGATGGACA
AGGATATGTATGACTTCATGGCTCTTGACGGACCATCTACTCCTCTCATAGATCGTGGAGTAGCATTGCAAAAAATGATCAGGCTTATTACCA
TGGGATTAGGCGGAGAAGGATATTTGAATTTTATGGGAAATGAATTTGGACACCCCGAGTGGATTGATTTTCCAAGAGGTGATCTACATCTTC
CCAGTGGTAAATTTGTTCCTGGGAACAATTACAGTTATGATAAATGCCGGCGTAGGTTTGATCTAGGCAATTCAAAGCGTCTGAGATATCATG
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TAAAGCCAGGAAAGTACAAGATAGTCTTGGATTCAGATGATCCTTTGTTTGGAGGCTTTGGCAGGCTTAGTCATGATGCAGAGCACTTCAGCT
TTGAAGGGTGGTACGATAACCGGCCTCGATCCTTCATGGTGTACACACCATGTAGAACAGCAGTGGTCTATGCTTTAGTGGAGGATGAAGTGG
AGAATGAAGTGGAACCTGTCGCCGGTTAAGATATATCTTAGCAACAGATTTCCATCCTGGTTCTTGGTATTTTGTTGTCATGATAAACATAAT
CAAAGACCAATAGGAAACGCAGGGTTACATGCCAGCTTCCATCATCATAGGCAGCTCAGACCTCCTAAACCATAAATCTTCAAGTTGCTTGCG
TTCGGTAGTATGTTATGTGGTACTTTGCAATCTTAAATTATCATGATCGCTGTGGATGCTAACTATGACAATTTTGTATATATGCCAACGAGG
ATTTTAAGTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

(B)

MGHYTISGIRFPCAPLCKSQSTGFHGDRRTSSCLSFNFKKEAFSRRVFSGKSSHESDSSNVMVTASKRVLPDGRIECYSSSTDQLEAPGTVSE
ESQVLTDVESLIMDDKIVEDEVNKESVPMRETVSIRKIGSKPRSIPPPGRGQRIYDIDPSLTGFRQHLDYRYSQYKRLREEIDKYEGGLDAFS
RGYEKFGFSRSETGITYREWAPGATWAALIGDFNNWNPNADVMTQNECGVWEIFLPNNADGSPPIPHGSRVKIRMDTPSGNKDSIPAWIKFSV
QAPGELPYNGIYYDPPEEEKYVFKNPQPKRPKSLRIYESHVGMSSTEPVINTYANFRDDVLPRIKKLGYNAVQLMAIQEHSYYASFGYHVTNF
YAASSRFGTPDDLKSLIDKAHELGLLVLMDIVHSHASTNTLDGLNMFDGTDGHYFHSGPRGHHWMWDSRLFNYGSWEVLRFLLSNARWWLDEY
KFDGFRFDGVTSMMYTHHGLQVDFTGNYNEYFGYATDVDAVVYLMLLNDMIHGLFPEAVTIGEDVSGMPTVCIPVEDGGVGFDYRLHMAVADK
WVEIIQKRDEDWKMGDIVHMLTNRRWLEKCVSYAESHDQALVGDKTIAFWLMDKDMYDFMALDGPSTPLIDRGVALQKMIRLITMGLGGEGYL
NFMGNEFGHPEWIDFPRGDLHLPSGKFVPGNNYSYDKCRRRFDLGNSKRLRYHGMQEFDQAIQHLEEAYGFMTSEHQYISRKDERDRIIVFER
GNLVFVFNFHWTSSYSDYRVGCLKPGKYKIVLDSDDPLFGGFGRLSHDAEHFSFEGWYDNRPRSFMVYTPCRTAVVYALVEDEVENEVEPVAG
*

Figure 3.2 Full-length *sbe*II cDNA from cassava

A) The coding region of *sbe*II cDNA was highlighted in grey.

A) The deduced amino acid sequences of *sbe*II cDNA were shown.

```
SBERI           ----MLGSLGLFPAPDFGSLSPSLAKN-----------SKRAVERNCQIVKQKQIELTGC 45
SBERII          MGHYTISGIRFPCAPLCKSQSTGFHGDRRTSSCLSFNFKKEAFSRRVFSGKSSHESDSSN 60
                    :..: :  **   * *..:  :           .*.*..*.    *..: . :.

SBERI           RKLPGCSRFLFLPRISIDKRVKQGLAISAAVADEKKMITSFE----------------- 87
SBERII          VMVTASKRVLPDGRIECYSSSTDQLEAPGTVSEESQVLTDVESLIMDDKIVEDEVNKESV 120
                 :....*.*   **.  .  .: *  ..:*::*.:::*..*

SBERI           ---EDMEITGLLS----------------IDPGLESFKDHFRYRMQRFTNQKQLIEKYEG 128
SBERII          PMRETVSIRKIGSKPRSIPPPGRGQRIYDIDPSLTGFRQHLDYRYSQYKRLREEIDKYEG 180
                  * :.*  : *               ***.* .*::*: ** .::.. :: *:****

SBERI           GLEEFSNGYLKFGFNREAGGIVYREWAPAAQEAQVIGDFNGWDGSNHRMEKNEFGVWSIN 188
SBERII          GLDAFSRGYEKFGFSRSETGITYREWAPGATWAALIGDFNNWNPNADVMTQNECGVWEIF 240
                **: **.** ****.*.  **.******.*  * :*****.*: . . * :** ***.*

SBERI           IPD-SGGNPAIHHNSRVKFRFKHGDGVWVDRIPAWIRYATVDPTKFGAPYDGVYWDPPPP 247
SBERII          LPNNADGSPPIPHGSRVKIRMDTPSGN-KDSIPAWIKFSVQAPGEL--PYNGIYYDPPEE 297
                :*: :.*.*.* *.****:*:.  .*   * *****:::.  * ::  **:*:*:***

SBERI           ERYQFKYPRPPKAQAPRIYEAHVGMSSSEPRINTYREFADDVLPRIRANNYNTVQLMAVM 307
SBERII          EKYVFKNPQPKRPKSLRIYESHVGMSSTEPVINTYANFRDDVLPRIKKLGYNAVQLMAIQ 357
                *:* ** *:* :.:: ****:******:** **** :* *******:  .**:*****:

SBERI           EHSYYGSFGYHVTNFFAVSSRSGTPEDLKYLIDKAHSLGLSVLMDVVHSHASNNITDGLN 367
SBERII          EHSYYASFGYHVTNFYAASSRFGTPDDLKSLIDKAHELGLLVLMDIVHSHASTNTLDGLN 417
                *****.*********:*.*** ***:*** ******.*** ****:******.*  ****

SBERI           GFDVGQSTQDSYFHTGDRGYHKLWDSRLFNYANWEVIRFLLSNLRWWLEEYKFDGFRFDG 427
SBERII          MFDG---TDGHYFHSGPRGHHWMWDSRLFNYGSWEVLRFLLSNARWWLDEYKFDGFRFDG 474
                **    *:. ***:* **:* :********..***:****** ****:***********

SBERI           VTSMLYHHHGINMAFTGDYNEYFSEATDIDAVVYLMLANSLIHNILPDATVIAEDVSGMP 487
SBERII          VTSMMYTHHGLQVDFTGNYNEYFGYATDVDAVVYLMLLNDMIHGLFPEAVTIGEDVSGMP 534
                ****:* ***::: ***:*****. ***:******** *.:**.::*:*..*.*******

SBERI           GLGRSVSEGGIGFDYRLAMAIPDKWIDYLKNKSDEEWSMKEISWSLTNRRYTEKCVAYAE 547
SBERII          TVCIPVEDGGVGFDYRLHMAVADKWVEIIQ-KRDEDWKMGDIVHMLTNRRWLEKCVSYAE 593
                :  .*.:**:****** **:.***:: :: * **:*.* :*   *****: ****:***
SBERI           SHDQAIVGDKTVAFLLMDKEMYYGMSCLTDASPMVDRGVALHKMVHLLTMALGGEGYLNF 607
SBERII          SHDQALVGDKTIAFWLMDKDMYDFMALDGPSTPLIDRGVALQKMIRLITMGLGGEGYLNF 653
                *****:*****:** ****:**  *:   ::*::******:**::*:**.*********

SBERI           MGNEFGHPEWIDFPR-----------EGNGWSYDKCRRQWNLVDTEHLRYRFMNAFDKAM 656
SBERII          MGNEFGHPEWIDFPRGDLHLPSGKFVPGNNYSYDKCRRRFDLGNSKRLRYHGMQEFDQAI 713
                ***************           **.:********:::* ::::***: *: **:*:

SBERI           NLLDEKYSFLASTKQIVSSTNEEDKVIVFERGDLVFVFNFHPENTYDGYKVGCDLPGKYR 716
SBERII          QHLEEAYGFMTSEHQYISRKDERDRIIVFERGNLVFVFNFHWTSSYSDYRVGCLKPGKYK 773
                : *:* *.*::* :* :* .:*.*::******:********  .:*..*:***  ****:

SBERI           VALDSDAWEFGGRGRVGHDVDHFTSPEGIPGVPETNFNNRPNSFKILSAARTCVVYYRVE 776
SBERII          IVLDSDDPLFGGFGRLSHDAEHFS--------FEGWYDNRPRSFMVYTPCRTAVVYALVE 825
                :.****   *** **:.**.:**:         *  ::***.** : :..**.***  **
```

(Continued)

```
SBERI            EKEGNHNSSDIGAANETLTDIAKLGDFEGINETSPADAVAKQEDLKAAQPSLIADDIATK 836
SBERII           D-------------------------EVENEVEPVAG---------------------- 837
                 :                          *  **..*. .

SBERI            ANTETEEIEEETSDDK 852
SBERII           ----------------
```

Figure 3.3 Alignment of deduced amino acid sequences of *sbe*I and *sbe*II cDNAs (SBERI and SBERII, respectively) using CLUSTALW program

Conserved residues are indicated by asterisk (*).

Same group of side chain and similar size are indicated by semi colon (:)

Same group of side chain but different size are indicated by full stop (.)

```
SBERI             95 LLSIDPGLESFKDHFRYRMQRFTNQKQLIEKYEGGLEEFSNGYLKFGFNR    144
                     :..|||.|..|:.|..||..::...::.|:||||||:.||.||.||||:|
SBERII           147 IYDIDPSLTGFRQHLDYRYSQYKRLREEIDKYEGGLDAFSRGYEKFGFSR    196

SBERI            145 EAGGIVYREWAPAAQEAQVIGDFNGWDGSNHRMEKNEFGVWSINIPDSG-    193
                     ...||.||||||.|..|.:|||||.|:.:...|.:||.|||.|.:|::.
SBERII           197 SETGITYREWAPGATWAALIGDFNNWNPNADVMTQNECGVWEIFLPNNAD    246

SBERI            194 GNPAIHHNSRVKFRFKHGDGVWVDRIPAWIRYATVDPTKFGAPYDGVYWD    243
                     |:|.|.|.||||.|.....|. .|.|||||::...|.:.  ||:|:|:|
SBERII           247 GSPPIPHGSRVKIRMDTPSGN-KDSIPAWIKFSVQAPGEL--PYNGIYYD    293

SBERI            244 PPPPERYQFKYPRPPKAQAPRIYEAHVGMSSSEPRINTYREFADDVLPRI    293
                     ||..|:|.||.|:|.:.::.||||:||||||:||.||||..|.|||||||
SBERII           294 PPEEEKYVFKNPQPKRPKSLRIYESHVGMSSTEPVINTYANFRDDVLPRI    343

SBERI            294 RANNYNTVQLMAVMEHSYYGSFGYHVTNFFAVSSRSGTPEDLKYLIDKAH    343
                     :...||.|||||:.|||||.|||||||||:|.|||.|||:|||.||||||
SBERII           344 KKLGYNAVQLMAIQEHSYYASFGYHVTNFYAASSRFGTPDDLKSLIDKAH    393

SBERI            344 SLGLSVLMDVVHSHASNNITDGLNGFDVGQSTQDSYFHTGDRGYHKLWDS    393
                     .|||.||||:||||||.|..||||.||   .|...|||:|.||:|.:|||
SBERII           394 ELGLLVLMDIVHSHASTNTLDGLNMFD---GTDGHYFHSGPRGHHWMWDS    440

SBERI            394 RLFNYANWEVIRFLLSNLRWWLEEYKFDGFRFDGVTSMLYHHHGINMAFT    443
                     |||||.:|||:||||||.||||:|||||||||||||||:|.|||:.:.||
SBERII           441 RLFNYGSWEVLRFLLSNARWWLDEYKFDGFRFDGVTSMMYTHHGLQVDFT    490

SBERI            444 GDYNEYFSEATDIDAVVYLMLANSLIHNILPDATVIAEDVSGMPGLGRSV    493
                     |:||||||..|||:||||||||.|.:||.:.|:|..|.|||||||.:...|
SBRII            491 GNYNEYFGYATDVDAVVYLMLLNDMIHGLFPEAVTIGEDVSGMPTVCIPV    540

SBERI            494 SEGGIGFDYRLAMAIPDKWIDYLKNKSDEEWSMKEISWSLTNRRYTEKCV    543
                     .:||:||||||.||:.|||::.:: |.||:|.|.:|...|||||:.||||
SBERII           541 EDGGVGFDYRLHMAVADKWVEIIQ-KRDEDWKMGDIVHMLTNRRWLEKCV    589

SBERI            544 AYAESHDQAIVGDKTVAFLLMDKEMYYGMSCLTDASPMVDRGVALHKMVH    593
                     :||||||||:|||||:||.||||:||..|:....::|::||||||.||:.
SBERII           590 SYAESHDQALVGDKTIAFWLMDKDMYDFMALDGPSTPLIDRGVALQKMIR    639

SBERI            594 LLTMALGGEGYLNFMGNEFGHPEWIDFPR-----------EGNGWSYDKC    632
                     |:||.|||||||||||||||||||||||           .||.:|||||
SBERII           640 LITMGLGGEGYLNFMGNEFGHPEWIDFPRGDLHLPSGKFVPGNNYSYDKC    689

SBERI            633 RRQWNLVDTEHLRYRFMNAFDKAMNLLDEKYSFLASTKQIVSSTNEEDKV    682
                     ||:::|.:::.|||..|..||:|:..|:|.|.|:.|..|.:|..:|.|::
SBERII           690 RRRFDLGNSKRLRYHGMQEFDQAIQHLEEAYGFMTSEHQYISRKDERDRI    739
```

(Continued)

```
SBERI             683 IVFERGDLVFVFNFHPENTYDGYKVGCDLPGKYRVALDSDAWEFGGRGRV    732
                      ||||||:||||||||..::|..|:|||..||||::.||||...|||.||:
SBERII            740 IVFERGNLVFVFNFHWTSSYSDYRVGCLKPGKYKIVLDSDDPLFGGFGRL    789

SBERI             733 GHDVDHFTSPEGIPGVPETNFNNRPNSFKILSAA RTCVVYYRVEEKEGN     781
                      .||.:|| |.||.       ::|||.||.:.:..||.|||..||::..|
SBERII            790 SHDAEHF-SFEGW-------YDNRPRSFMVYTPCRTAVVYALVEDEVEN     830
```

Figure 3.4 Alignment of deduced amino acid sequences of *sbe*I and *sbe*II cDNAs (SBERI and SBERII, respectively) using EMBOSS align.

Conserved residues are indicated by asterisk (|).

Same group of side chain and similar size are indicated by semi colon (:)

Same group of side chain but different size are indicated by full stop (.)

```
SBERI           MLGSLGLFPAPDFGSLSPSLAKNSKRAVERNCQIVKQKQ---------------------- 39
Potato          MEINFKVLSKPIRGSFPSFSPKVSSGASRNKICFPSQHS---------------------- 39
Wheat           MLCLTASSSPSPSPSLPPRPSRPAADRPGPGISVSHSGSSFLFFSFVSGLRSASRRFPDA 60
Maize           MLCLVSPSS-SPTPLPPPRRSRSHADRAAP------------------------------ 29
                *       . .     ..  .:

SBERI           -IELT-GCRKLPGCSRFLFLPRISIDKRVKQGLAISAAVADEKKMITSFEEDMEIT--GL 95
Potato          -TGLKFGSQERSWDISSTPKSRVRKDERMKHSSAISAVLTDDNSTMAPLEEDVKTENIGL 98
Wheat           MPRAQGGGNVRLSAVPAPSSLRWSWPRKAKSKFSVPVSAPRDYTMATAEDGFGDLP---I 117
Maize           -PGIAGGGNVRLSVLSVQCKARRSGVRKVKSKFATAATVQEDKTMATAKGDVDHLP---I 85
                      * .           *    .: *   : ..    : .  :.     .     :

SBERI           LSIDPGLESFKDHFRYRMQRFTNQKQLIEKYEGGLEEFSNGYLKFGFNREAGGIVYREWA 155
Potato          LNLDPTLEPYLDHFRHRMKRYVDQKMLIEKYEGPLEEFAQGYLKFGFNREDGCIVYREWA 158
Wheat           YDLDPKFAGFKDHFSYRMKKYLEQKHSIEKYEGGLEEFSKGYLKFGINTENDATVYREWA 177
Maize           YDLDPKLEIFKDHFRYRMKRFLEQKGSIEENEGSLESFSKGYLKFGINTNEDGTVYREWA 145
                 .:** :  : *** :**::: :**  **: ** **.*::******:* : .  ******

SBERI           PAAQEAQVIGDFNGWDGSNHRMEKNEFGVWSINIPDSGGNPAIHHNSRVKFRFKHGDGVW 215
Potato          PAAQEDEVIGDFNGWNGSNHMMEKDQFGVWSIRIPDVDSKPVIPHNSRVKFRFKHGNGVW 218
Wheat           PAAKDAQLIGDFNNWNGSGHRMTKDNFGVWSIRISHVNGKPAIPHNSKVKFRFHRGDGLW 237
Maize           PAAQEAELIGDFNDWNGANHKMEKDKFGVWSIKIDHVKGKPAIPHNSKVKFRFLHG-GVW 204
                ***:: ::*****.*:*:.* * *::******.* .  .:*.* ***:***** :* *:*

SBERI           VDRIPAWIRYATVDPTKFGAPYDGVYWDPPPPERYQFKYPRPPKAQAPRIYEAHVGMSSS 275
Potato          VDRIPAWIKYATADATKFAAPYDGVYWDPPPSERYHFKYPRPPKPRAPRIYEAHVGMSSS 278
Wheat           VDRVPAWIRYATFDASKFGAPYDGVHWDPPTGERYVFKHPRPRKPDAPRIYEAHVGMSGE 297
Maize           VDRIPALIRYATVDASKFGAPYDGVHWDPPASERYTFKHPRPSKPAAPRIYEAHVGMSGE 264
                ***:** *:*** *.:**.******:****. *** **:*** *. ************..

SBERI           EPRINTYREFADDVLPRIRANNYNTVQLMAVMEHSYYGSFGYHVTNFFAVSSRSGTPEDL 335
Potato          EPRVNSYREFADDVLPRIKANNYNTVQLMAIMEHSYYGSFGYHVTNFFAVSSRYGNPEDL 338
Wheat           KPEVSTYREFADNVLPRIKANNYNTVQLMAIMEHSYYASFGYHVTNFFAVSSRSGTPEDL 357
Maize           KPAVSTYREFADNVLPRIRANNYNTVQLMAVMEHSYYASFGYHVTNFFAVSSRSGTPEDL 324
                :* :.:******:*****:***********:******.*************** *.****

SBERI           KYLIDKAHSLGLSVLMDVVHSHASNNITDGLNGFDVGQSTQDSYFHTGDRGYHKLWDSRL 395
Potato          KYLIDKAHSLGLQVLVDVVHSHASNNVTDGLNGFDIGQGSQESYFHAGERGYHKLWDSRL 398
Wheat           KYLVDKAHSLGLRVLMDVVHSHASSNMTDGLNGYDVGQNTQESYFHTGERGYHKLWDSRL 417
Maize           KYLVDKAHSLGLRVLMDVVHSHASNNVTDGLNGYDVGQSTQESYFHAGDRGYHKLWDSRL 384
                ***:******** **:********.*:******:*:**.:*:****:*:***********

SBERI           FNYANWEVIRFLLSNLRWWLEEYKFDGFRFDGVTSMLYHHHGINMAFTGDYNEYFSEATD 455
Potato          FNYANWEVLRFLLSNLRWWLEEYNFDGFRFDGITSMLYVHHGINMGFTGNYNEYFSEATD 458
Wheat           FNYANWEVLRYLLSNLRYWMDEFMFDGFRFDGVTSMLYNHHGINMSFAGNYKEYFGLDTD 477
Maize           FNYANWEVLRFLLSNLRYWLDEFMFDGFRFDGVTSMLYHHHGINVGFTGNYQEYFSLDTA 444
                ********:*:******:*::*: ********:***** *****:.*:*:*:***.  *

SBERI           IDAVVYLMLANSLIHNILPDATVIAEDVSGMPGLGRSVSEGGIGFDYRLAMAIPDKWIDY 515
Potato          VDAVVYLMLANNLIHKIFPDATVIAEDVSGMPGLGRPVSEGGIGFDYRLAMAIPDKWIDY 518
Wheat           VDAVVYMMLANHLMHKILPEATVVAEDVSGMPVLCRSVDEGGVGFDYRLAMAIPDRWIDY 537
Maize           VDAVVYMMLANHLMHKLLPEATVVAEDVSGMPVLCRPVDEGGVGFDYRLAMAIPDRWIDY 504
                :*****:**** *:*:::*:***:******** * *.*.***:************:****
```

(Continued)

```
SBERI           LKNKSDEEWSMKEISWSLTNRRYTEKCVAYAESHDQAIVGDKTVAFLLMDKEMYYGMSCL 575
Potato          LKNKNDEDWSMKEVTSSLTNRRYTEKCIAYAESHDQSIVGDKTIAFLLMDKEMYSGMSCL 578
Wheat           LKNKDDLEWSMSAIAHTLTNRRYTEKCIAYAESHDQSIVGDKTMAFLLMDKEMYTGMSDL 597
Maize           LKNKDDSEWSMGEIAHTLTNRRYTEKCIAYAESHDQSIVGDKTIAFLLMDKEMYTGMSDL 564
                ****.* :***  :: :**********:********:******:********** *** *

SBERI           TDASPMVDRGVALHKMVHLLTMALGGEGYLNFMGNEFGHPEWIDFPREGNGWSYDKCRRQ 635
Potato          TDASPVVDRGIALHKMIHFFTMALGGEGYLNFMGNEFGHPEWIDFPREGNNWSYDKCRRQ 638
Wheat           QPASPTIDRGIALQKMIHFITMALGGDGYLNFMGNEFGHPEWIDFPREGNNWSYDKCRRQ 657
Maize           QPASPTIDRGIALQKMIHFITMALGGDGYLNFMGNEFGHPEWIDFPREGNNWSYDKCRRQ 624
                  *** :***:**:**:*::******:************************.*********

SBERI           WNLVDTEHLRYRFMNAFDKAMNLLDEKYSFLASTKQIVSSTNEEDKVIVFERGDLVFVFN 695
Potato          WNLADSEHLRYKFMNAFDRAMNSLDEKFSFLASGKQIVSSMDDDNKVVVFERGDLVFVFN 698
Wheat           WSLSDIDHLRYKYMNAFDQAMNALDDKFSFLSSSKQIVSDMNEEKKIIVFERGDLVFVFN 717
Maize           WSLVDTDHLRYKYMNAFDQAMNALDERFSFLSSSKQIVSDMNDEEKVIVFERGDLVFVFN 684
                *.* * :****::*****:*** **:::***:* *****. ::::.*::************

SBERI           FHPENTYDGYKVGCDLPGKYRVALDSDAWEFGGRGRVGHDVDHFTSPEGIPGVPETNFNN 755
Potato          FHPKNTYEGYKVGCDLPGKYRVALDSDAWEFGGHGRTGHDVDHFTSPEGIPGVPETNFNG 758
Wheat           FHPSKTYDGYKVGCDLPGKYKVALDSDALMFGGHGRVAHDNDHFTSPEGVPGVPETNFNN 777
Maize           FHPKKTYEGYKVGCDLPGKYRVALDSDALVFGGHGRVGHDVDHFTSPEGVPGVPETNFNN 744
                ***.:**:************:*******  ***:**..** ********:*********.

SBERI           R--PNSFKILSAARTCVVYYR--VEEKEGNHNSSDIGAANETLTDIAKLGDFEGINETSP 811
Potato          RQIPSKCCLLREHVWLITELMNACQKLKITRQTFVVSYYQQPISRRVTRNLKIRYLQISV 818
Wheat           R--PNSFKVLSPPRTCVAYYR--VEEKAEKPKDEGAASWGKAAPGY---------IDVEA 824
Maize           R--PNSFKVLSPPRTCVAYYR--VDEAGAGRRLHAKAETGKTSPAES--------IDVKA 792
                *  *..  :*      :.      ::     .    .   :. .           : .

SBERI           ADAVAKQEDLKAAQPSLIADDIATKANTETEEIEEETSDDK-- 852
Potato          TLTNACQKLKFTRQTFLVSYYQQPILRRVTRKLKDSLSTNIST 861
Wheat           TRVKDAADGEATSGSKKASTGGDSSKKGINFVFGSPDKDNK-- 865
Maize           SRASSKEDKEATAGGK----------KGWKFARQPSDQDTK-- 823
                : .   .   :            . .      .
```

Figure 3.5 Alignment of SBERI from cassava with SBEI from other plants

Conserved residues are indicated by asterisk (*).

Same group of side chain and similar size are indicated by semi colon (:)

Same group of side chain but different size are indicated by full stop (.)

```
SBERII          MGHYTISGIRFPCAP-LCKSQSTG--FHGDRR-TSSCLSFNFKKEAFSRRVFSGKSSHES 56
Potato          -MVYTLSGVRFPTVPSVYKSNGFS--SNGDRR-NAN-VSVFLKKHSLSRKILAEKSSYNS 55
Wheat           MATFAVSGATLGVARPAGAGGGLLPRSGSERRGGVDLPSLLLRKKDSSRAVLSR------ 54
                   :::**  :  .    . .     .:**   .  *. ::*.  ** :::

SBERII          DSSNVMVTASKRVLPDG-RIECYSS--STDQLEAPGTVSEESQVLTDVESLIMDD----K 109
Potato          EFRPSTVAASGKVLVPGTQSDSSSS--STDQFEFTETSPENSPASTDVDSSTMEHASQIK 113
Wheat           ------AASPGKVLVPDGESDDLASPAQPEELQIPEDIEEQT-AEVNMTGGTAEKLESSE 107
                     .::. :**  . . :  :*  ..:::: .    *:: . .:: .   :.    :

SBERII          IVEDEVNK--------ESVPMRETVSIRKIG---------------------SKPRSIPP 140
Potato          TENDDVEPSSDLTGSVEELDFASSLQLQEGGKLEESKTLNTSEETIIDESDRIRERGIPP 173
Wheat           PTQGIVET--------ITDGVTKGVKELVVG---------------------EKPRVVPK 138
                  :. *:           . . :.    *                      : * :*

SBERII          PGRGQRIYDIDPSLTGFRQHLDYRYSQYKRLREEIDKYEGGLDAFSRGYEKFGFSRSETG 200
Potato          PGLGQKIYEIDPLLTNYRQHLDYRYSQYKKLREAIDKYEGGLEAFSRGYEKMGFTRSATG 233
Wheat           PGDGQKIYEIDPTLKDFRSHLDYRYSEYRRIRAAIDQHEGGLEAFSRGYEKLGFTRSAEG 198
                ** **:**:*** *..:*.*******:*:::*  **::****:********:**:**  *

SBERII          ITYREWAPGATWAALIGDFNNWNPNADVMTQNECGVWEIFLPNNADGSPPIPHGSRVKIR 260
Potato          ITYREWALGAQSAALIGDFNNWDANADIMTRNEFGVWEIFLPNNVDGSPAIPHGSRVKIR 293
Wheat           ITYREWAPGAHSAALVGDFNNWNPNADTMTRDDYGVWEIFLPNNADGSPAIPHGSRVKIR 258
                ******* **  ***:******:.*** **::: **********.****.**********

SBERII          MDTPSGNKDSIPAWIKFSVQAPGELPYNGIYYDPPEEEKYVFKNPQPKRPKSLRIYESHV 320
Potato          MDTPSGVKDSIPAWINYSLQLPDEIPYNGIHYDPPEEERYIFQHPRPKKPKSLRIYESHI 353
Wheat           MDTPSGVKDSISAWIKFSVQAPGEIPFNGIYYDPPEEEKYVFQHPQPKRPESLRIYESHI 318
                ****** ****.***::*:* *.*:*:***:********:*:*::*:**:*:********:

SBERII          GMSSTEPVINTYANFRDDVLPRIKKLGYNAVQLMAIQEHSYYASFGYHVTNFYAASSRFG 380
Potato          GMSSPEPKINSYVNFRDEVLPRIKKLGYNALQIMAIQEHSYYASFGYHVTNFFAPSSRFG 413
Wheat           GMSSPEPKINSYANFRDEVLPRIKRLGYNAVQIMAIQEHSYYASFGYHVTNFFAPSSRFG 378
                ****.** **:*.****:******:*****:*:********************:*.*****

SBERII          TPDDLKSLIDKAHELGLLVLMDIVHSHASTNTLDGLNMFDGTDGHYFHSGPRGHHWMWDS 440
Potato          TPDDLKSLIDKAHELGIVVLMDIVHSHASNNTLDGLNMFDCTDSCYFHSGARGYHWMWDS 473
Wheat           TPEDLKSLIDRAHELGLLVLMDIVHSHSSNNTLDGLNGFDGTDTHYFHGGPRGHHWMWDS 438
                **:*******:*****::*********:*.******* ** **  ***.*.**:******

SBERII          RLFNYGSWEVLRFLLSNARWWLDEYKFDGFRFDGVTSMMYTHHGLQVDFTGNYNEYFGYA 500
Potato          RLFNYGNWEVLRYLLSNARWWLDAFKFDGFRFDGVTSMMYIHHGLSVGFTGNYEEYFGLA 533
Wheat           RLFNYGSWEVLRFLLSNARWWLEEYKFDGFRFDGVTSMMYTHHGLQMTFTGNYGEYFGFA 498
                ******.*****:*********: :*************** ****.: ***** **** *

SBERII          TDVDAVVYLMLLNDMIHGLFPEAVTIGEDVSGMPTVCIPVEDGGVGFDYRLHMAVADKWV 560
Potato          TDVDAVVYLMLVNDLIHGLFPDAITIGEDVSGMPTFCIPVQEGGVGFDYRLHMAIADKRI 593
Wheat           TDVDAVVYLMLVNDLIHGLHPDAVSIGEDVSGMPTFCIPVPDGGVGLDYRLHMAVADKWI 558
                **********:**:****.*:*::**********.**** :****:*******:*** :

SBERII          EIIQKRDEDWKMGDIVHMLTNRRWLEKCVSYAESHDQALVGDKTIAFWLMDKDMYDFMAL 620
Potato          ELLKKRDEDWRVGDIVHTLTNRRWSEKCVSYAESHDQALVGDKTIAFWLMDKDMYDFMAL 653
Wheat           ELLKQSDESWKMGDIVHTLTNRRWLEKCVTYAESHDQALVGDKTIAFWLMDKDMYDFMAL 618
                *:::: **.*::***** ****** ****:******************************
```

(continued)

```
SBERII          DGPSTPLIDRGVALQKMIRLITMGLGGEGYLNFMGNEFGHPEWIDFPRGDLHLPSGKFVP 680
Potato          DRPSTSLIDRGIALHKMIRLVTMGLGGEGYLNFMGNEFGHPEWIDFPRAEQHLSDGSVIP 713
Wheat           DRPSTPRIDRGIALHKMIRLVTMGLGGEGYLNFMGNEFGHPEWIDFPRGPQTLPTGKVLP 678
                * ***. ****:**:*****:****************************.   *. *.:*

SBERII          GNNYSYDKCRRRFDLGNSKRLRYHGMQEFDQAIQHLEEAYGFMTSEHQYISRKDERDRII 740
Potato          GNQFSYDKCRRRFDLGDAEYLRYRGLQEFDRPMQYLEDKYEFMTSEHQFISRKDEGDRMI 773
Wheat           GNNNSYDKCRRRFDLGDADFLRYHGMQEFDQAMQHLEEKYGFMTSEHQYVSRKHEEDKVI 738
                **: ************::. ***:*:****:.:*:**: * *******::***.* *::*

SBERII          VFERGNLVFVFNFHWTSSYSDYRVGCLKPGKYKIVLDSDDPLFGGFGRLSHDAEHFSFEG 800
Potato          VFEKGNLVFVFNFHWTKSYSDYRIACLKPGKYKVALDSDDPLFGGFGRIDHNAEYFTFEG 833
Wheat           IFERGDLVFVFNFHWSNSFFDYRVGCSRPGKYKVALDSDDALFGGFSRLDHDVDYFTTEH 798
                :**:*:*********:.*: ***:.* :*****:.*****.*****.*:.*:.::*: *

SBERII          WYDNRPRSFMVYTPCRTAVVYALVEDEVEN---EVEPVAG--------- 837
Potato          WYDDRPRSIMVYAPCKTAVVYALVDKEEEEEEEEEEEVAAVEEVVVEEE 882
Wheat           PHDNRPRSFSVYTPSRTAVVYALTE----------------------- 823
                 :*:****: **:*.:*******.:
```

Figure 3.6 Alignment of SBERI from cassava with SBEII from other plants

Conserved residues are indicated by asterisk (*).

Same group of side chain and similar size are indicated by semi colon (:)

Same group of side chain but different size are indicated by full stop (.)

3.1.3 Cloning of *sbe* cDNA fragments into pET expression vector

The pET-28 vector that is suitable for cloning of *sbe*I cDNA, pre-digested with *Sal*I and *Xho*I, would be pET-28c, while pET-28b would be needed for cloning of *Sal*I- and *BamH*I-digested *sbeII* cDNA. Ligation and transformation of the recombinant plasmid into the *E. coli* expression host strain rossetta-gami (DE3) were carried out as described in section 2.4. The presence of *sbe*I and *sbe*II cDNAs in pET-28 vectors was confirmed by PCR (Figure 3.7) and by digesting the recombinant plasmids with appropriate restriction enzymes (Figure 3.8).

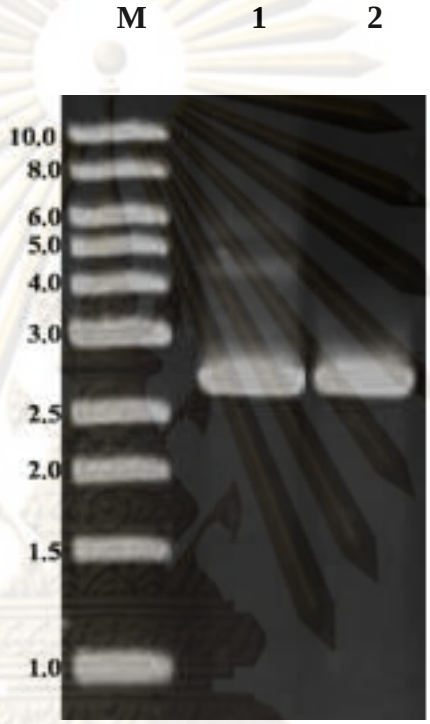


Figure 3.7 PCR amplification of the recombinant pET-28 vectors

Lane M = 1-kb DNA ladder

Lane 1 = *sbe*I amplified product

Lane 2 = *sbe*II amplified product

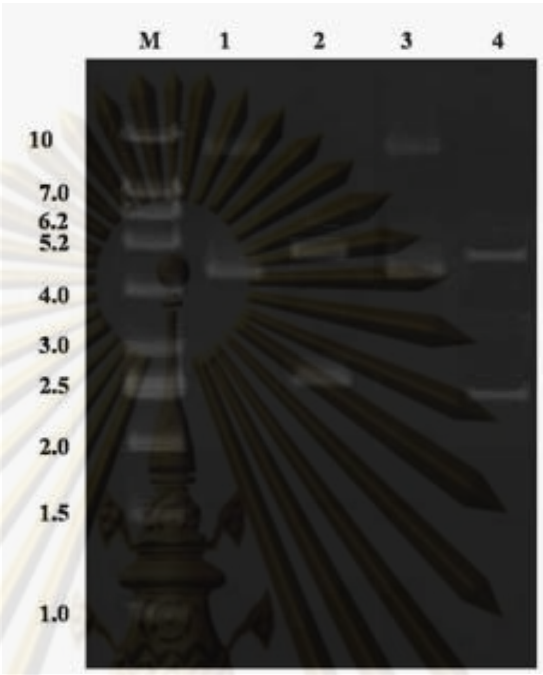


Figure 3.8 Restriction analysis of the recombinant pET-28 vectors

Lane M = 1-kb molecular weight marker

Lane 1 = recombinant *sbeI*

Lane 2 = *SalI* and *XhoI*- digested products of *sbe* I-pET 28c vector

Lane 3 = recombinant *sbeII*

Lane 4 = *SalI* and *BamHI*-digested products of *sbe*II-pET 28b vector

3.1.4 Production of the recombinant polypeptides

To obtain the optimal concentration of IPTG suitable for the production of recombinant proteins, various concentrations of IPTG (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM) were added to the transformed cells from section 3.1.3. After induction, the cells were incubated for a certain time and then collected for preparation of soluble proteins used in SBE activity assay. The result showed that at 0 to 0.2 mM IPTG very little activity of SBERI could be detected, while induction with 0.4 mM IPTG could significantly increase SBE activity (Figure 3.9A). However, if the IPTG was increased to 0.6, 0.8 and 1 mM IPTG, the results were not significantly altered, thus indicating that 0.4 mM was the lowest concentration of IPTG to give the highest enzyme expression. For SBERII, similar results were observed (Figure 3.9B). After 4 hours of induction with 0.4 mM IPTG, the specific activity of SBERI and SBERII was observed at 1.2 and 0.79 units/mg proteins, respectively.

3.1.5 Protein pattern of cell and crude extracts

The transformed cells from section 3.1.3 were induced with various concentrations of IPTG as described in section 3.1.4. After a period of time, the cells were harvested by centrifugation, resuspended in sample buffer for SDS-PAGE, and analyzed by 10% SDS-PAGE (section 2.5.2). The results in Figure 3.10-3.11 showed that both SBERI and SBERII were expressed as 90 kDa proteins.

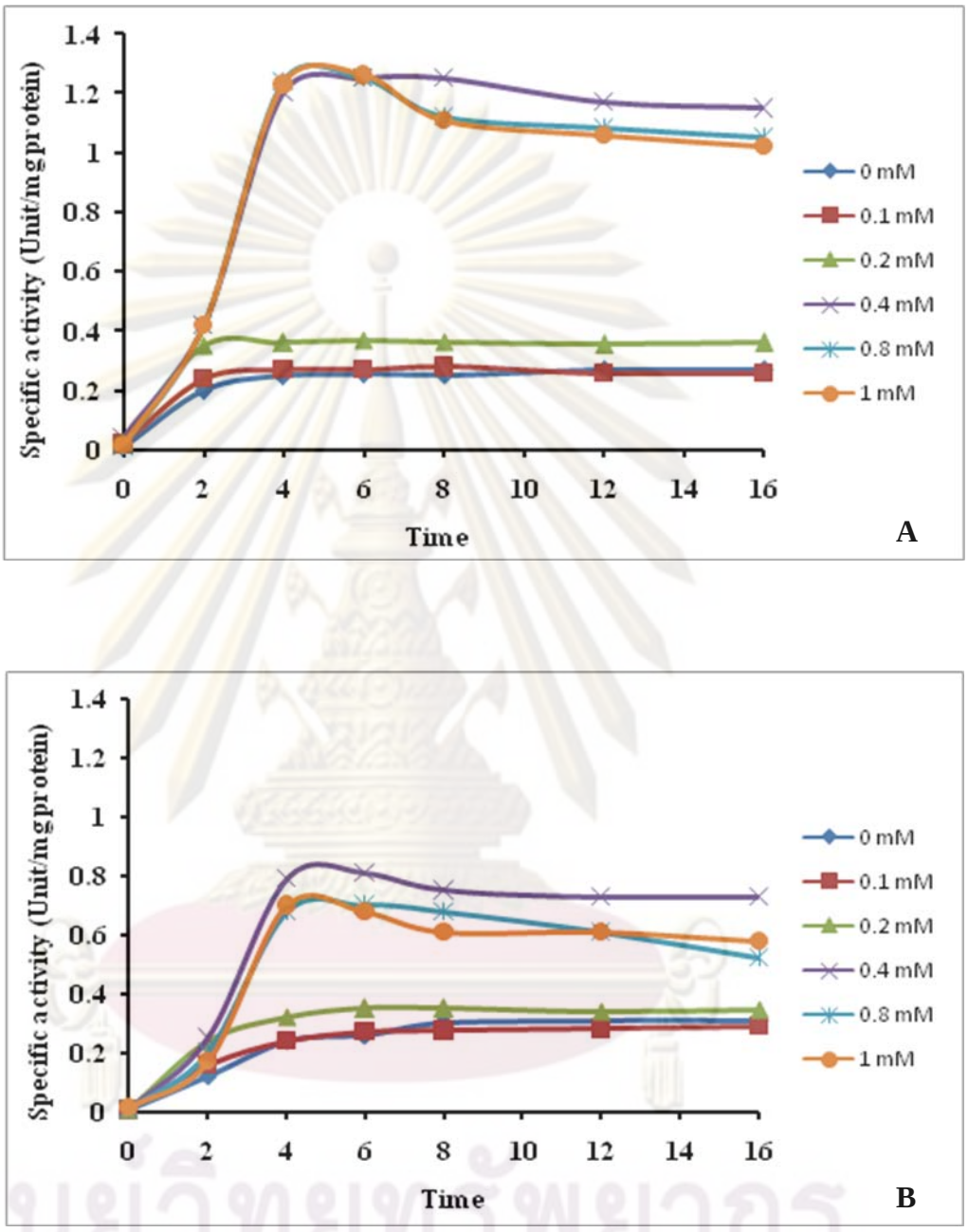
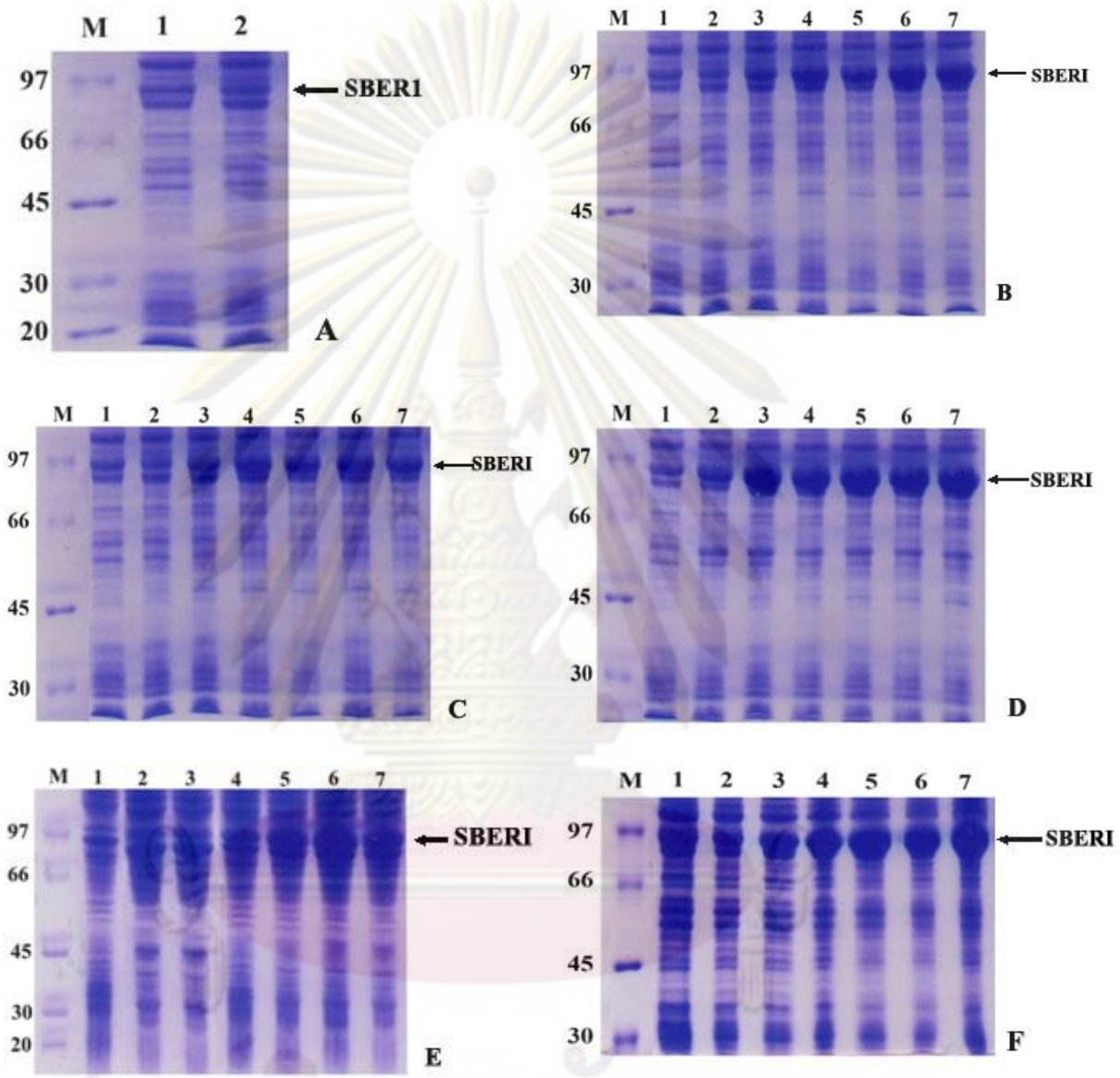


Figure 3.9 The effect of IPTG concentration and induction time on the specific activity of SBE produced in *E. coli* expression host

A = SBERI

B = SBERII



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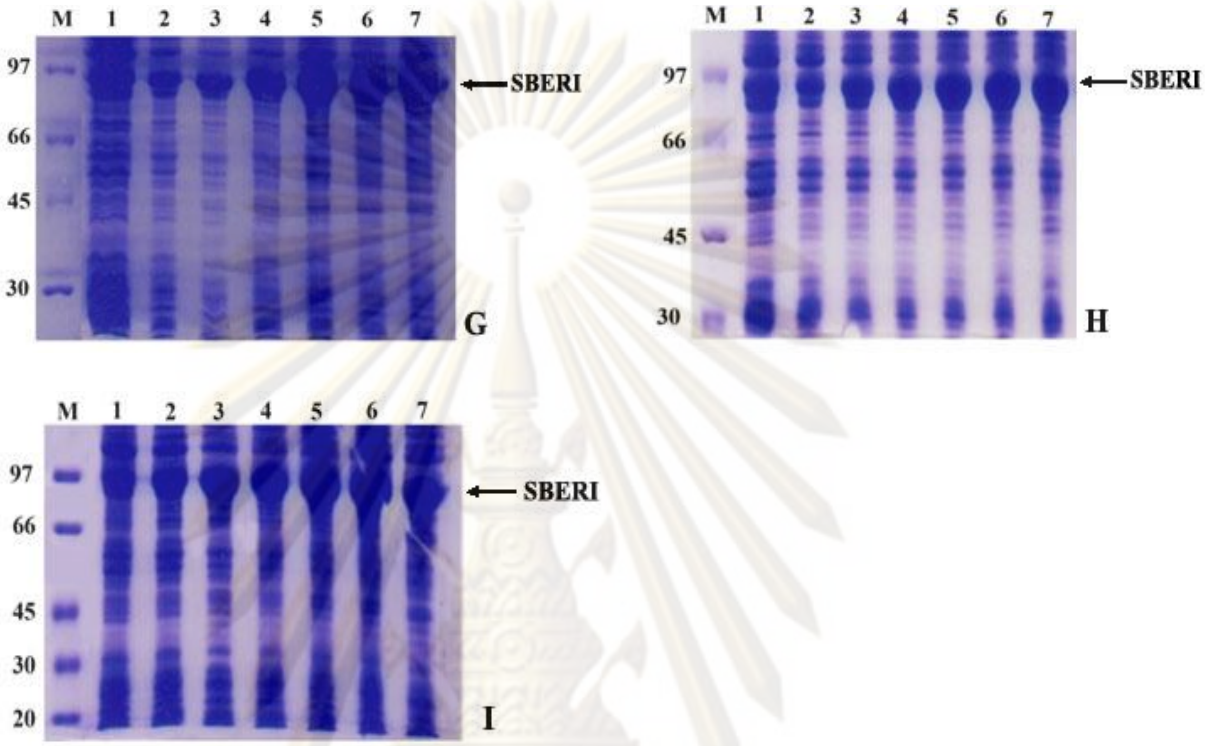


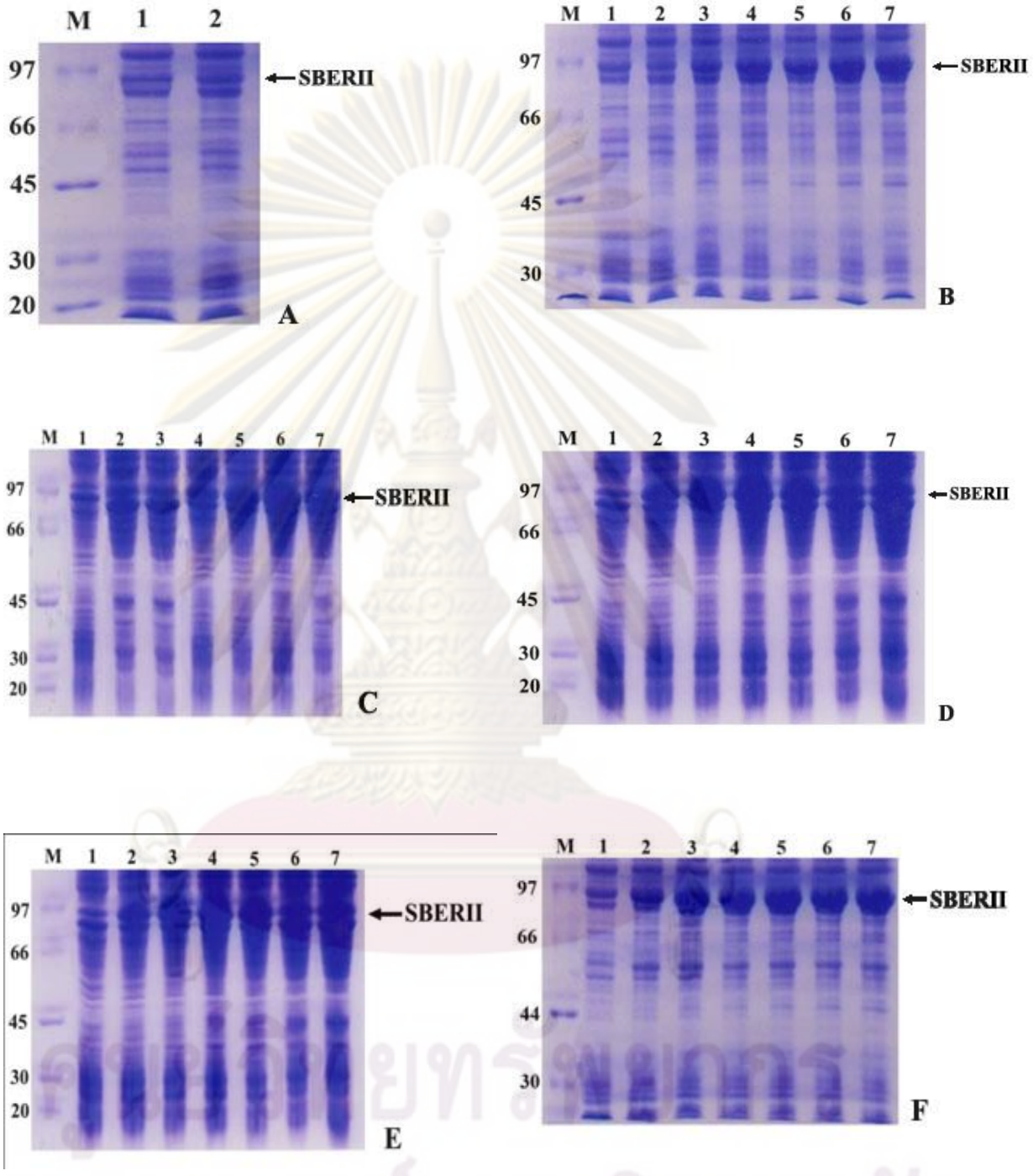
Figure 3.10 SDS-PAGE analysis of the total cellular proteins from *E. coli* rosetta-gami harboring *sbe*I-pET28c recombinant plasmid

A = Control of SBER ; 1= *E. coli* rosetta-gami 2 = recombinant SBERI

B – I = SDS-PAGE of cellular proteins after induction with 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM IPTG, respectively

Lane M = LMW marker

Lane 1-7 = Cells were collected at 0, 2, 4, 6, 8, 12, 16 hours, respectively



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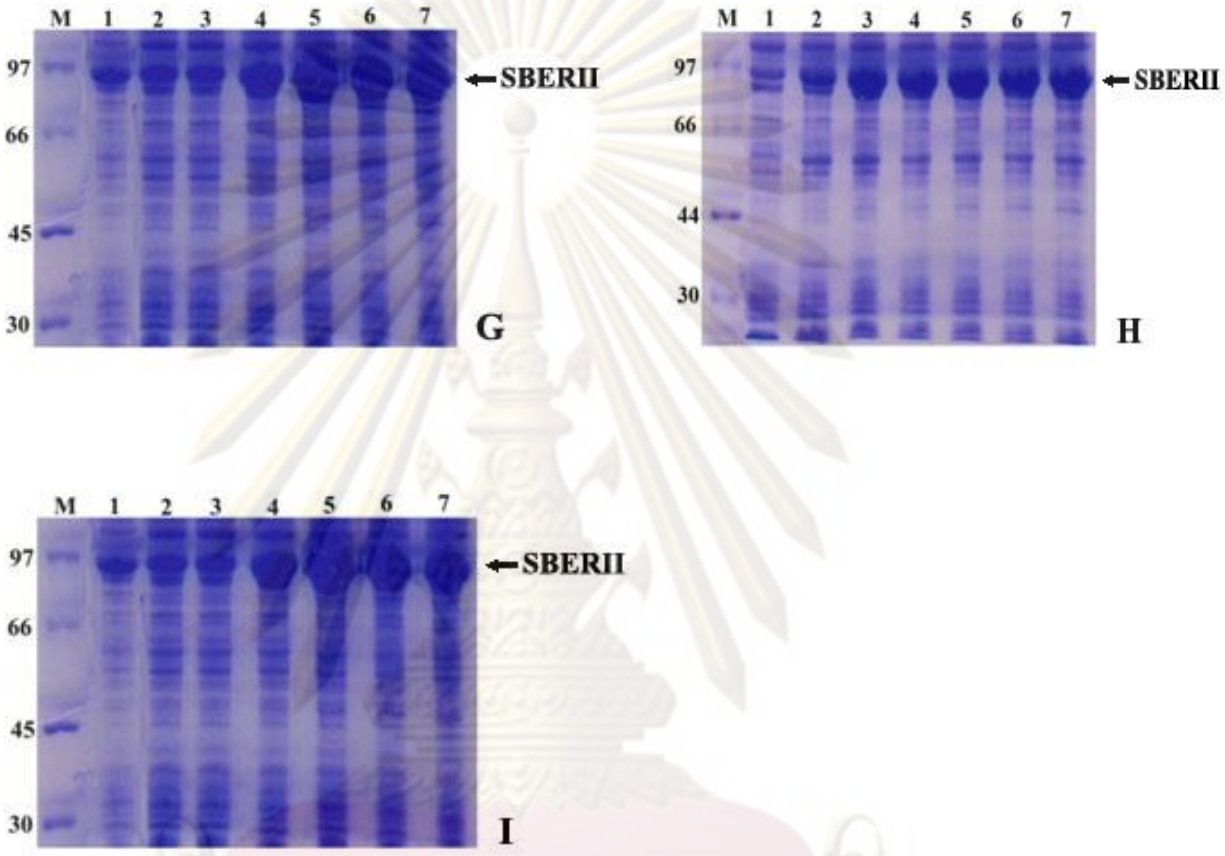


Figure 3.11 SDS-PAGE analysis of the total cellular proteins from *E. coli* rosetta-gami harboring *sbe*II-pET28b recombinant plasmid.

A = Control of SBER ; 1= *E. coli* rosetta-gami 2 = recombinant SBERII

B – I = SDS-PAGE of cellular proteins after induction with 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM IPTG, respectively

Lane M = LMW marker

Lane 1-7 = Cells were collected at 0, 2, 4, 6, 8, 12, 16 hours, respectively.

3.2 Purification of the recombinant proteins

3.2.1 Preparation of crude enzymes

The recombinant enzymes were purified from 5 g of the *E. coli* expression host strain rossetta-gami(DE3) grown in 1-litre medium (section 2.6). Crude preparation of SBERI and SBERII contained 704 and 626 units of enzyme activity and specific activity of 1.6 and 1.0 units/ mg protein. SBERs were further purified.

3.2.2 Purification of SBERI and SBERII

Since the recombinant proteins obtained from pET-28 expression system carry histidine-tagging sequence at the N-terminal region, this therefore allows purification of the recombinant enzymes by Hitrap nickel column chromatography (section 2.6). Elution of the recombinant enzymes from the column was carried out using buffer containing 0.5 M imidazole and 0.3 M NaCl. According to the chromatographic profiles of each SBER (Figure 3.12), only the fractions exhibiting activity peak were collected. Table 3.1 summarized the purification results of each SBER.

After purification, 7% native starch PAGE analysis was carried out. To identify the location of SBERI and SBERII, the gel was stained with I_2 as described in section 2.5.1. As shown in Figure 3.13B, reddish activity bands of both SBERI and SBERII were observed, thus indicating that the purified proteins carried SBE activity.

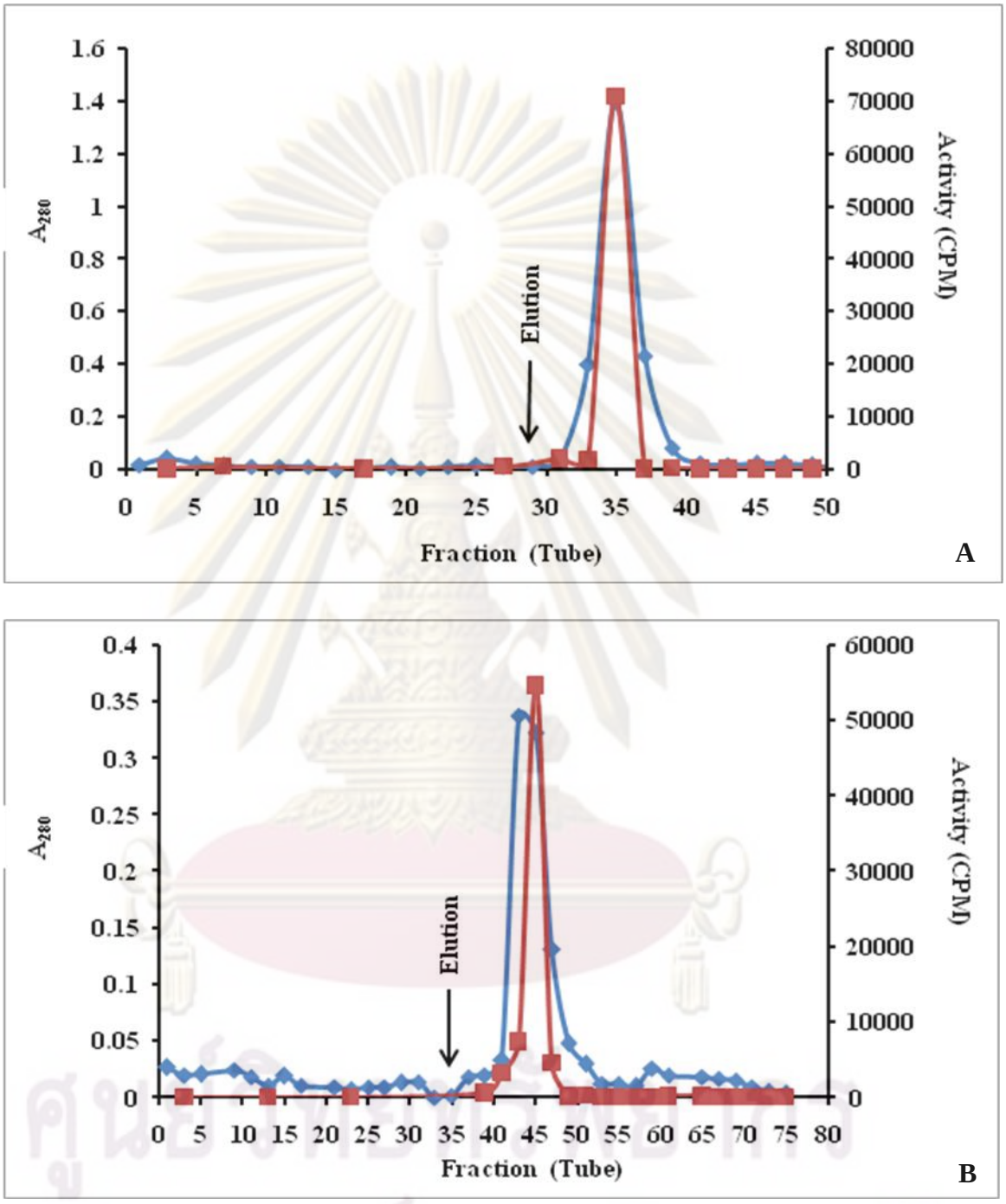


Figure 3.12 Chromatographic profiles of SBERI and SBERII on Hitrap-His tag column chromatography.

A = SBERI

B = SBERII

Table 3.1 Purification table of transformed cassava starch branching enzymes

	Total Protein	Total activity	Specific activity	Fold	%Recovery
Recombinant starch branching enzyme I					
Crude	440	704	1.6	1	100
Hitrap - Histag	135	1134	8.4	5	161
Recombinant starch branching enzyme II					
Crude	402	626	1.5	1	100
Hitrap - Histag	114	866	7.6	5	138

3.3 Characterization of recombinant SBE

3.3.1 Molecular weight determination by SDS-PAGE

Recombinant SBE was applied to 10% SDS-PAGE as described in section 2.5.2. Standard proteins were run in parallel and a standard curve was constructed from their molecular weight and relative mobility (Figure 3.13A). SBERI and SBERII showed one band with molecular weight 90 kDa as determined from standard curve (Appendix 3).

3.3.2 Optimum pH of recombinant of SBE

Each recombinant SBEs were incubated in various pHs's using appropriate buffer as described in section 2.7.3. The results were shown in Figure 3.14 A and B). Both recombinants SBE showed highest activity at pH 7.0 in all buffers, with highest activity observed in citrate buffer than phosphate buffer and Tris-HCl buffer.

3.3.3 Optimum temperatures of recombinant SBE

Each SBER was assayed at different temperatures as described in section 2.7.4. SBE activity of both recombinants enzymes showed similar profiles with highest activity at 37°C. Both recombinant SBEs loss activity to less than 90% at 50°C (Figure 3.15).

3.3.4 Substrate specificity of recombinant SBE

To compare the substrate specificity, amylose and amylopectin from potato were used as substrates and assayed as described in section 2.4.2. The results were shown in Figure 3.16 A and B. SBERI utilized amylose better than SBERII while SBERII was more active towards amylopectin.

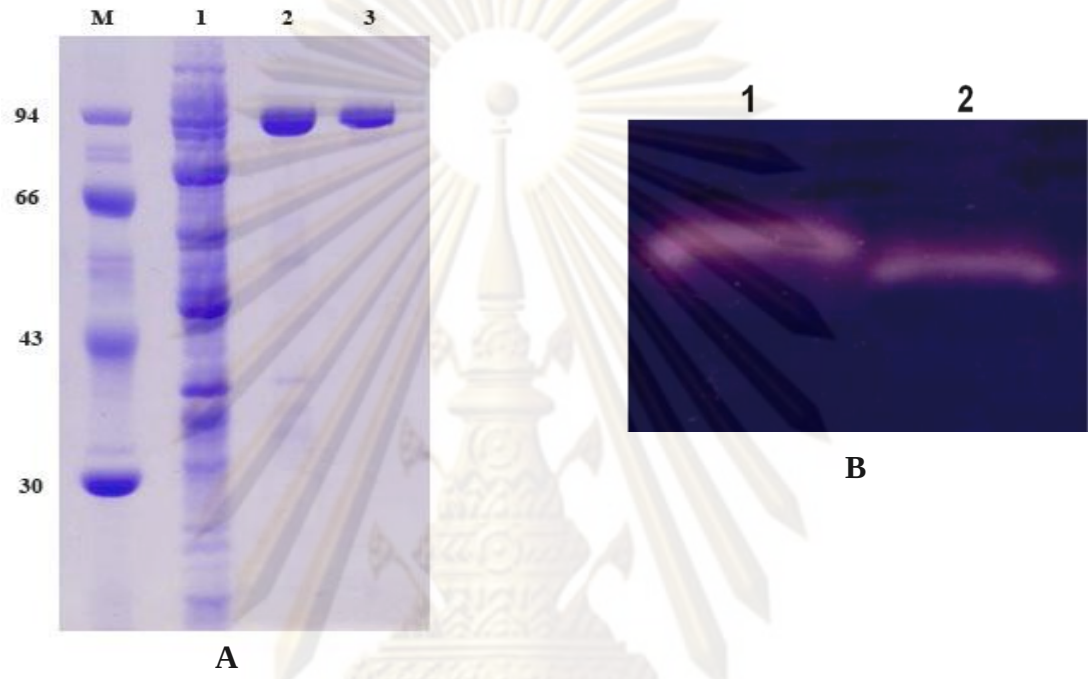


Figure 3.13 Zymograms of recombinant starch branching enzymes.

A = SDS-PAGE of recombinant starch branching enzymes

Lane M = Low molecular weight protein marker

Lane 1 = Crude enzyme

Lane 2 = Purified SBERI

Lane 3 = Purified SBERII

B = Native starch-PAGE of recombinant starch branching enzymes

Lane 1 = Purified SBERI

Lane 2 = Purified SBERII

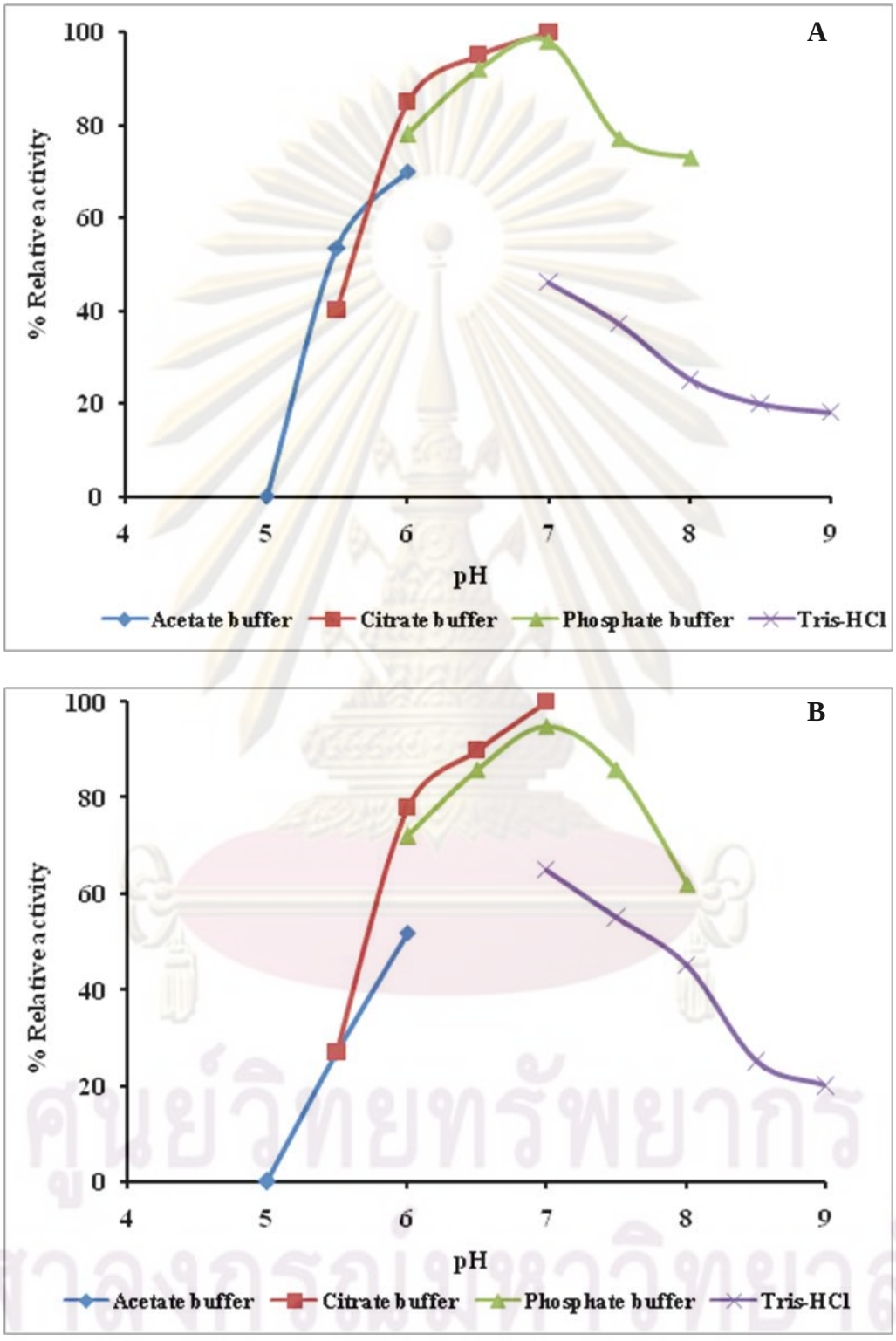


Figure 3.14 Optimum pH of recombinant SBEs

A = SBERI B = SBERII

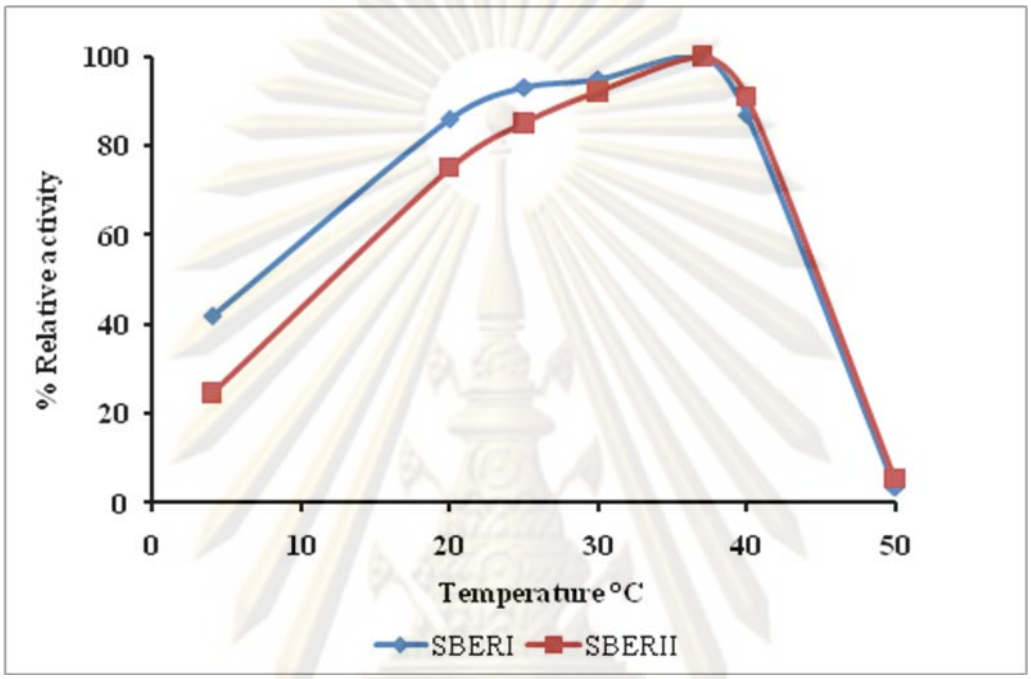


Figure 3.15 Optimum temperatures of recombinant SBE.

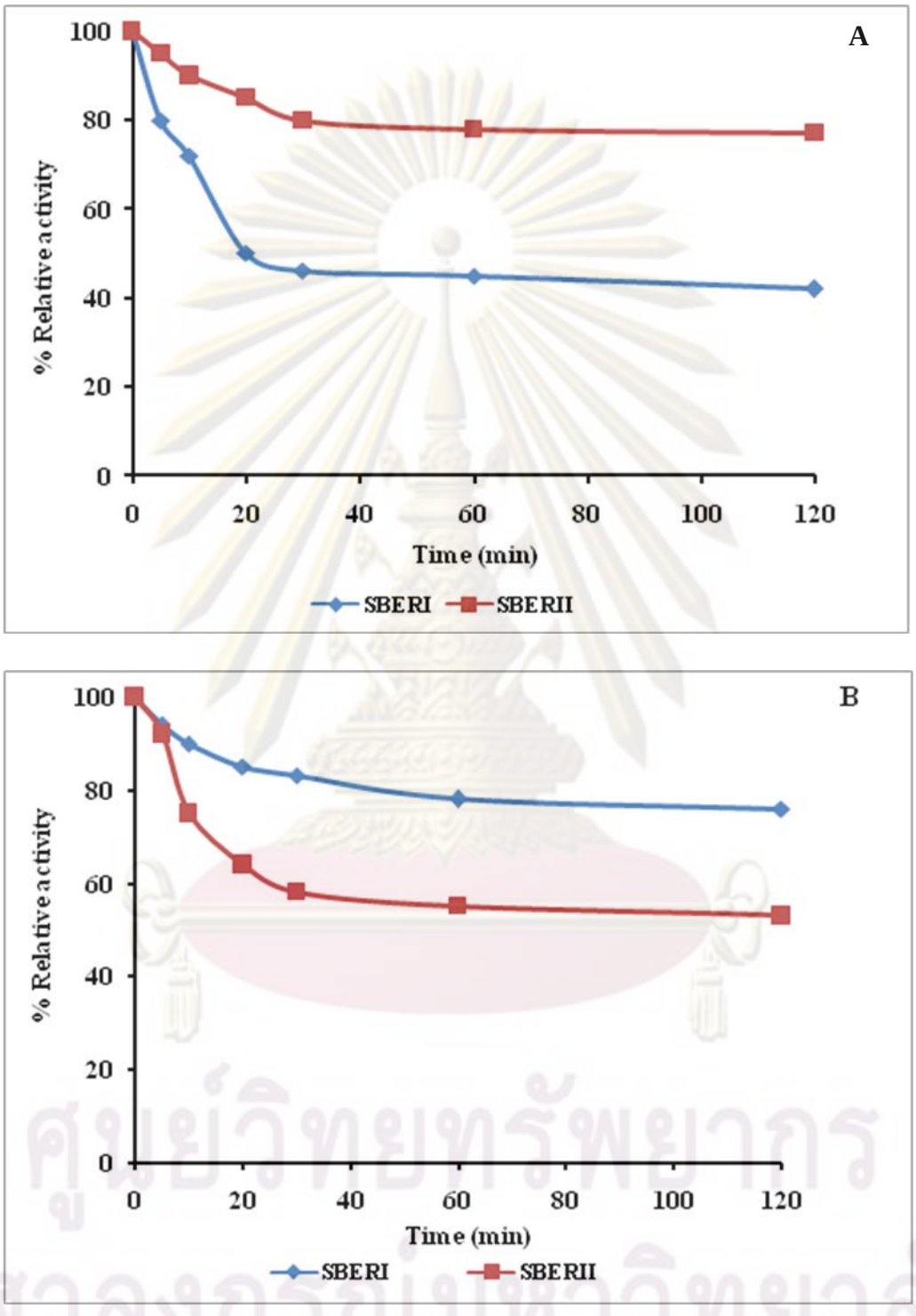


Figure 3.16 Substrate specific of recombinant SBEs

A = Amylose as substrate

B = Amylopectin as substrate

3.3.5 Kinetic study of recombinant SBE with amylose

Two recombinant SBE were assayed for SBE activities with amylose as substrate as described in section 2.9 at optimum pH and temperature as determined in section 3.3.2 and 3.3.3. The results were shown in 3.17-3.18 as saturation curve and Lineweaver-Burke plots of SBERI and SBERII, respectively. K_m for amylose were calculated at 2.13 and 3.46 mg/ml and V_{max} were 1.7 and 3.4 ΔA_{660}/min for SBERI and SBERII, respectively.

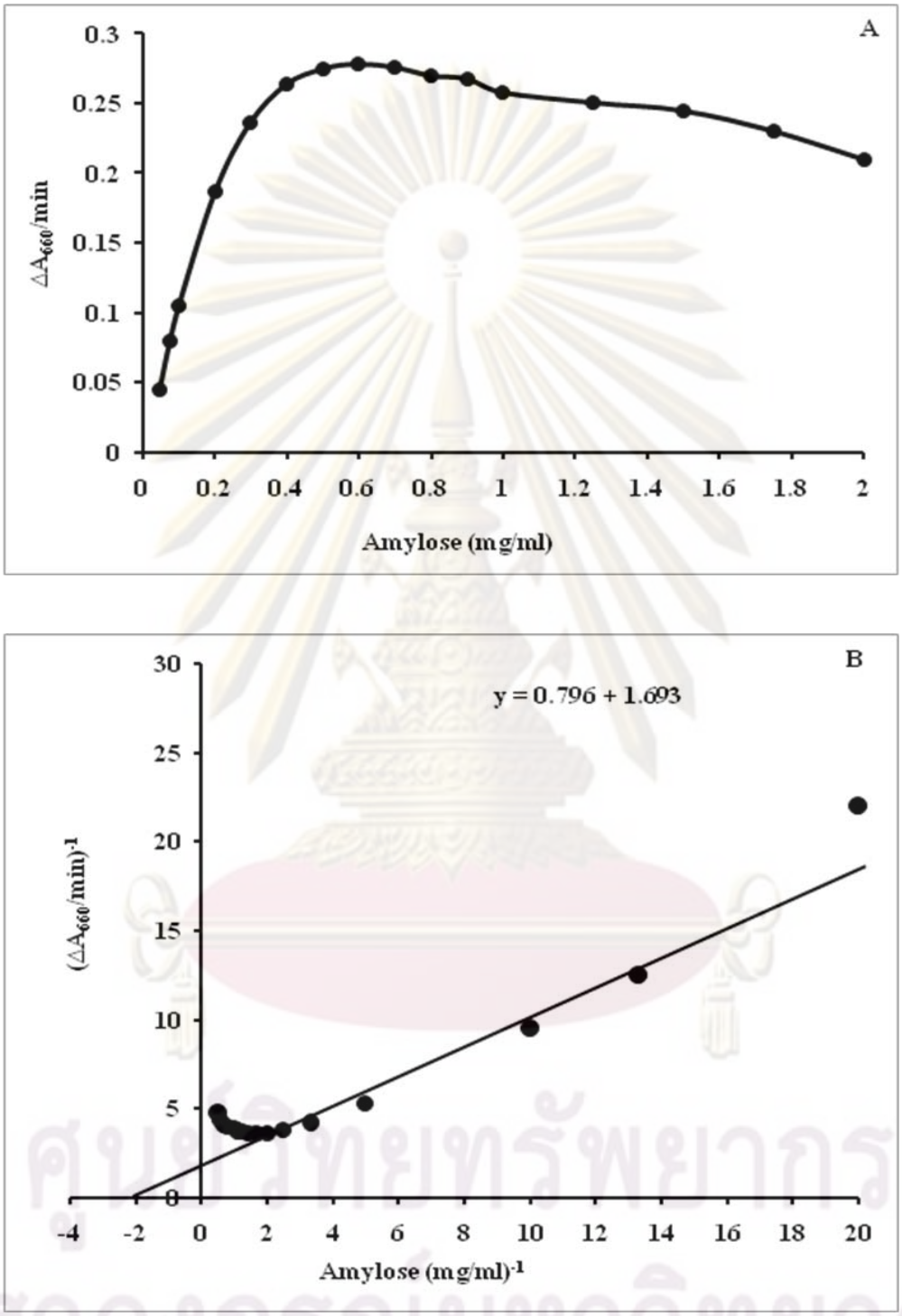
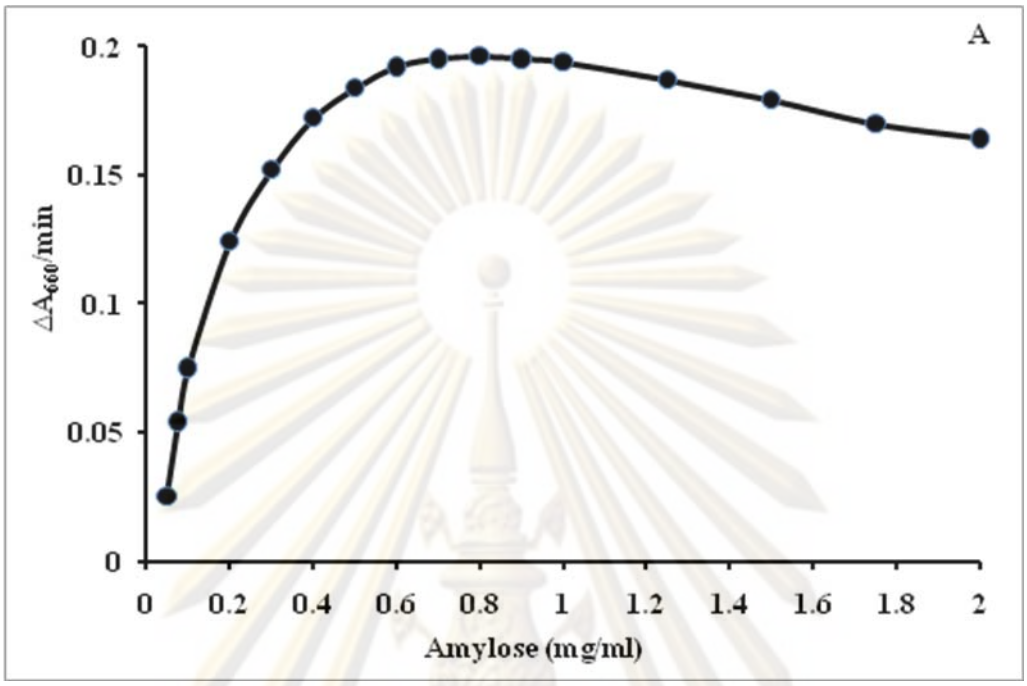


Figure 3.17 Kinetic studies of SBERI with amylose as substrate

A = Saturation curve B = Lineweaver –Burke plot of amylose



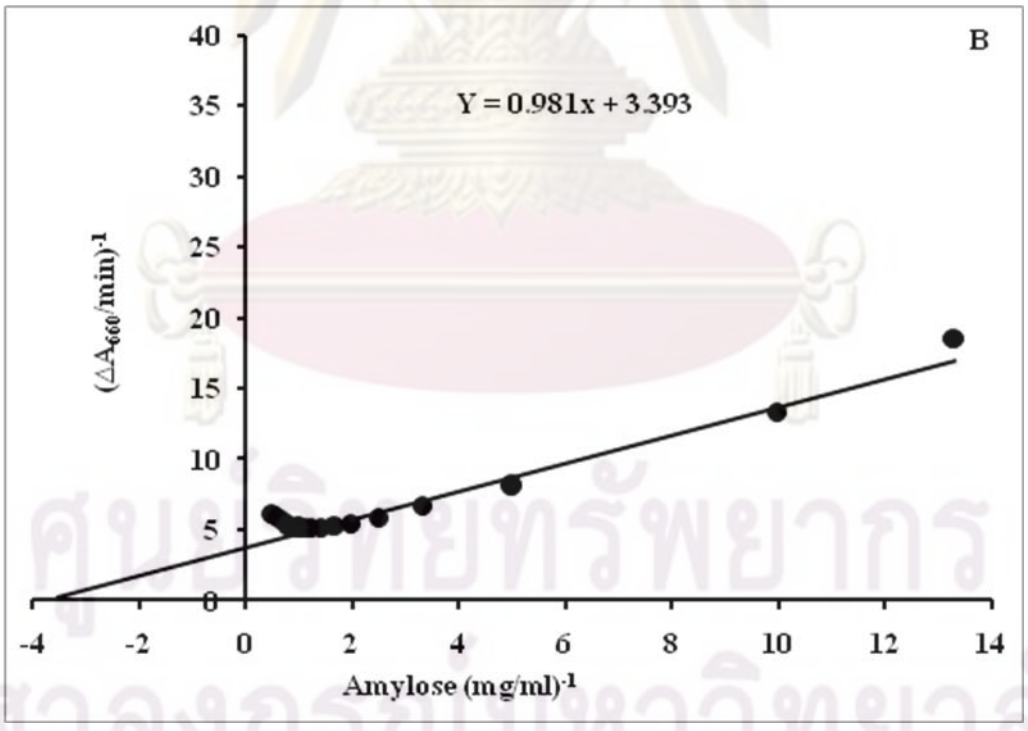


Figure 3.18 Kinetic studies of SBERII with amylose as substrate

A = Saturation curve B = Lineweaver –Burke plot of amylose

3.3.6 Amino acid compositions

The amino acid compositions of three isoforms of native SBE from cassava tuber cv. KU50 and SBERI and SBERII were determined with the method described in section 2.7.7. The amino acid compositions of recombinant enzymes were calculated from the deduced amino acid sequences. The results were expressed as percentage of total amino acid and tabulated in Table 3.2. The amino acid compositions of SBE1 and SBE2 were similar to the deduced amino acid from SBERI whereas SBE3 was more similar to deduced amino acids of SBERII. The amino acids such as alanine, glutamic acid, lysine, proline, arginine and tyrosine which showed some difference were in bold .

3.3.7 Effect of chemical modifying reagents

Each purified isoforms of SBE and recombinant SBEs were incubated with modifying reagents namely IAA (iodoacetic acid), NEM (N-ethyl-maleimide), EDC (1-Ethly-3-(3-diaminopropyl) carbodiimide), TNBS (Trinitrobenezene sulphonic acid), PGO (Phynylglyoxal), DEPC (Diethypyrocarbonate), NAM (N-Acetylimidazol) and NBS (N-Bromo-succinimide) as described in section 2.7.2. The result shown in Figure 3.19 revealed that SBE1, SBE2 and SBERI were affected by DEPC and NAM while SBE3 and SBERII were not.. On the other hand, EDC, NBS, PGO and TNBS affected the activity of all SBE. IAA and NEM showed no effect on all enzymes. PGO showed more prominent effect on recombinant enzymes.

Table 3.2 Amino acid compositions of native and recombinant SBEs

Amino acid		Native enzyme*			Recombinant enzyme**	
		SBE1	SBE2	SBE3	SBER1	SBER2
A	**ala**	**8.6**	**8.9**	**4.2**	**6.9**	**4.5**
C	cys	1.2	1.7	2.2	0.9	1.2
D	asp	9.2	9.2	9.5	7.3	7.6
E	**glu**	**9.55**	**9.4**	**8.4**	**7.3**	**6.3**
F	phe	7.4	7.1	7	5.5	5.6
G	gly	10	10.2	11.3	8	8.4
H	his	5	5.2	5.3	3	3.5
I	ile	7.2	7.1	6.9	5	4.9
K	**lys**	**7.9**	**7.5**	**6.5**	**5.3**	**4.5**
L	leu	9.3	9.2	9	7	6.8
M	met	1.7	2.5	3.2	2.8	3.3
P	**pro**	**6.3**	**6.5**	**7.4**	**4.3**	**5.1**
R	**arg**	**3.82**	**4.1**	**5.2**	**5.4**	**6.2**
S	ser	6.6	8.7	7.2	6.9	7.9
T	thr	3.05	3.29	3.5	4.3	4.2
V	val	3.07	3.6	2.1	5.9	6.3
W	trp	0.44	0.55	0.6	2	2
Y	**tyr**	**1.53**	**2.2**	**1.4**	**4.6**	**5.1**

Native enzyme* means starch branching enzyme from cassava tuber

Recombinant enzyme** means starch branching enzyme from recombinant clone

Amino acids were expressed as percentage by weight of total amino acids in the molecule

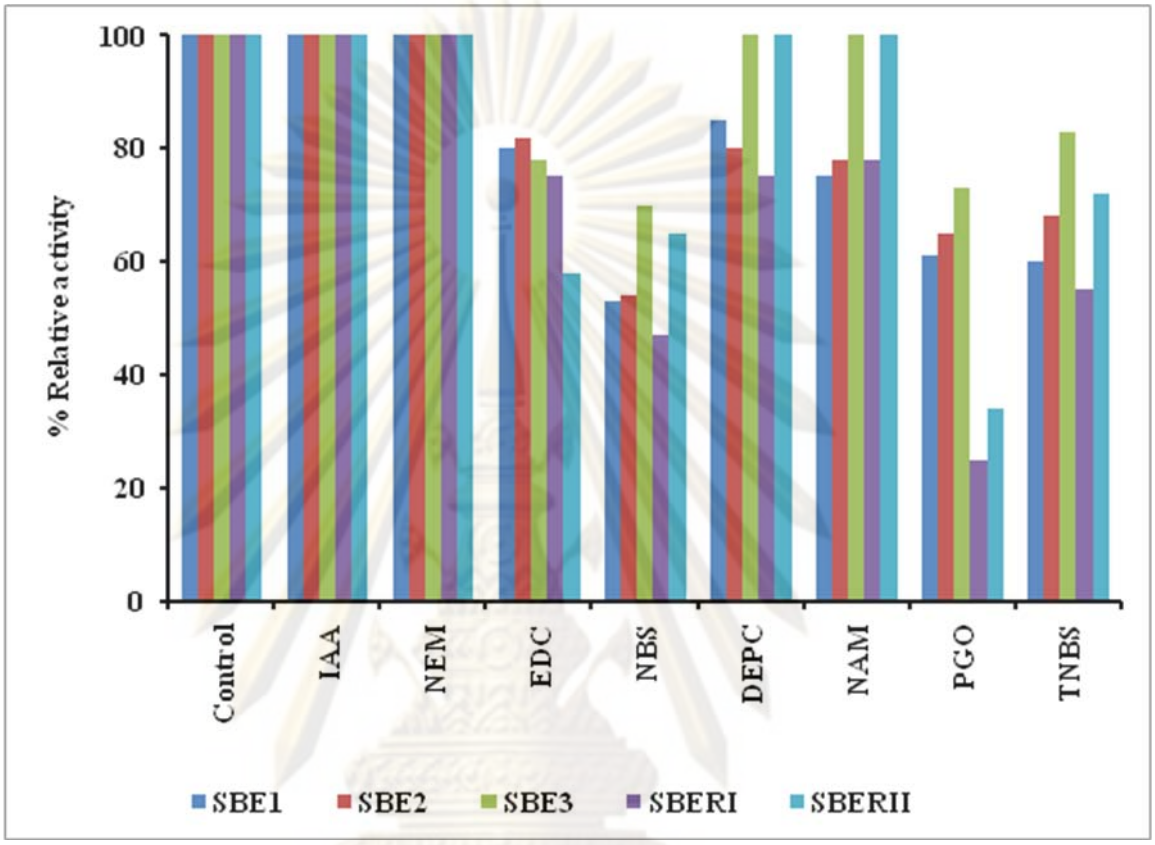


Figure 3.19 Chemical modifying effects in SBEs

SBE1-3 = native enzymes from cassava

SBERI and SBERII = recombinant enzymes

3.4 Immunological study of SBE

3.4.1 Western blot analysis

The purified SBEs from tubers of cassava cv. KU50 and recombinant enzyme were used to raise polyclonal antibodies at Department of Medical technology, Chiang Mai University. The antibodies were conjugated with horse radish peroxidase for western blot analysis. The purified native and recombinant SBEs were electrophoresed by SDS-PAGE and western blot performed as described in section 2.12. The nitrocellulose membrane were incubated with their polyclonal antibodies prepared according to section 2.13.

Each conjugated of SBEs prepared according to section 2.12. The results showed that the purified native isoforms reacted with the polyclonal antibodies from SBERs. Bands were detected at 100,60 and 60kDa for SBE1,SBE2 and SBE3 respectively(Figure 3.20).Cross activity of the recombinant SBEs with antibodies of native isoforms were also observed (Figure 3.21). However, only positive bands at 94 kDa (Figure 3.21) were detected for both SBERI and SBERII.

3.4.2 Monitor of enzyme development by immunological method

Native crude SBE from 6, 9 and 12 months tubers were prepared and electrophoresed on SDS-PAGE and were monitored by polyclonal antibody. The SDS-PAGE of the enzyme at different ages was immunoblotted against antibodies from SBERI and SBERII. Antibody from SBERI stained more intensely with both bands of 108 kDa and 60 kDa. whereas antibody from SBERII showed less intense bands at both molecular weight intensity of both bands increased with ages (Figure 3.22).

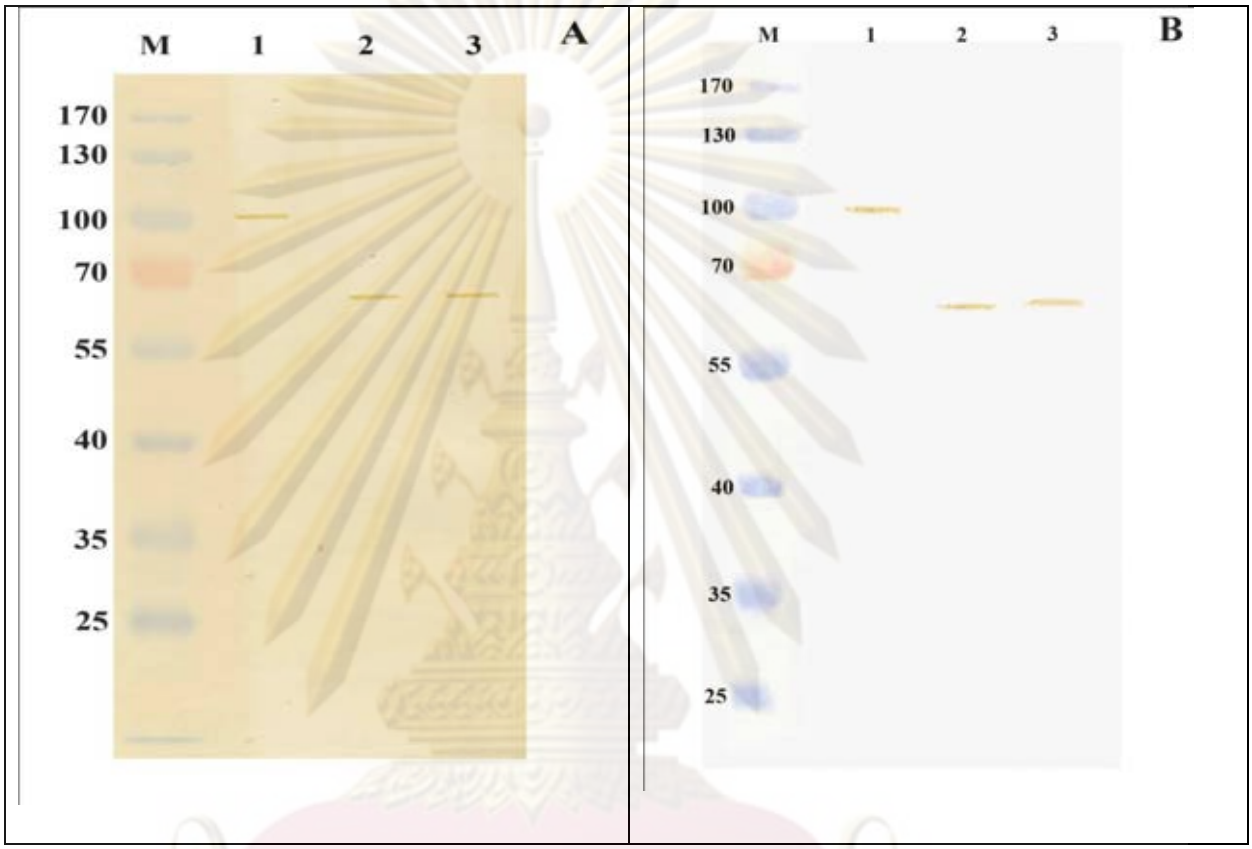


Figure 3.20 Immunoblot pattern of SBE isoforms against antibodies of recombinant SBE.

Lane M = Protein marker (kDa)

Lane 1 = SBE1

Lane 2 = SBE2

Lane 3 = SBE3

A = SBE isoforms against antyibody of SBERI

B = SBE isoforms against antibody of SBERII

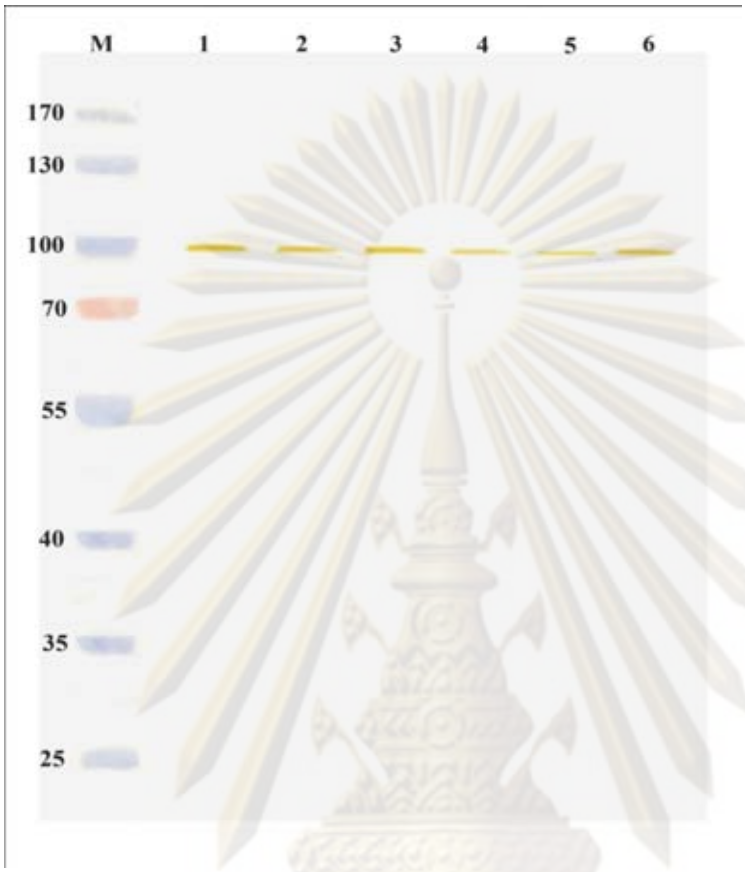


Figure 3.21 Immunoblot pattern of recombinant SBE against with antibodies of recombinant SBE

Lane M = Protein marker (kDa)

Lane 1 = SBERI against antibody of Isoform 1

Lane 2 = SBERII against antibody of Isoform 1

Lane 3 = SBERI against antibody of Isoform 2

Lane 4 = SBERII against antibody of Isoform 2

Lane 5 = SBERI against antibody of Isoform 3

Lane 6 = SBERII against antibody of Isoform 3

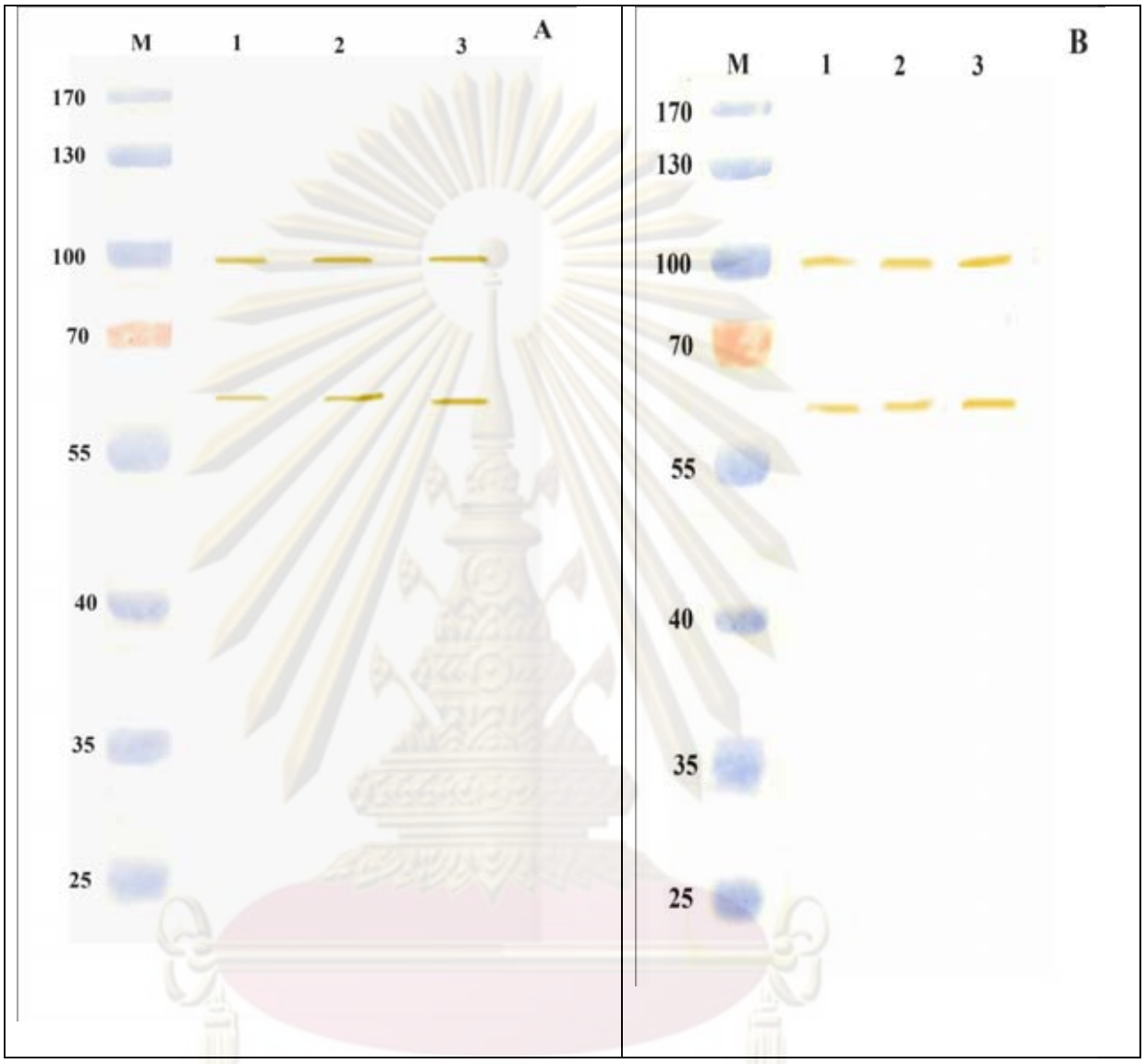


Figure 3.22 Immunoblot pattern of SBE at different ages by recombinant SBE antibody

Lane M = Protein marker (kDa)

Lane 1 = Crude extract from 6 month age

Lane 2 = Crude extract from 9 month age

Lane 3 = Crude extract from 12 month age

A = against with SBERI B = against with SBERII

CHAPTER 4
DISCUSSION

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 in 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 (Hamada *et al*., 2002). The presence of multiple isoforms of starch branching enzyme 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 into 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 (Burton *et al*., 1995), and II and I of potato tuber (Martin *et al.*, 1995) 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 (Guan and Preiss, 1993). 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 (Munyikawa *et al*., 1997).

Plant branching enzyme (Q-enzyme) was first identified in potato (Drummond *et al.,* 1972), the purified SBE had monomeric molecular weight ranging from 83-103kD. Protein sequencing showed that two isoforms of branching enzyme were present in the starch of potato tuber (Larsson *et al.,* 1976). The cDNA of potato SBE was cloned and identified as B class (major form) (Kobmann *et al.,* 1991) and A class (minor form)(Jobling *et al.,* 1999). Study of purified starch branching enzyme I and II expressed in *E.coli* showed that SBE I was more active on amylose substrate whereas SBE II was more active on amylopectin (Larsson 2001). SBE was also found in many cereal crops such as BE I, IIa and IIb in developing kernels of maize (Boyer and Preiss, 1978), QE I and QE II developing rice endosperm (Yamaonichi and Nakamura, 1992). Previously, cDNA coding for Cassava (*Manihot esculenta* Crantz.) branching enzyme was cloned from λgt11 cDNA library using a potato cDNA probe (Salehuzzaman *et al*., 1992). Next, the cloned encoding SBE II was isolated and examined on the spatial and temporal expression of the *sbeII* gene (Jossons et al., 2003). For Thai cultiva , SBE containing two subunits of 80 kD was purified from five minutes cultivar (Aroonrungsawadi, 1999). Recently, three isoforms were isolated from tubers of cassava KU50. Characterizations of the 3 isoforms suggested that isoforms 1 and 2 (SBE, SBE2) should be grouped in class B. whereas isoform 3 which is specific for amylopectin should be grouped in class A.

In this work, cloning and expression of cassava *sbe* genes were attempted. The full length cDNA of *sbe* genes extracted from cassava tubers cultiva KU50

were used. The expressed transformant SBEs (SBERI and SBERII) were purified, characterized and compared to the 3 native isoforms.

4.1 Characterization of cDNA of *sbe* gene

The cDNA of *sbe* genes from cassava tubers (*Manihot esculenta* Crantz.) encoded to two forms of SBE: SBERI and SBERII. Their deduced amino acid sequences were compared with other plants SBE: cereal/grain SBE type (Maize, rice and wheat) and tuber SBE type (sweet potato, potato). In the comparison with those in EMBL-GENBank-DDBL database, the percentages of amino acid sequence similarity were higher than ClustalW2 program because the similarity were especially at a specific domain, the glycogen branching domain (Figure 4.1). The glycogen branching domain were found associated with different types of catalytic domains at either the N-terminal or C-terminal ends and may be involved in homodimeric/tetrameric/dodecameric interactions. Members of this family included the alpha amylase super-families (Preiss et al., 1986). Several other identical regions were also observed in the comparison. Conserved amylolytic regions found in other amylolutic enzymes from other plants were also found (Jespersen et al., 1993). The structural distinction extended to the isoforms of branching enzymes from other species. Although the sizes of the SBERI and SBERII were very close, the alignment showed that they were not the same protein. Previously, cDNA coding for Cassava (*Manihot esculenta* Crantz.) branching enzyme was cloned from λgt11 cDNA library using a potato cDNA probe (Salehuzzaman *et al.*, 1992). The cloned encoding SBE II was isolated and examined on the spatial and temporal expression of the *sbeII* gene (Baguma *et al.*, 2003). Report (Salehuzzaman *et al.*, 1992) found cDNA clones coding for branching enzyme were isolated from a λgt11 cDNA library of cassava about 3.0 kb in size. The full-length cDNAs cloned from potato and pea were also around 3.0 kb in size. in *sbeI* (Martin *et al.*,1990; Kobmann *et al.*, 1990). Jossons (Jossons *et al.*, 2003) cloned partial cDNA for *sbeII* from mRNA of leaves cassava by a cDNA-specific fragment of *sbeI*, the longest clone of 1838 bp. The

overall identity between the cassava sbeII clone and other plant *sbeII* and *sbeI* genes was >76 and <56%, respectively. The deduced amino acid sequence of the cassava *sbeII* clone showed the highest degree of identity with SBEII from pea (81%) and sweet potato (79%). The resemblance between cassava SBEII and SBEI was considerably lower (52 and 68% identity and similarity, respectively).

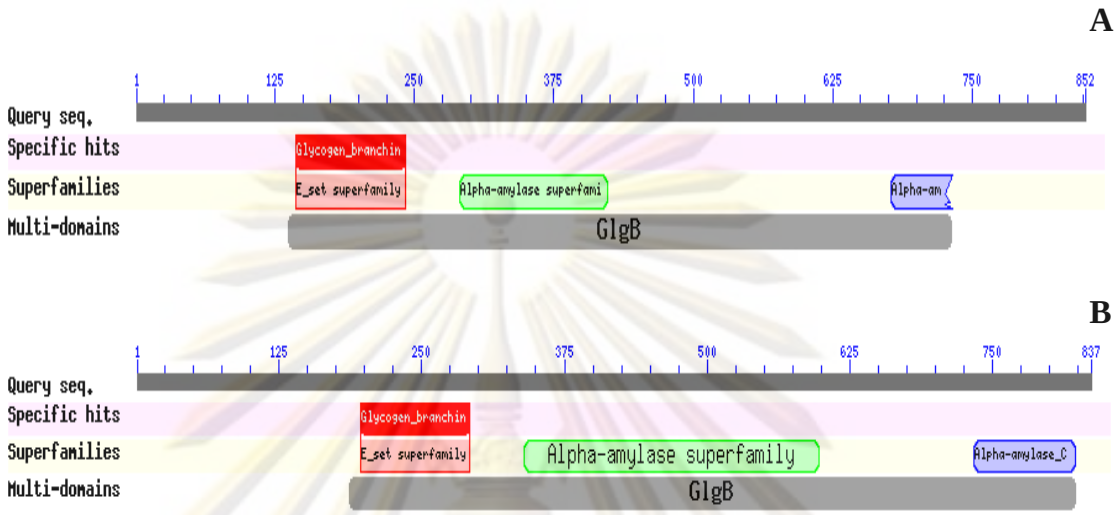


Figure 4.1 Schematic presentation of conserved domain of SBERs' deduced amino acid sequences

A = SBERI B = SBERII

Red = Glycogen branching enzyme-like N-terminus domain.

Pink = "Early" set of sugar utilizing enzymes domain.

Green = α-amylase superfamily conserve domain

Blue = α- amylase, catalytic domain. C-terminal all-beta domain.

Grey = Glycogen branching enzyme domain.

4.2 Cloning and expression of *sbe* genes

Efficient cloning and expression of eukaryotic genes especially plant genes in bacteria were hard to achieve because most amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. In each cell, the tRNA population closely reflects the codon bias of the mRNA population. When the mRNA of heterologous target genes is over expressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frame shifting and amino acid mis-incorporation.

The choice of vectors were made according to the need for restriction sites and open reading frame compatibility. The pET-28a-c(+) vector carries an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. The sequence is numbered by the pBR322 convention, so the cloning/expression region of the coding strand was transcribed by T7 RNA polymerase. The addition of T7•Tag® is peptide for easy detection on Western blots. Upstream primers were specificly designed close to open reading with suitable restriction sites in order that suitable pET 28 type couls be selected for transcription of frame shift to encode this enzyme. Thus, *sbeI* gene which contained *Xho* I site was compatible to pET 28c whereas *sbeII* gene contained *BamH* I site which suited pET 28b. Moreover, downstream primers were designed to contain stop condon and restriction site for their plasmid: the *Sal*I site. The recombinant plasmids were cloned into *E. coli* rosetta gami (DE) and expressed under T7 promotor. The Rosetta-gami strains are resistant to kanamycin, tetracycline,streptomycin, and chloramphenicol. They contained a chromosomal copy of the gene for T7 RNA polymerase. The upstream region of the inserted genes contained highly efficient ribosome binding site from the phage T7 major capsid protein (Novagen, 2002). The plasmids containing T7 promoter driven expression which were repressed until IPTG induction of T7 RNA polymerase from a lac promoter. The recombinant plasmid is transferred to an These hosts are lysogens of bacteriophage DE3, a lambda derivative that has the immunity region

of phage 21 and carries a DNA fragment containing the lacI gene, the lacUV5 promoter, and the gene for T7 RNA polymerase (Studier and Moffatt, 1986; Novy and Morris, 2001). Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the lacUV5 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). Addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase production, which in turn transcribes the target DNA in the plasmid.

The *E.coli* Rosetta-gammi (DE) was chosen as expression host for the *sbe* genes. The Rosetta strains were designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* (Brinkmann *et al*., 1989; Seidel *et al.,* 1992; Kane, 1995; Kurland and Gallant, 1996). Expression of such proteins can be dramatically increased when the level of rare tRNA is increased within the host (Brinkmann *et al*., 1989; Seidel *et al.,* 1992; Rosenberg *et al*., 1993; Del Tito *et al.,* 1995). Rosetta-gami host strains are Origami derivatives that combine the enhanced disulfide bond formation resulting from trxB/gor mutations with enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for AGG, AGA, AUA, CUA,CCC, GGA on a compatible chloramphenicol-resistant plasmid. The tRNA genes are driven by their native promoters. Many proteins require the formation of stable disulfide bonds to fold properly into a native conformation. Disulfide bonds are usually formed only upon export into the periplasmic space, (Prinz *et al*., 1997; Aslund *et al*., 1999). Without disulfide bonds, these proteins may be degraded or accumulate as inclusion bodies.The enhancement of the formation of disulfide bonds in this *E. coli* systein the cytoplasm made the proteins more soluble and were not accumulated in the form of inclusion body.

The crude extract from recombinant clone was assay for SBE activity. They were studied of induction time and final concentration of IPTG, which showed activity in their conditions. Even in absence of IPTG, the expression of *sbe* gene was occurred because there are some expression of T7 RNA polymerase from *lacUV5* promoter in the DE lysogen from *E. coli* genome. Highest SBE

activity were achieved at 0.4-0.6 M IPTG, thus, final concentration of 0.4 M IPTG was used for induction.

4.3 Purification of recombinant SBE

The purification of protein is the preparation of extract containing the protein in a soluble form and extraction procedures should be selected according to source of the protein. SBE is intracellular enzyme, so ultrasonication was employed to release the enzymes from the cells. The recombinant SBE contained His•Tag sequence at N-terminus of enzymes, polyhistidine-tag is an amino acid motif in proteins that consists of at least six histidine (*His*) residues. The His•Tag sequence is very useful for affinity purification such as Ni Sepharose. Affinity media contain bound metal ions, nickel to which the polyhistidine-tag binds with micromolar affinity.

SBERI and SBERII were expressed with His-tag at N-terminus. The his-tag were not removed. Attempt to remove the histidine with thrombin treatment led to significant activity lost. Search through the amino acid sequences showed existence of thrombin reactive sites within the peptides at three positions on SBERI i.e. at residues 59, 292 and 729 and two positions on SBERII i.e. residues 143 and 342 . There were also reports that the presence of his-tag did not or slightly affect activity of some proteins and enzymes. Since the SBE activities of the recombinant enzymes obtained were at satisfactory levels, the His tag were left intact. After, purification by nickel column, the activities of both recombinant enzymes increased significantly, resulting in the apparent recovery of enzymes greater than 100%.This was probably resulted from the removal of some activity interfering factors by the column (Amersham, 1999).

4.4 Characterization of SBE isoforms

4.4.1 Molecular weight and determination

The molecular weights calculated from the deduced from amino acid sequence, cDNA was encoded *sbe* genes were approximately 95 kDa. The molecular weight of purified recombinant SBEs were determined to be approximately 90 kDa by SDS-PAGE. Previously, purified SBE isoforms from cassava tuber KU. 50 were determined of molecular weight 108, 60 and 60 kDa (Yaiyen *et. al*., 2008) Many proteins are synthesized as inactive precursors that are activated under proper physiological conditions by limited proteolysis of enzyme or post-translational modification. The process can occur with native SBE expressed in the the eukaryotic system whereas this process was about in the transformants, recently in only one form of SBE. In kidney bean, demonstrates that two SBE isoforms encoded by a single gene have different sub-cellular localization and protein profiles as well as distinct enzymatic properties (Hamada *et.al*.,2002). In addition to its unusually large molecular size, it was associated with the granule. Because the wheat SBEI is located only in the soluble fraction of the endosperm (Samuel *et al.*, 1997).

4.4.2 Effect of pH and temperature on SBE activity

Each isoform was incubated in various buffers and appropriate pH its activity measured. It was found that, citrate buffer is highest activity than other buffer in same pH, indicating that citrate has a significant influence on the catalytic activity but has no effect on its substrate affinity for amylose (Matsui et.al.,2001). When the SBERI and II was incubated at various temperatures, the activity showed the highest temperature at 37°C, after which the enzyme activity dropped steadily to lower than 50%. The observation was close of those separated in native SBE from KU50 tuber and also in the other plant.

4.4.3 Kinetic constants of SBERI and SBERII

Starch branching enzyme employed amylose as main substrate. Experiments were carried out to investigate the K_m and V_{max} for amylose of each recombinant. The K_m were 2.13 and 3.46 mg/ml and V_{max} were 1.7 and 3.4 Δ A_{660} / min for SBERI and SBERII respectively. This indicated that SBERI was more specific towards amylose than SBERII. Kinetic study of purified native enzymes were also performed previously (Yaiyen *et al.*, 2006).

The K_m constant were 1.12, 1.37 and 2.17 mg/ml and V_{max} were 1.03,0.83 and 0.57 Δ A_{660} / 30 min for SBE 1,SBE2 and SBE3, respectively (Table 4.1) This indicated that isoforms 1 and 2 were more active towards amylose than isoform 3.Therefore, the kinetic data of SBERI was more correlated to SBE1 and SBE2 whereas SBERII was more correlated to SBE3. It was noted that the K_m and V_{max} of SBERI and SBERII were both higher than those observed in the three native isoforms. It was reported that C-terminal domain of SBE was involved in substrate specificity where as N-terminal domain is important for specificity of transferred chain length and require for maximum enzyme activity. The recombinant enzymes were tagged with histidines at both terminals. The higher K_m and V_{max} observed could be the effect from the presence of his-tag which might hinder the substrate binding to some extent. Similar substrate inhibition characteristics were observed in the saturation curves of SBERI and SBERII when compared to those reported for SBE1,SBE2 and SBE3 by Yaiyen(2006).

Table 4.1 Comparison of characterization of native and recombinant enzymes

	Molecular weight (kDa)	pH Optimum	Substrate specific	K_m in amylose(mg/ml)
SBE1	108	7.0	Amylose	1.12
SBE2	60	7.0	Amylose	1.37
SBE3	60	7.0	Amylopectin	2.17
SBERI	90	7.0	Amylose	2.13
SBERII	90	7.0	Amylopectin	3.46

4.4.4 Comparison of SBE isoform activities with amylose and amylopectin as substrate

There have been reports that some SBE isoforms, although catalyzed Branching of amylose, preferentially used amylopectin as substrate. So experiments were carried out to investigate if any of the recombinant SBEs displayed such activities. The results showed SBERI utilized amylose better than SBERII while SBERII was more active towards amylopectin. In previous report, native cassava SBE1 and SBE2 were more active to amylose than SBE3, indicating similarity of SBERI to SBE1 and SBE2 and SBERII to SBE3.This in agreement with the kinetic studies ins section 4.4.3. Starch branching enzymes have been classified to two classes by amino acid sequences, class A preferentially branches amylopectin whereas class B preferentially branches amylose. From the above information, SBERI should be grouped in class B whereas SBERII which is specific for amylopectin should be grouped in class A. According to Hamada *et al* (2002), SBERI which had high affinity for amylose might be localized in the starch granule whereas SBERII in the soluble fraction of the plastid.

4.4.5 Effect of amino acid modifications of starch branching enzymes

The results from treatment of each form of SBE with several amino acid modifying chemicals showed that only SBE1, SBE2 and SBERI were affected by DEPC and NAM by approximately 20-30%. NAM affected tyrosine residue by causing acetylation at at –OH group of tyrosine. Tyrosine played role as catalytic residue in amylase domain (Chibbar *et al.,* 1999 and Myers *et al.*,1995). DEPC reacted with histidine residues, probably by cleavage of imidazole ring rendering the modification of histidine reversible. It was reported that histidine residues were important for substrate binding (Funane *et al*., 1998). The substrates amylose and amylopectin provided significant protection against BE inactivation by DEPC **(**Loosemore and Pratt, 1976). At least one histidine residue was responsible for the loss of BE activity in Maize (Preiss *et al*.,1998). EDC, NBS, PGO and TNBS affected the activity of all SBE inthis study. 1-Ethly-3-(3-diaminopropyl) carbodiimide (EDC) play role on tyrosine modification at –COOH group. It was previous reported to have effect on catalytic residue in amylase domain (Nakamura *et al.*,1996). N-Acetylimidazole (NBS) affected with tryptophan by cleaving the peptides at carboxyl side of tryptophan residues. Tryptophan was important at starch binding site in α-amylase (SÖgaard et al., 1993). Phenylglyoxal (PGO) can react specifically with guanido groups of arginine residues under mild conditions. The only comparably rapid reaction is with α-amino groups. The arginine was conserved was important in catalysis of SBE in C-terminus (Cao and Preiss, 1996). In our experiment PGO caused dramatic reduction in SBE activity. Trinitrobenzenes sulphonic acid (TNBS) or picryl sulphonic affected hydrophilic amino acid such as serine, threonine, asparagine and glutamine. SBE is a member of α-amylase family with catalytic domain in the form of a $(\alpha/\beta)_8$ barrel, with the active site being at the C-terminal end of the barrel β-strands, one glutamic acid and two aspartic acid residues necessary for activity (Mac Gergor *et al.,* 2001).

From this study, tyrosine and histidine were important for activity of SBE, SBE2 and SBERI. Moreover, arginine may be more important at the active

site of the SBE especially SBE1, SBE2 and SBERI. These finding complied with previous reports on SBE in other plants and also further support the similarity of SBE1, SBE2 and SBERI.

4.4.6 Amino acid compositions of SBE

The analysis of amino acid composition of native enzymes were calculated as % of total amino acids by weight and compared with deduced amino acid compositions of recombinant starch branching enzymes. It was found that, alanine, glutamic acid, lysine, proline, arginine and tyrosine were significant group of amino acids which were similar in SBE1 and SBE2 to SBERI whereas SBE3 was more similar to deduced amino acids from *sbe*II genes. Again, this support the prior experiments.

4.5 Immunological study of SBE

Polyclonal antibodies of purified native isoforms and transformant SBE were raised. The antibodies were conjugated with horse radish peroxidase. When western blots of the antibodies and the starch branching enzymes were performed, antibosies of SBERI and SBERII can bind and showed positive staining of purified SBE1, SBE2 and SBE3 bands at the molecular weight of 108, 60 and 60 kDa, respectively. When SBERI and SBERII were incubated with polyclonal antibodies from each native isoforms, positive band appeared at 90 kDa. The results suggested that all native SBE isoforms and recombinant SBE contained similar epitope sequences which resulted in cross reactivity of the antibodies. Slight difference were observed that antibodies to SBE1 and SBE2 recognized SBERI better than SBERII whereas SBE3 antibody bind better with SBERII. When crude enzyme at different ages were immunoblotted against antibodies from SBERI and SBERII. Antibody from SBERI stained more intensely with both bands of 108 kDa and 60 kDa. whereas antibody from SBERII showed less intense bands at both molecular weight. The intensity of both bands increased with ages, with highest staining at 12 months. Yaiyen (2008) reported the specific

activity of SBE in cassava tubers started to accumulate and increase from 6 months up to 12 months old. Very small amount was detected at 3 months.

4.6 Correlations of sbe genes to native SBE isoforms

Only two sbe genes could be isolated from cDNA of cassava, the *sbeI* and *sbeII.* Both genes were of almost the same size but were expressed into transformant enzymes with some different characteristics, especially concerning substrate specificities and affinity. When sequences alignment were performed, frame shift of the open reading frames were observed, rendering some difference in amino acid sequences and compositions. When the two genes were expressed in *E.coli*, only one protein band around 95 kDa was observed for both transformant enzymes which was close to the molecular weight of SBE1.The smaller molecular weight SBE2 and SBE3 should most likely be the post translational –modified products of both *sbeI* and *sbeII* genes expressed in the parenchymal cells of cassava tubers, which would not be produced in the transformants. In such case, the difference observed in the substrate specificity may be cuased by the cleaved portion. Considering the alignment shown in figure 4.1, it was also speculated the cleavage occurred at N-terminal since 125-350 residue. This speculation can only be proved if the amino acid sequences of the smaller isoforms are known. On the other hand, the observed characteristics, such as amino acid modification, amino acid compositions and kinetic parameters, suggested the relationships of SBE1 and SBE2 with SBERI and SBE3 with SBERII. At this point, we would propose that SBE1 and SBE2 were the expression product of *sbeI* gene and classified as class B SBE. On the other hand, SBE3 was the expression product of *sbeII* gene and was the class A SBE (Guan and Preiss, 1993). Since both *sbe* genes were successfully cloned and expressed, they can be used for more detailed study by gene manipulation. In addition, the sequences of both *sbe* genes at N-terminal which was speculated to be the binding domain with different substrates. May be used to raise antibodies which will be able to detect isoforms of SBE more specificly.

CHAPTER 5

CONCLUSIONS

1. The cDNA of *sbe*I and *sbe*II contained 2559 and 2514 bp open reading frame, which encoded for polypeptides of 852 and 837 amino acids.
2. Both cassava showed similarity of 49% by CLUSTALW2 program and 72.2% by EMBOSS alignment program when their deduced amino acid sequences were compared.
3. PCR products of *sbeI* and *sbeII* genes were 2.8 and 2.7 kb and were ligated to pET 28c for *sbeI* and pET 28b for *sbeII* and transformed into *E.coli* Rosetta gami(DE). The expression of SBERI and SBERII were induced by 0.4 mM IPTG at 4 hours.
4. SBERI and SBERII were purified 5 folds by His-trap chromatography column.
5. SBERI moved slower than SBERII when electrophoresis by 7% native starch-PAGE and stained with I_2. Purified SBERI and SBERII showed single band with molecular weight 90 kDa by SDS-PAGE.
6. SBERI and SBERII showed optimum pH at 7.0 and optimum temperature at 37°C.
7. SBERI utilized amylose better than SBERII while SBERII was more active on amylopectin. K_m for amylose was calculated at 2.13 and 3.46 mg/ml and V_{max} were 1.7 and 3.4 ΔA_{660}/min for SBERI and SBERII, respectively.

8. Amino acid composition showed native SBE1 and SBE2 were similar to the deduced amino acid from *sbe*I gene whereas SBE3 was more similar to deduced amino acids from *sbe*II gene.
9. SBE1, SBE2 and SBERI were affected by DEPC and NAM only while EDC, NBS, PGO and TNBS affected the activity of all SBE. IAA and NEM showed no effect on all enzymes. PGO showed more prominent effect on recombinant enzyme. The tyrosine, histidine, arginine residue played role in the activities of native and transformed starch branching enzyme.
10. Polyclonal antibodies of native SBE and transformed SBE cross-reacted with each other. Anti- SBERs showed positive bands at 108 and 60 kDa when blotted with SBE1, SBE2 and SBE3 while anti-SBE1, anti-SBE2and anti-SBE3 showed positive band at 90 kDa when blotted with purified SBERI and SBERII.
11. When anti-SBERI and anti-SBERII were used to monitor SBE in crude extracts prepared from 6, 9 and 12 months tubers, anti-SBERI stained more intensely with the both bands at 108 kDa and 60 kDa compared to anti-SBERII. The band intensity increased with tuber age.

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APPENDICES

APPENDIX A

Luria-Bertani (LB) broth media

Peptone from casein	1%
Yeast extract	0.5%
NaCl	0.5%
pH	6.8-7.2

The LB plate was added the 1.0-2.0% Agar

The selective antibiotic drug was supplement

APPENDIX B

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 7.8-8.3).

APPENDIX C

Immunoblotting assay

Tank-blotting transfer buffer, 1 liter

25 mM Tris(Hydroxymethyl)-aminomethane

150 mM Glycine

20% Methanol

pH should be approximately 7.8-8.3

Phosphate buffer saline (PBS) buffer

0.2 M Phosphate buffer pH 7.4 containing 0.15 M NaCl

Blocking buffer

3% (w/v) Bovine serum albumin in PBS buffer.

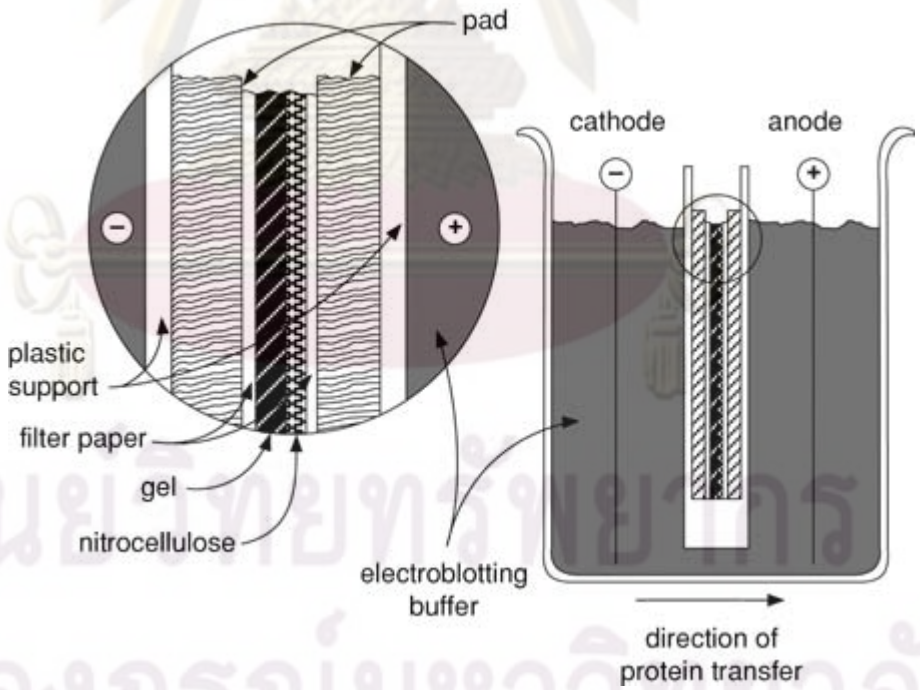


Figure Appendix C Immunoblotting with a tank blotting unit.

APPENDIX D

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	

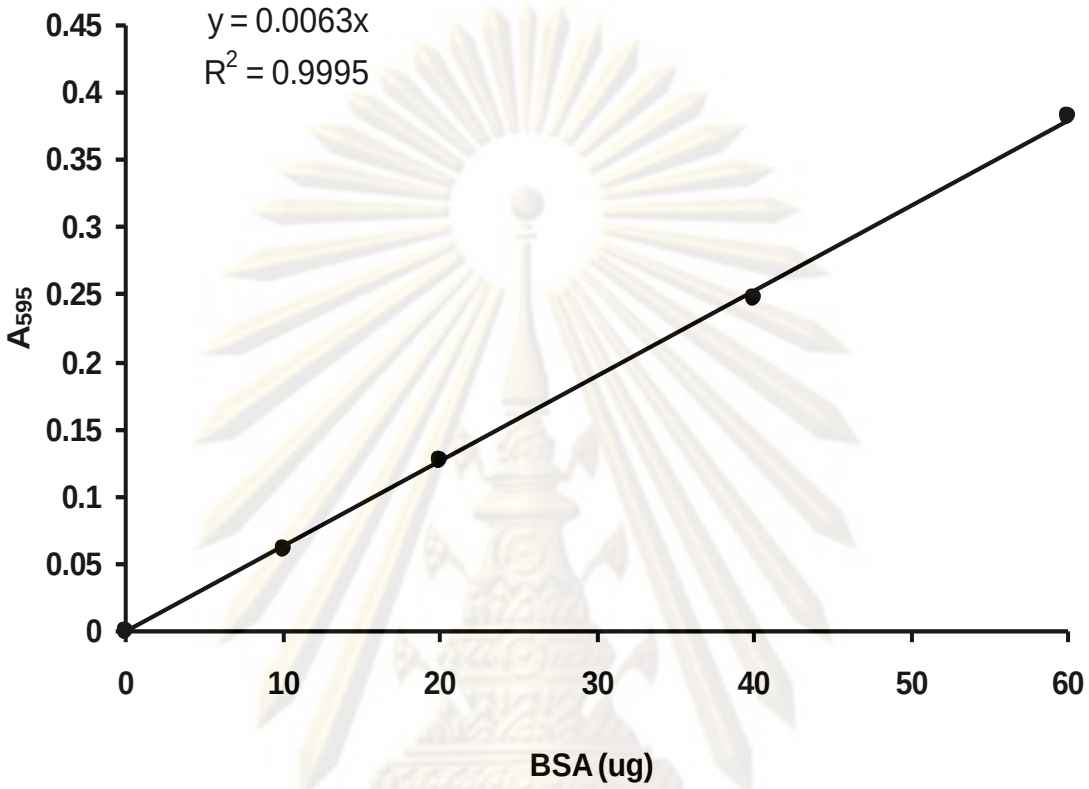
Iodine solution II

1% Potassium iodide; 0.1% Iodine

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

APPENDIX E

Calibration curve of protein concentration



APPENDIX F

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 Department of Biotechnology, Faculty of Science, Ramkhamhaeng University in 1994. After gradating with degree of Bachelor of Science, He was enrolled in the Mater degree of Biochemistry Faculty of Science Chulalongkorn University during 2000-2004. Then, He continued studying for Ph.D. of Biotechnology Faculty of Science Chulalongkorn University in 2005.