การแยกและลักษณะสมบัติของเอนไซม์ตัดโซ่กิ่งจากหัวมันสำปะหลัง
Manihot esculenta CRANTZ

นาย วศิน ศิลปวิสุทธิ์

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ISOLATION AND CHARACTERIZATION OF STARCH DEBRANCHING ENZYME FROM TUBER OF CASSAVA *Manihot esculenta* CRANTZ

Mr. Wasin Sinlapawisut

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วศิน ศิลปวิสุทธิ์ : การแยกและลักษณะสมบัติของเอนไซม์ตัดโซ่กิ่งจากหัวมันสำปะหลัง *Manihot esculanta* CRANTZ. (ISOLATION AND CHARACTERIZATION OF STARCH DEBRANCHING ENZYME FROM TUBER OF CASSAVA *Manihot esculenta* CRANTZ.) อ. ที่ปรึกษา : รศ.ดร. ทิพาพร ลิมปเสนีย์, 100 หน้า. ISBN 974-53-2205-9.

เอนไซม์ตัดโซ่กิ่ง (Debranching enzyme) เป็นเอนไซม์ตัวหนึ่งในกระบวนการสังเคราะห์แป้ง เอนไซม์ตัดโซ่กิ่งมีแอคติวิตีสองแบบคือ ไอโซอะไมเลส (Isoamylase) และ พูลลูแลนเนส (pullulanase) ในการทดลองนี้ ทำการสกัดและศึกษาสมบัติของเอนไซม์ตัดโซ่กิ่งในหัวมันสำปะหลังสายพันธุ์ 5 นาที พบแอคติวิตีของเอนไซม์ตัดโซ่กิ่งในส่วน parenchyma ของหัวมันเท่านั้น ได้ทำให้เอนไซม์ตัดโซ่กิ่งที่พบนี้บริสุทธิ์ขึ้นด้วยการตกตะกอนที่ความเข้มข้นของแอมโมเนียมซัลเฟตอิ่มตัว 20-50% และวิธีคอลัมน์โครมาโตกราฟี โดยใช้ DEAE-Sepharose และ Sephadex G-150 เอนไซม์ที่เตรียมได้เป็นชนิดพูลลูแลนเนส ที่มีความบริสุทธิ์ขึ้น 18 เท่า น้ำหนักโมเลกุลของเอนไซม์นี้ซึ่งคำนวณจากการแยกด้วย Sephadex G-150 มีค่าเท่ากับ 103 กิโลดาลตัน เมื่อวิเคราะห์ด้วยอิเล็กโทรโฟเรซีสแบบเสียสภาพที่มีเอสดีเอส พบแถบโปรตีนหลักที่มีขนาด 35 กิโลดาลตัน ซึ่งคาดว่าพูลลูแลนเนสอาจประกอบด้วยหน่วยย่อยที่มีขนาดเท่ากัน 3 หน่วยและมีค่า pI เท่ากับ 4.75 พูลลูแลนเนสที่เตรียมได้สามารถเร่งปฏิกิริยาได้ดีที่ค่าความ เป็นกรด-ด่างเท่ากับ 6.0 และอุณหภูมิ 37 องศาเซลเซียส และทนต่ออุณหภูมิในช่วง 4-40 องศาเซลเซียส เอนไซม์มีความจำเพาะต่อ พูลลูแลน (pullulan) สูงกว่า อะไมโลเพคติน และน้ำแป้ง(soluble starch) พูลลูแลนเนสมีค่า K_m ต่อ พูลลูแลน เท่ากับ 0.88 มิลลิกรัม/มิลลิลิตร การศึกษาผลของ sulfhydryl reagents พบว่า DTT สามารถกระตุ้นแอคติวิตีของเอนไซม์ แต่ NEM และ IAA มีผลยับยั้ง แสดงว่าหมู่ –SH มีบทบาทในการเกิดปฏิกิริยาของพูลลูแลนเนส สำหรับผลของ divalent metal ions พบว่า Ni^{2+}, Hg^{2+} และ Cu^{2+}มีผลยับยั้ง ในขณะที่ Mn^{2+} และ Co^{2+} มีผลกระตุ้นแอคติวิตีของพูลลูแลนเนส

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....
สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD: DEBRANCHING ENZYME / CASSAVA / STARCH BIOSYNTHESIS

Wasin Sinlapawisut : ISOLATION AND CHARACTERIZATION OF STARCH DEBRANCHING ENZYME FROM TUBER OF CASSAVA *Manihot esculenta* CRANTZ. THESIS ADVISOR : Assoc.Prof. Tipaporn Limpaseni, Ph.D, 100 pp. ISBN 974-53-2205-9.

Debranching enzyme, one of enzymes involved in starch biosynthesis in plants, catalyzes the hydrolysis of α-1,6-glucosidic linkages specifically in α-glucan. The enzyme can show either pullulanase or isoamylase activity or both. Debranching enzyme was detectable in parenchyma of cassava tuber but not in cortex. Starch debranching enzyme was purified from parenchyma of cassava tuber by 20-50% saturated ammonium sulfate precipitation followed by chromatographies on DEAE-Sepharose and Sephadex G-150, which resulted in purity of 18 folds. The chromatographic profile showed a peak of pullulanase activity with molecular weight of 103 kDa on Sephadex G-150 and 35 kDa on SDS-PAGE. The debranching enzyme had a pI of 4.75, a pH optimum of 6.0, optimum temperature 37 ^{o}C, and stable at 4-40 ^{o}C. The enzyme showed high specificity for pullulan as a substrate, but lower activity with soluble starch and amylopectin. The K_m for pullulanase was 0.88 mg/ml. It can be activated by DTT but inhibited by NEM and IAA, indicating the involvement of SH-group on pullulanase activity. The enzyme was activated by Mn^{2+} and Co^{2+} but inhibited by Ni^{2+}, $Hg^{2+}$and Cu^{2+}.

Department…….. Biochemistry…… Student's signature……
Field of study…….. Biochemistry…….Advisor's signature……
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CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I

CHAPTER III

Figure **Page**

LIST OF ABBREVIATION

A	Absorbance
BSA	Bovine serum albumin
Da	Dalton
DBE	Debranching enzyme
DP	Degree of polymerization
DTT	Dithiothreitol
EDTA	Ethelenediaminetetraacetic acid
GBSS	Granule-bound starch synthase
GSH	L-Glutathione reduced
HCl	Hydrochloric acid
IAA	Iodoacetic acid
IEF	Isoelectric focusing
kDa	kilo Dalton
L	Litre
M	Molar
ml	Millilitre
mM	Millimolar
MW	molecular weight
NEM	*N*-ethylmaleimide
NaCl	Sodiumchloride
NaOH	Sodium hydroxide
pI	Isoelectric point

PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
SBE	Starch branching enzyme
TCA	Trichloroacetic acid
TEMED	*N*,*N*,*N*$^{/}$,*N*$^{/}$-tetramethyl ethylene diamine

CHAPTER I

Introduction

1.1 Cassava

Cassava (*Manihot esculenta* Crantz) is a root crop belonging to section fructicosae of family Euphorbiaceae, Dicotyledonae [1]. It is one of the world's most important, and under-exploited staple food crops and source of income. It is the third most important source of calories in the tropics consumed by some 600 million people on a daily basis in Africa, Asia, and Latin America. It provides a cheap source of dietary carbohydrate energy ranking fourth after rice, sugarcane and maize, and sixth among crops in global production.

Cassava is a shrubby, tropical, perennial plant that is not well known in the temperate zone. For most people, cassava is commonly associated with tapioca. The plant grows very tall, at times reaching 15 feet, with leaves varying in shape and size. The edible parts are the tuberous roots and the leaves. The tuber (root) is some-what dark brown in color and grows up to 2 ft. long sometimes longer, depending on the cultivar and the soil conditions (Figure 1.1). Apparently, the plant adapted very well to the local growing conditions and was less susceptible to the locust. Cassava thrives better on poor soils than any other major food plant. As a result, fertilization is rarely necessary. Due to its ability to grow well on marginal soils. However, yields can be increased by planting cuttings on well drained soil with adequate organic matter. Cassava is a heat-loving plant that requires a minimum temperature of 80 degrees F to grow. Since many cultivars are

drought resistant, cassava can survive even during the dry season, when the soil moisture is low, but humidity is high. Cassava is divided into two groups: the sweet and the bitter types. The bitter type contains higher concentrations of cyanogenetic glucosides, poisonous plant compounds that often cause headaches in humans, than the sweet type. The sweet type is popular due to its edible tubers and leaves; however, the bitter type is also grown for the production of animal feed and as raw material in many industries.

Cassava is a high starch producer with levels between 73.7 and 84.9% of its total storage root dry weight. This attribute together with the unique properties of its starch creates demand for particular food and nonfood applications. For example, cassava starch readily gelatinizes on cooking with water and the solution remains comparatively fluid after cooling. The excellent clarity of its starch is desirable for transparent gels, its bland flavour in pharmaceuticals, and its resistance to shear stress and freezing in the film-forming industries. Besides, cassava starch is used in baby foods, gari, chips, sago, pappads, paints, corrugated boxes, plastics and the tanning of leather. More recently, cassava has found a speciality in the production of synthetic rice. In spite of its potential, cassava starch remains under-exploited mainly because of considerable fluctuations in starch grades and qualities supplied.

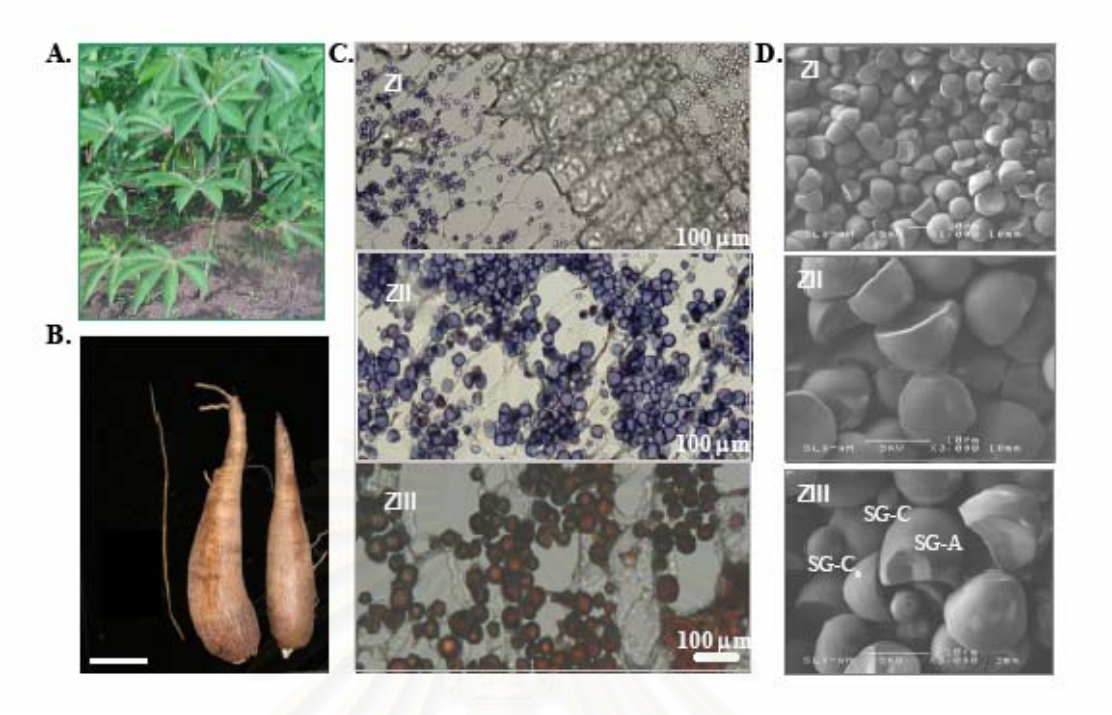


Figure 1.1. Display of a cassava plant and its starch granule architecture. **A**. Cassava plant. **B**. Cassava storage roots. **C**. Iodine stained map showing cellular distribution of amylase (blue-black stained) and amylopectin (reddish-brown stained fractions). **D**. The scanning electron micrograph of starch granule architecture. Abbreviations: ZI, periderm region;ZII, cortical region; ZIII, parenchyma region; SC-A, starch granule type A; SC-C, starch granule type C; SC-Ca, starch granule type Ca as described [2].

1.2 Starch

Starch is the principal carbon-reserve polysaccharide in plants, and the major source of carbohydrates in the diets of both human and animal. It is a glucose polymer of α-glucans linked by α-1,4 bonds and branched at α-1,6 positions [3]. It exists in the leaf chloroplasts as transitory starch (i.e. the primary product of photosynthesis) and in the amyloplasts as storage starch [4]. It is a huge (0.1 > 50 μm in diameter) complex quaternary structure made up of two major glucan polymers: amylose (15 - 30%) and amylopectin (70 - 85%) [2].

Amylose is considered to be an essentially linear polymer composed almost entirely of α-1,4-linked D-glucopyranose (Figure 1.2). Recent evidence, however, has suggested that some branches are present on the amylose polymer [5]. Simplified models for the structure of amylose are shown in Figure 1.3. Although typically illustrated as a straight chain structure for the sake of simplicity, amylose is actually often helical. The interior of the helix contains hydrogen atoms and is therefore hydrophobic, allowing amylose to form a type of clathrate complex with free fatty acids, fatty acid components of glycerides, some alcohols, and iodine [6]. Iodine complexation is an important diagnostic tool for the characterization of starch. Complexation with lipids, particularly mono- and diglycerides, is a well known property of the amylose helix. The formation and structural integrity of amylose-lipid complexes are functions of various factors, including temperature, pH, contact and/or mixing time between the host amylose polymer and the “guest” molecule, and the structure of the fatty acid or glyceride. The resulting “inclusion complex” (Figure 1.4), as it is often called, can alter the properties of the starch.

As depicted, the hydrophobic core of the amylose helix complexes with the hydrophobic constituent. Amylose complexation with fats and food emulsifiers such as mono- and diglycerides can shift starch gelatinization temperatures, alter textural and viscosity profiles of the resultant *paste*, and limit retrogradation. Another well-known attribute of amylose is its ability to form gel after the starch granule has been cooked, i.e., gelatinized and pasted. This property is evident in the behavior of certain amylose-containing starches. Corn starch, wheat starch, rice starch, and particularly high-amylose corn starch isolated from hybrid corn plants are usually considered gelling starches. Gel formation is primarily the result of the reassociation (i.e., retrogradation) of solubilized starch polymers after cooking and can occur quite rapidly with the linear polymer amylose.

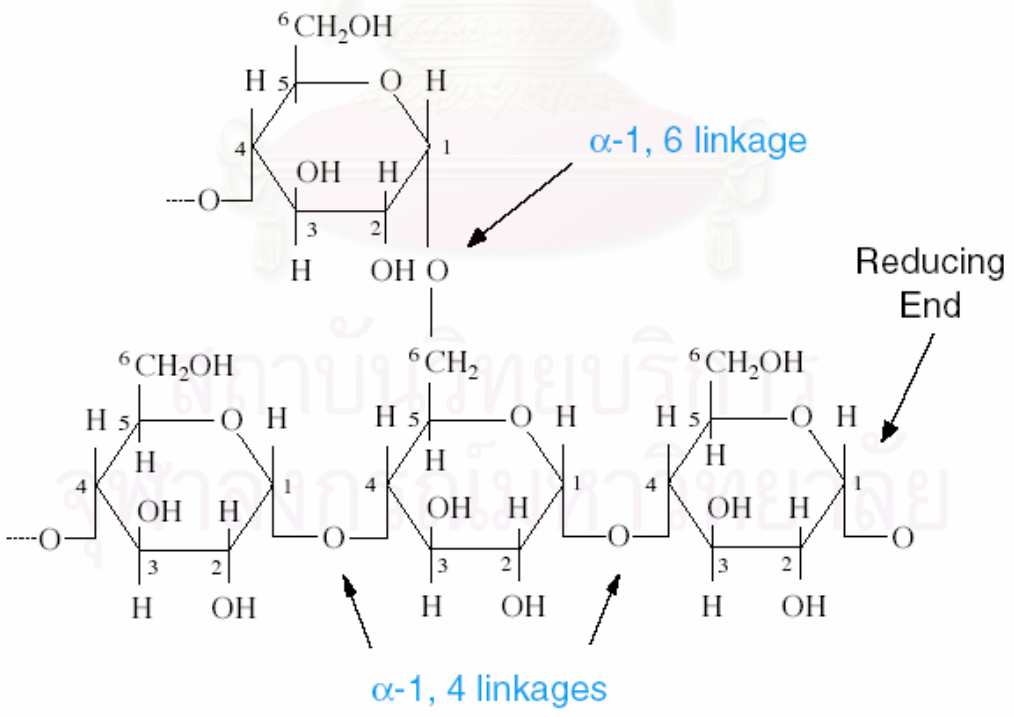


Figure 1.2 α-1,4and α-1,6 glycosidic bonds of starch [6].

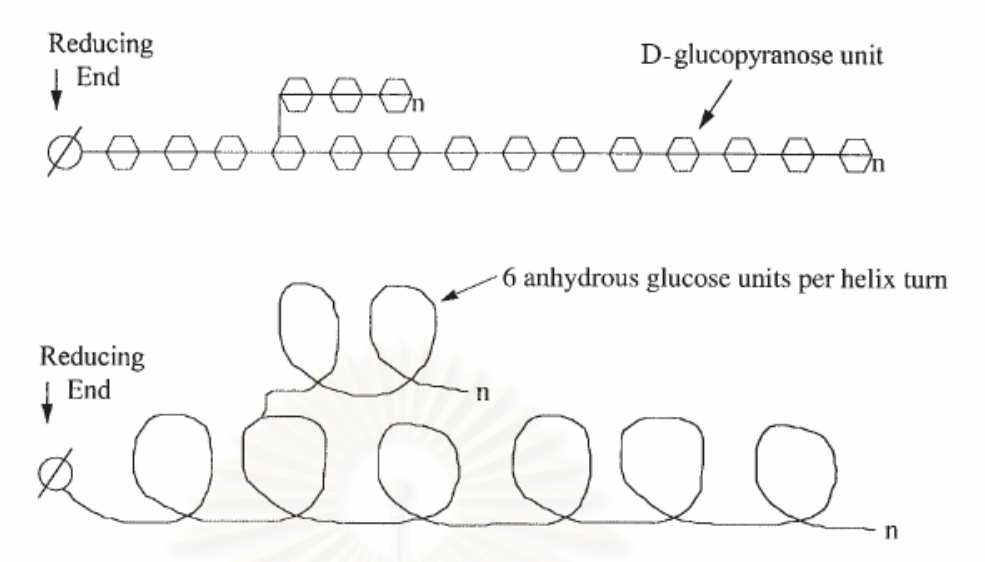


Figure 1.3 Amylose models. Amylose can be depicted as either a straight chain or a helix [6].

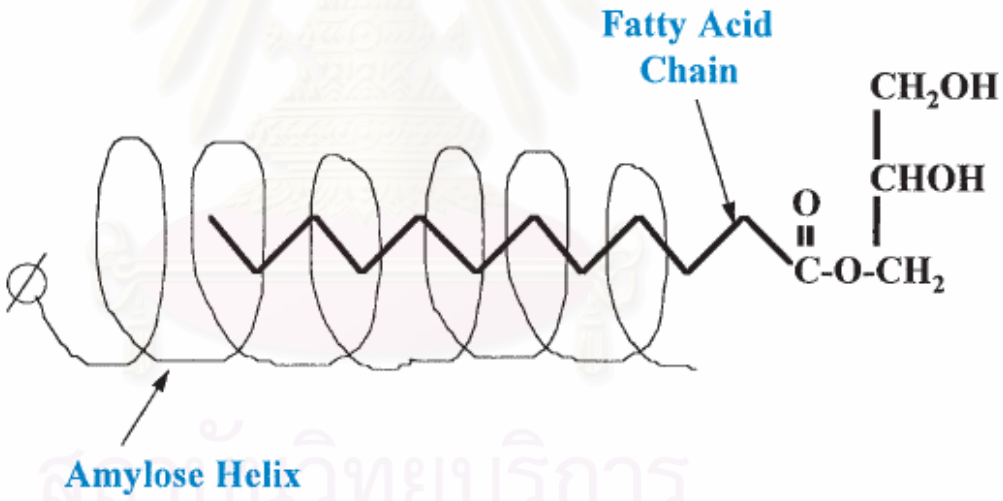


Figure 1.4 Starch-lipid inclusion complex. An amylose helix is complexed with the fatty acid chain of a monoglyceride[6].

Amylopectin, the predominant molecule in most normal starches, is a major constituent of starch in plant tissues (Table1.1). It usually comprises about 75-80%, ranging 60% up 90%, and reaches about 100% in waxy cultivars of rice, maize, sorghum, barley, millet, foxtail millet, pea and potato. In general, amylopectin is similar to glycogen (Table 1.2.), except for its fewer branch points. Amylopectin is composed of α -1,4-linked glucose segments connected by α -1,6-linked branch points. It has been estimated that about 4–6% of the linkages within an average amylopectin molecule are α -1,6 linkages [7]. This may appear to be a small percentage, but it results in more than 20,000 branches in an average molecule, although the branches themselves are not large (Fig 1-5). Studies suggest a bimodal size distribution of polymer chains, namely small and large chains [8,9]. The small chains have an average degree of polymerization (DP) of about 15, whereas that of the larger chains is about 45. This unique configuration contributes to the crystalline nature of amylopectin and an ordered arrangement of amylopectin molecules within the starch granule. The behavior of branch chains of amylopectin is similar to that of amylose chains in that entire chains or, more commonly, portions of chains can be helical. Because of the highly branched nature of amylopectin, its properties differ from those of amylose. For example, given the size of the molecule and its “tumbleweed-like” structure, retrogradation is slow and gel formation can either be delayed or prevented. Pastes from starches that contain essentially all amylopectin (waxy starches) are considered to be non-gelling but typically have a cohesive and gummy texture.

Amylose from various botanical sources has a DP of about 1,500–6,000. The much larger amylopectin molecule has a DP of about $3x10^6$–$3x10^7$ [10]. On the basis of these numbers and given a molecular weight (MW) for anhydroglucose of 162, the MW of amylose can range from about $2.43x10^5$ to $9.72x10^5$. Although amylose from potato starch has been reported to have a MW of up to about 10^6, the MW of amylose is typically less than $5x10^5$. The MW of amylopectin can range from about $10^7$to $5x10^8$. Differences in the MW of amylose and amylopectin fractions are directly related to the plant source, the method of polymer isolation (usually a solvent precipitation method is used), and the method of MW determination. The ratio of amylose to amylopectin within a given type of starch is a very important point to consider with respect to starch functionality in foods. The amylose and amylopectin content and structure affect the architecture of the starch granule, gelatinization and pasting profiles, and textural attributes. The approximate amylose and amylopectin contents of several starches are shown in Table 1.1. By using classical breeding techniques as well as sophisticated molecular biology, it is now possible to obtain starches from various hybrid plant sources that contain essentially all amylose, all amylopectin, or various ratios in between.

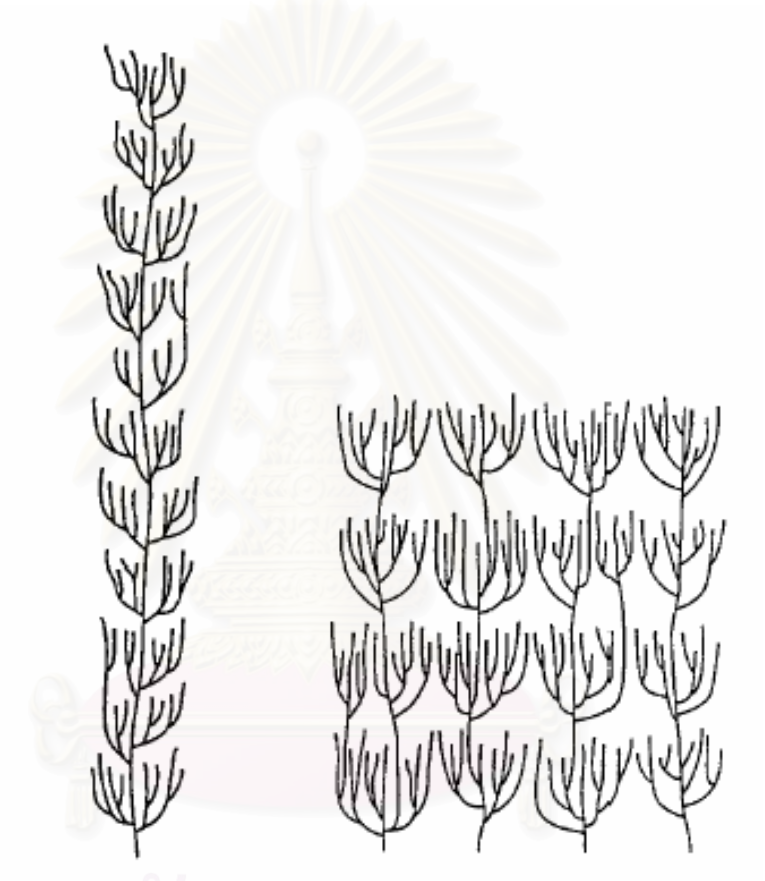


Figure 1.5 Amylopectin molecule (left) and enlargement of typical packed clusters (right). Individual chains are helical, and pairs of chains are double helical[6].

Table 1.1 Approximate amylose and amylopectin content of common food starches [6].

Starch Type	Amylose Content (%)	Amylopectin Content (%)
Dent corn	25	75
Waxy corn	<1	>99
Tapioca	17	83
Potato	20	80
High-amylose corn	55–70 (or higher)	45–30 (or lower)
Wheat	25	75
Rice	19	81

Table 1.2 Properties of amylase, amylopectin and glycogen [10].

Property	Amylose	Amylopectin	Glycogen
Branch (%)	0.2-1.0	4.0-5.5	8-10
Chain length (C.l.n.)	100-550	18-25	12-15
D.p.n.	~10^3	10^5-10^6	~10^5
Molecular size	10^5-10^6	10^7-10^8	~10^7
Distribution of branches		irregular	irregular
α-Amylolysis limit (%)	~70	~55	~45
Solubility in water	insoluble	insoluble	soluble
Iodine coloration (λ_{max})	660	530-550	430-450
Structure	helix	cluster	tree-like

C.l.n., number-average chain length; D.p.n., number-average molecular weight.

Amylose and amylopectin do not exist free in nature, but as components of discrete, semicrystalline aggregates called starch granules. The size, shape, and structure of these granules vary substantially among botanical sources. The diameters of the granules generally range from less than 1 μm to more than 100 μm, and shapes can be regular (e.g., spherical, ovoid, or angular) or quite irregular. Wheat, barley, and rye granules exhibit two separate distributions of granule sizes and distinctly different shapes. For example, large, oval granules (type A) of approximately 35 μm (major axis) and smaller, more spherical granules (type B) about 3 μm in diameter can both be extracted from wheat flour. Some granules, such as those in oats and rice, have a higher level of structure in which many small, individual granules are cohesively bound together in an organized manner. These are called compound starch granules. Although the major components of all types of starch granules are amylose and amylopectin polymers, there is great diversity in the structure and characteristics of native starch granules.

The arrangement of amylose and amylopectin within the starch granule is not completely understood. The “packaging” of these two polymers in the native starch granule is not random but is very organized. When heated in the presence of water, however, starch granules become much less ordered. This loss of internal order occurs at different temperatures for different types of starch. Depending on the starch, if it is heated in water indefinitely, the native granule swells until its structure eventually disintegrates, and amylose and amylopectin are released into an aqueous suspension. Much of what is known about the internal structure is the result of microscopic evaluation of partially degraded granules. Compared with crystalline areas, amorphous areas of the granule are generally degraded more

easily by acid and enzymes, such as α-amylase. Granules exhaustively treated in this manner demonstrate a ringed pattern analogous to the growth rings on a crosscut piece of wood from a tree trunk (Figure 1.6). This pattern indicates that the crystalline and amorphous areas of the granule alternate. It is thought that this configuration results from alternating periods of growth and rest during the synthesis of the starch granule [11]. As discussed previously, amylopectin is a large molecule composed of two distinct populations of chain lengths. The smaller chains are thought to be in such close proximity that they interact strongly, resulting in crystalline regions that are quite extensive and arranged regularly with respect to each other throughout the granule. The model in Figure 1.7, describing the arrangement of the amylopectin molecule within a growth ring of a starch granule, has been proposed by French [12]. These radially oriented amylopectin "clusters" are also believed to be associated with amylose, which is interwoven throughout the crystalline and amorphous areas. The location of amylose within the granule remains one of the unknown facts required to complete our picture of the internal structure of the starch granule.

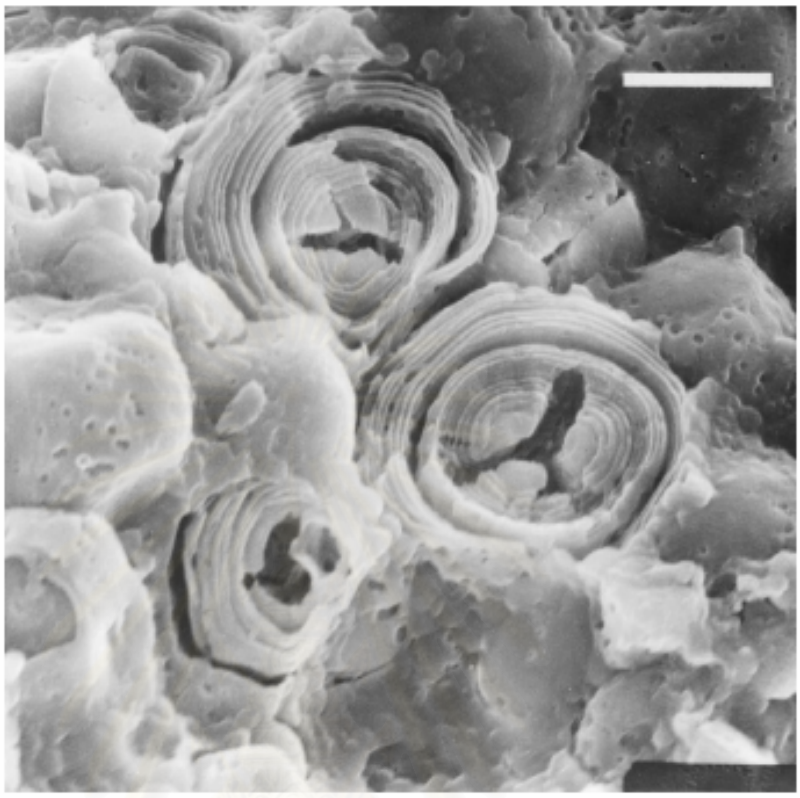


Figure1.6. Cross section of a sorghum kernel treated with α -amylase. Note the concentric rings in the broken starch granules. Bar = 10 μm. [11]

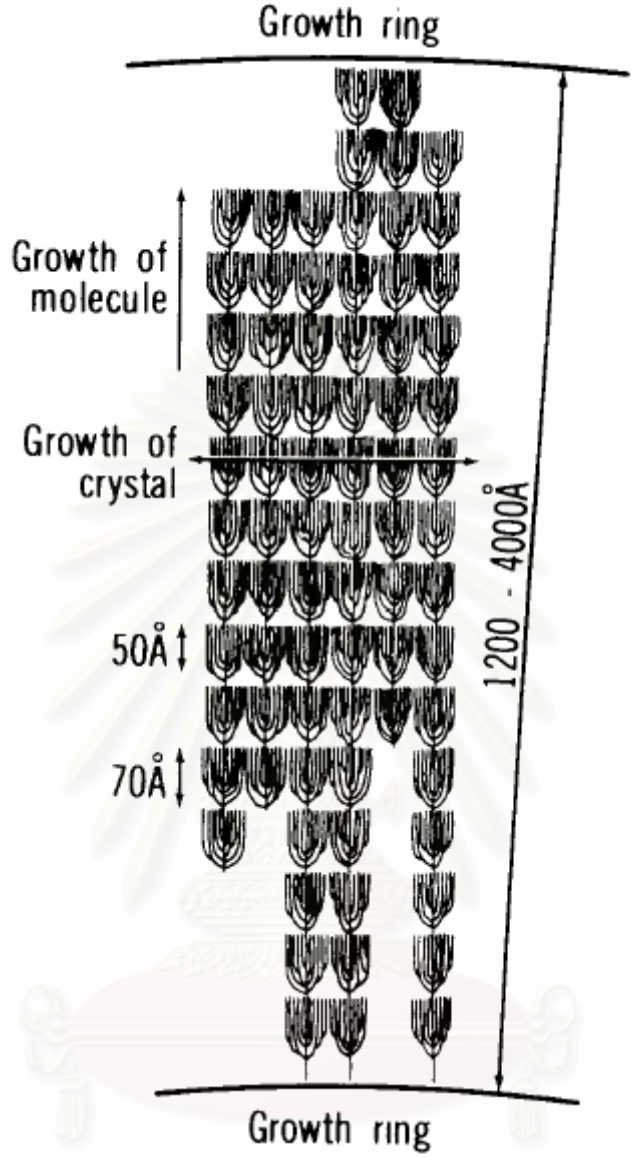


Figure 1.7 Model of the structure of amylopectin in starch granules (1 Å = 0.1 nm)[12]

Pullulan is a linear homopolysaccharide of glucose that often is described as an α-1,6 linked polymer of maltotriose subunits. This unique linkage pattern endows pullulan with distinctive physical traits. Pullulan has adhesive properties and can be used to form fibers, compression moldings, and strong oxygen-impermeable films. Pullulan is derivatized easily to control its solubility or provide reactive groups. Consequently, pullulan and its derivatives have numerous potential food, pharmaceutical, and industrial applications. Pullulan is essentially a linear glucan containing α-1,4 and α-1,6 linkages in a ratio of 2:1. Partial acid hydrolysates of pullulan include isomaltose, maltose,panose, and isopanose .The discovery of the enzyme pullulanase provided a critical tool for the analysis of the structure of pullulan [14]. Pullulanase specifically hydrolyzes the α-1,6 linkages of pullulan and converts the polymer almost quantitatively to maltotriose . Based on this result, pullulan frequently is described as a polymer of α-1,6 linked maltotriose subunits (Figure 1.8).

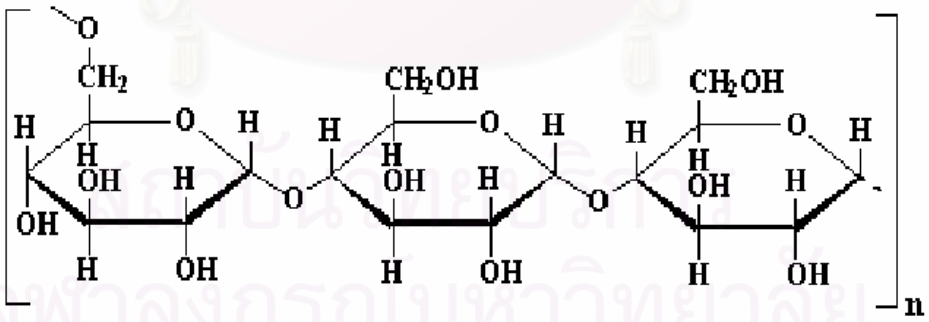


Figure 1.8 Chemical structure of a representative portion of pullulan, illustrating the primary structure of repeating linkages [14]

1.3. Starch biosynthesis and the enzymes involved

1.3.1. The overall pathway

The synthesis of α-1,4 glucans consists of three critical steps in the chloroplast and amyloplast [15] (Figure 1.9); the supply of glucose-6-phosphate (Glc-6-P) into the plastid, the synthesis of ADP-glucose (ADPG) from Glc-1-P, and the synthesis of starch from ADPG. Briefly, the first committed and rate-limiting step involves the synthesis of ADPG from Glc-1-P and ATP, catalysed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.23). Once activated, the nucleotide sugar (ADPG) is transferred by the starch synthase (SS; EC 2.4.1.21) to the nonreducing end of an α-1,4 glucan resulting in the generation of linear α-1,4 glucans. The linear α-1,4 glucans are then used as substrates by starch branching enzyme (SBE or Q-enzyme; EC 2.4.1.18) which introduces α-1,6 interlinear chain linkages producing amylopectin. Subsequently, amylopectin is crystallized into starch by the concerted effort of starch debranching enzymes (DBE; EC 2.4.1.41), phosphorylase (P-enzyme; EC: 2.4.1.1) and glucanotransferase (D-enzyme, EC 2.4.1.25). Finally, UDP-glucose: protein glucosyltransferase or amylogenin (38 or 45 kDa, EC 3.6.1 category) has been speculated to be involved in the initial priming process of starch synthesis. In subsequent sections, the role and subcellular localisation of each of these enzymes is described.

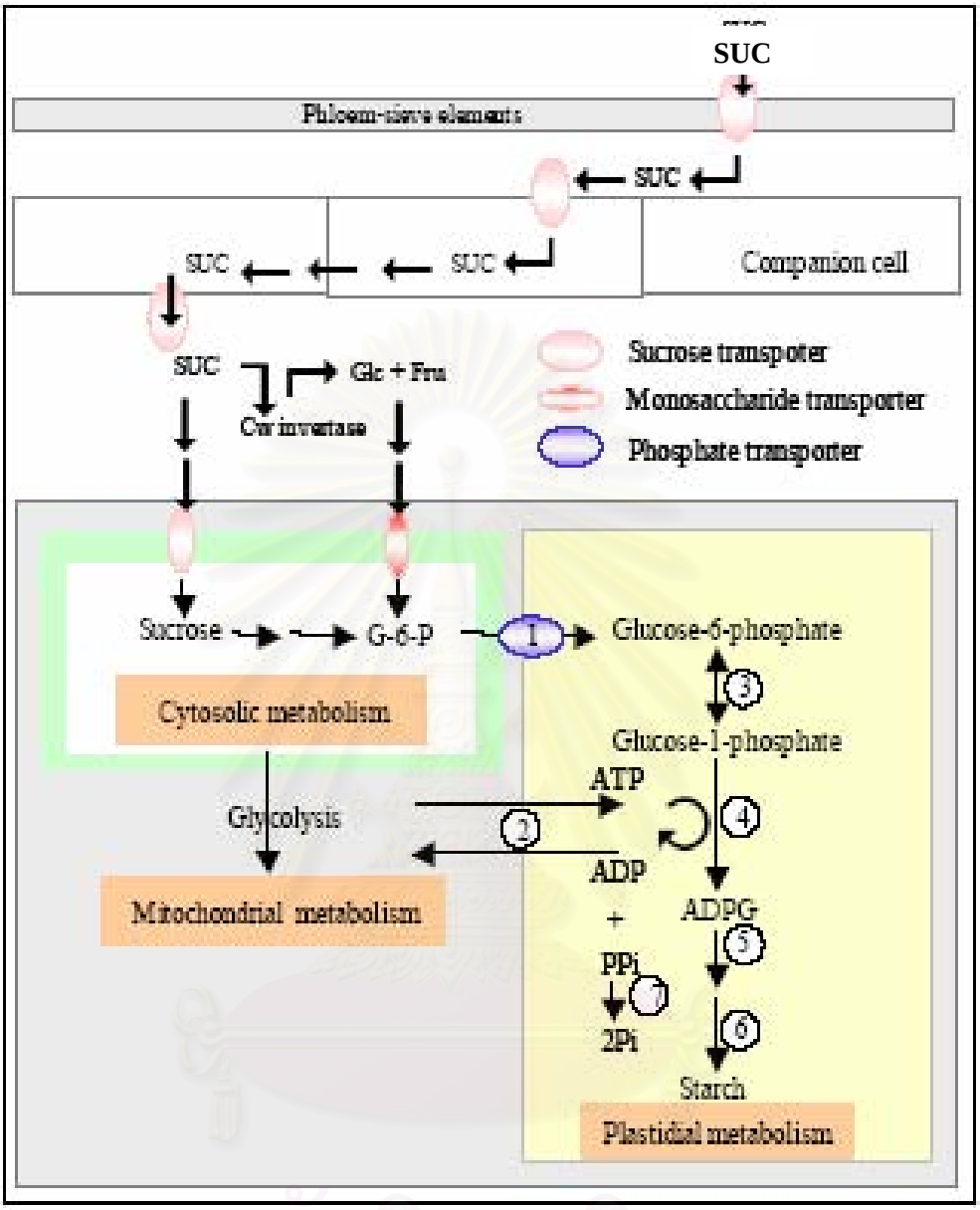


Figure 1.9 Phloem unloading and plastidial carbon metabolism. **1**, G-6-P transporter; **2**, amyloplast adenylate transporter; **3**, plastidial phosphoglucomutase; **4**, ADP-glucose pyrophosphorylase; **5**, starch synthases; **6**, starch branching enzymes; **7**, inorganic pyrophosphatase; Cw invertase, cell wall invertase; Pi, inorganic phosphate; PPi, pyrophosphate[3].

1.3.2. The synthesis of ADPG through ADP-Glucose pyrophosphorylase (AGPase)

ADP-glucose pyrophosphorylase catalyses a rate-limiting reaction in prokaryotic glycogen and plant starch synthesis [16]. In either case, all share similar molecular size, catalytic and allosteric regulatory properties but differ in higher-order protein structure. As for bacterial AGPase (*glgC*), it is a homotetrameric enzyme encoded by a single gene. Conversely, higher plant AGPase is a heterotetrameric enzyme (α2β2) composed of a pair of large subunits and a pair of small subunits, encoded by different genes [17]. At the regulatory level, small effector molecules whose nature reflects the major carbon assimilatory pathway of the organism modulate the enzyme catalytic activity. Bacterial AGPase are activated by intermediates of glycolysis [e.g. pyruvate, fructose-6-phosphate (Fru-6-P), fructose-1,6-bisphosphate (Fru-1,6-bisP)] and inhibited by AMP. Likewise, blue-green algae and higher plants AGPase are activated by 3-phosphoglyceric acid (3-PGA) and inhibited by Pi, key intermediates in CO_2 assimilation by the C3 pathway. Structure-function analysis of AGPase indicates that the amino acid sequences of the small subunits particularly the lysine residue near the *C*-terminus are highly conserved between species, whereas those of the large subunits are more divergent [18], suggesting different roles in enzyme function. Actually, the small subunit is crucial for catalytic activity while the large subunit is important for its regulatory properties. In addition, the small subunit by itself is capable of forming a homotetrameric enzyme exhibiting near-normal catalytic properties but with impended allosteric regulatory properties. In contrast, the large subunit, is incapable of forming an active enzyme, but enhances the

allosteric properties of the small subunit. Recently, it has been shown through phylogenetic studies that plant AGPase's are distinctly localised and subdivided into leaf, stem, root and endosperm types. At plastid level, AGPase are further classified into two groups based on subcellular localisation, i.e. plastidial and cytosolic forms [19]. Extended phylogenetic analysis of the small subunit proteins suggest that the cytosolic type probably evolved from the leaf localised type [20]. Such lineage raises the question of a modulating factor between the two spatially localised active types: the photosynthetic and sink storage tissue types. It has been shown that photosynthetic tissue has a pool of triose phosphates that accumulate in the chloroplast during the light cycle. Likewise, in sink tissue, 3-PGA pool is generated as an intermediate in the respiratory process of glycolysis and may be quite low whereas Pi peaks during starch accumulation. Taken together, this shows that in sink tissue, Pi inhibition may dominate over 3-PGA activation of AGPase, and phosphate may be the key physiological factor that modulates AGPase activity. This is further corroborated by the fact that maize endosperm AGPase, which is mainly cytosolic, exhibits significant sensitivity to Fru-6-P activation atypical of chloroplast or tuber AGPase [17]. Conversely, tissues without an extraplastidial AGPase, glucose-6-phosphate (Glc-6-P) is transported into the amyloplast, where plastidic phosphoglucomutase converts it to Glc-1-P for ADPG synthesis by plastidic AGPase (Figure 1.7). Insight into the role of cytosolic and plastidial AGPase in starch synthesis has been gained through studies involving mutants of maize, and barley.

1.3.3. The synthesis of amylose by granule bound starch synthase I (GBSSI)

GBSSI belongs to the class of starch synthases. All members appear to share the same basic structure, consisting of a glass domain (substrate-binding site), a typical transit peptide [21] and eight motifs [22,23]. The class is classified into three distinctly localised fractions in the plastids: those bound exclusively to the granule (granular-bound starch synthases, GBSS); those with exclusive or nearly exclusive activity in the soluble phase (starch synthases, SS); and ones present in both the granule-bound and soluble phase. In addition, the fractions are further subdivided into four subclasses based upon cDNA and amino acid sequences, i.e. GBSS (60 kDa), SSI (57 kDa), SSII (77 kDa) and SSIII (110-140 kDa). More recently, pea GBSSI has been further subdivided into GBSSIa and GBSSIb isoforms [24]. Likewise, in monocots, there is evidence to suggest that SSII further diverged into two subdivisions, SSIIa and SSIIb [21]. The synthesis of amylose was first attributed to the major granule-bound starch synthase I (GBSSI) many decades ago [25]. Since then, this discovery has been corroborated by several independent studies involving *waxy* mutants with a defective *gbssI* gene product. Such mutants have been identified in various species e.g., rice [26], maize [27], wheat [28], barley [29], *amf* potato [30] and *Iam* pea [31]. To corroborate the *waxy* phenotype, *gbssI* in potato was antisensed and analyses of the corresponding amylose content in the transgenic lines showed a marked decrease. Unexpectedly, despite compelling evidence that GBSSI is the sole enzyme in amylose synthesis, graminea *waxy* mutants were recently shown to accumulate normal starch granules in tissues such as pericarp, leaf, stem and root indicating that another gene(s)

controls amylose production. For example, the leaves and stem of *waxy* rice, the leaves and pericarp of *waxy* maize, the pericarp of *waxy* wheat, and the pods, leaves and nodules of *Iam* pea all contain amylose. This observation raised the possibility that although GBSSI has been considered to be solely involved in the synthesis of amylose, other enzymes also play role. This was recently strengthened by the isolation of a second isoform of GBSSI from *waxy* wheat [32] and pea leaves [24], designated GBSSII and GBSSIb respectively. Indeed, both isoforms were demonstrated to be involved in the synthesis of amylose.

1.3.4. The synthesis of amylopectin by the soluble starch synthases (SSS)

Various studies have demonstrated that SSI, SSII and SSIII are involved in amylopectin synthesis, although the role of SSI and SSIIb remains unclear. In pea, studies of *rug5* [33], which is closely allied with a defect in *ssII* gene showed altered amylopectin branching pattern with decreased intermediate-sized glucans (dp 15 - 25) and increased short-chain glucans (dp < 10) [33]. In potato tubers, antisense inhibition of SSII and SSIII singly [34] or in combination [35] resulted into a significant shift from longer to shorter chains. In wheat, elimination of the *ssIIa* gene product (SGP-1) manifested a phenotype with reduced starch content and altered starch structure [36]. Likewise, the fairly recently discovered *sugary–2-like* phenotype in maize mutants defective in SSIIa, demonstrate that SSIIa is involved in starch synthesis. Overall, present understanding indicate that loss of SSII (dicots) or SSIIa (monocots) results in reduced starch content, reduced amylopectin chain length distribution, deformation of the starch granules, altered physicochemical properties of starch and perturbed crystallisation. It has also been shown through studies of the maize mutant, *dull1*

[37], *Chlamydomonas reinhardtii* mutant, *STA3* [38] and transgenic potato carrying an antisense SSIII construct [34]that SSIII contributes to amylopectin branch-length distribution.

1.3.5. The role of starch branching enzymes (SBE or Q-enzyme)

Starch branching enzymes (SBE, 40–152 kDa) are involved in amylopectin synthesis. They catalyze the hydrolysis of α-1,4 linkage and subsequent formation of α-1,6 glucosidic bond between the cleaved chain and a hydroxyl group on **C**6 of a glucosyl moiety of an α-1,4 glucan template. They belong to the α-amylase family characterised by a catalytic (β/α) 8-barrel domain [39]. The domain encompasses specific active sites that arise from the interconnecting β-loops providing for substrate binding and catalytic activity. To date, two classes [referred to as A (SBEII) and B (SBEI)] have been identified based on amino acid sequences and *in vitro* catalytic properties of purified enzymes. In monocots, SBEII have further been subdivided into SBEIIa and SBEIIb filiations depending on specific catalytic properties, length of amino acid residues in the *N*-terminal domain and *C*-terminal polyglutamic acid repeats. Type I SBE, has been identified in maize, rice, pea , cassava, and wheat. More recently, a *novel* SBEI cDNA, *sbeIc*, was isolated from developing endosperm of wheat [40], uncovering the wide diversity of type I SBEs. Type II SBE, has been identified in pea, maize, rice, barley, wheat, cassava and sorghum. Notably, SBEI and SBEII isoforms exhibit different substrate preferences. SBEII isoforms have lower affinity for amylose than SBEI, implying that SBEI isoforms uses longer glucan chains than SBEII isoforms [41]. Also, it has been found that SBEI isoforms have greater branching activity and preferentially use amylose as

a substrate. Moreover, their protein structure exhibits further contrasting architectural features, i.e. SBEII possess an extended serine rich *N*-terminal domain while SBEI have a lengthy *C*-terminus of 100 amino acid residues. The *N*- and *C*-terminal domains have been associated with protein anchoring and regulatory functions, respectively. It has been shown from several studies that SBE isoforms are differentially and independently expressed during organ/tissue development and within the amyloplast. Genes encoding SBEI are commonly and constitutively expressed in photosynthetic and vegetative tissues while SBEII are preferentially expressed in starch storage compartments. The reverse is rare but common. For example, potato SBEII is predominantly expressed in leaves with very low but detectable levels in the tuber, whereas SBEI is the major isoform in the tuber. This disparity is widened by the expression pattern of *sbeII* in monocots such as wheat [42], barley [43], maize [44] and rice [45] where maximal expression of *sbeII* is attained early in kernel maturation. By contrast, SBEI is strongly expressed in the second half of embryo development in maize [46], wheat [47] and barley [48], while maize SBEIIa is more highly expressed in leaves than in endosperm. In pea embryo, both forms are present at comparable levels in the soluble fraction, whereas in potato, SBEI is the most abundant soluble fraction than SBEII [49]. Most significantly, whereas both SBEIIa and SBEIIb share similar patterns of action, similar expression profiles, and are both distributed between the granule and stroma, only mutations in SBEIIb cause a high amylose phenotype in cereal grains. This raises the question of different function for different isoforms. In elucidating the function of the different SBE isoforms, insight has been gained from analysis of starches derived from contrasting *sbe*

mutants. In both monocots and dicots, mutational and gene suppression of SBEI cause minimal effects on general starch synthesis and composition in tubers, leaves and endosperm. However, it has been shown that loss of SBEI protein in rice results into significant changes in the fine structure of amylopectin and physicochemical properties of the resulting starch in the rice endosperm [50]. As for SBEII, exclusive elimination of SBEII in potato led to increased levels of amylose [49], although, combined suppression of both SBEII and SBEI markedly increased the amylose content of the resulting starch phenotype [51]. This showed that the seemingly obscure function of SBEI in starch synthesis might be reflective of overlapping specificities and complementation between SBEI and SBEII (SBEIIa and SBEIIb). It has also been proposed that the enzyme might not interact with the substrate until SBEII (SBEIIa and SBEIIb) have acted [52]. In spite of these efforts, the question of function for the different isoforms remains not clearly resolved. Notably, isoform substrate specificities (cleavage and branching junctions), configuration of the substrate molecules (helical structure or interchain) and enzyme complexes during starch synthesis require to be examined.

1.3.6. The role of debranching enzymes (DBE)

Starch debranching enzymes belong to the α-amylase super family [53]. They hydrolyse the α-1,6 glucan branches of amylopectin. The group is subdivided into two classes. The direct DBE, involved in the hydrolysis of α-1,6-linkages of α-polyglucans and the indirect DBE, engaged in hydrolysis of α-1,6-branches by 4-α -glucanotransferase and amylo-1,6-glucosidase. Direct DBE are further subdivided into pullulanase-type or R-enzyme (EC: 3.2.1.41) and isoamylase (EC: 3.2.1.68). The defining difference is their substrate specificity in

which pullulanases debranch pullulan and amylopectin but not glycogen, whereas isoamylase debranch both glycogen and amylopectin. Moreover, pullulanases generates maltosyl groups, while isoamylase releases maltotriosyls and large oligosaccharides. In cereals, isoamylase is a larger (400 kDa) multimeric enzyme composed of one type of isoamylase subunit [54]. Conversely, in potato, two distinct subunits define an equally large heteromultimeric enzyme [55]. There is good accumulated evidence that DBEs plays a crucial role in starch biosynthesis. This has been accumulated through the analysis of *sugary-1* mutants of maize and rice endosperm [56], *sta7* mutant of *Chlamydomonas* [57], *dbe1* mutant in *Arabidopsis* [58], which accumulate a water-soluble polysaccharide (WSP), designated phytoglycogen. This analysis led to the *trimming model* [59], which asserts that the synthesis of amylopectin, its organisation and incorporation into a starch granule is the result of 'trimming' by DBE of the highly branched glucans synthesised by SS and SBE. This model hypothesizes discontinuous synthesis by way of preamylopectin, *a theoretical intermediate of* amylopectin, through the activity of DBEs that results in altered efficiency of the enzyme complex. In spite of this account, the trimming model has some limitations as observed in *Arabidopsis dbe1* mutants, which accumulate starch and phytoglycogen suggesting that the accumulation of phytoglycogen in wild-type is presumably forestalled by the action of a specific isoamylase enzyme, perhaps in concert with other glucan-degrading enzymes. To date, analysis of the *Arabidopsis* genome has revealed that there are three genes encoding isoamylase like proteins (*ISA1*, *ISA2*, and *ISA3*). These are conserved in divergent plants and evidence from potato and *Arabidopsis* indicate that the protein encoded by *ISA1* and *ISA2* are subunits of one

heteromultimeric isoamylase protein *in vivo* [60]. Loss of *ISA1* and *ISA2* in *Arabidopsis* manifest a phytoglycogen accumulating phenotype suggesting that both are essential in starch synthesis. This leaves *ISA3* with undefined role but presumably might be important in starch degradation. Taking this knowledge into consideration and from the point of view that during mobilisation of transitory starch, both starch synthesis and degradation occurs concurrently, it seems plausible that *ISA3* is required to debranch glucan structures that arise during starch degradation, while *ISA1* and *ISA2* are essential for amylopectin synthesis. Taken together, the latter prompted the development of a new model *"simultaneous processing"*, where specific isoamylase, in this case *ISA1* and *ISA2*, clears the stroma of WSPs generated by *ISA3* and other starch degrading enzymes, leading to simultaneous stalled phytoglycogen accumulation but increased amylopectin synthesis. In spite of these exciting models, it remains unresolved whether glucan trimming, WSP clearing or some other mechanism explains the mode of action of DBE.

Pullulanase have attracted much attention as enzymes that are highly important for brewery [61]. From the biochemical viewpoint they merit interest, because they are necessary not only for starch degradation but also for starch synthesis where they may play an essential role in determining the fine structure of the amylopectin molecule [62]. Only a few primary structures of Pullulanase have been reported from plants and microorganisms which all are multidomain proteins containing a catalytic $(\beta/\alpha)_8$ barrel [63] and therefore are members of the glycoside hydrolase family 13 [64]. Starch-degrading enzymes are not only active in storage tissue like cereal grains but also in leaves and there especially in the chloroplasts

where they participate in the metabolism of the assimilatory starch produced by photosynthesis [65]. Protein polymorphism is a feature frequently found with starch-metabolising enzymes [65]. The β -limit and α -limit dextrin hydrolysing R-enzyme from spinach leaf chloroplasts, a limit dextrinase of the pullulanase type (EC 3.2.1.41), however, exhibits a special type of protein polymorphism, termed protein microheterogeneity. It has been shown to result from the capability of one and the same 100 kDa polypeptide to produce several interconvertible tertiary structures [66]. Since these can be separated by isoelectric focusing, different overall charges of the isoforms have been assumed. Up to seven isoforms have been shown to coexist in an aqueous solution of the protein and each isomer produces again the whole set of isoforms when subjected to a transitory pH shock. Protein modification by phosphorylation, limited proteolysis or other chemical changes could be ruled out as possible causes of the protein polymorphism [67]. The idea of a polypeptide chain folding into several defined structures has finally been confirmed by cloning the respective gene from spinach into Escherichia coli, isolating the protein and demonstrating that the recombinant pullulanase shows an identical microheterogeneity as the original polypeptide [67].

1.4 Objectives

Although cassava has entered the modern market economy, being used as food, feed product and other industrial products, its market price has been very low. It is, therefore, essential to add value to the crops by improving the quality of its major product i.e. cassava starch. The quality of starch is the result of ratio between amylose and amylopectin. Many enzymes are involved in process of starch biosynthesis in plants. Cassava tubers are the organ for starch storage, therefore, starch biosynthesis is an important metabolic pathway in the tubers. Our laboratory has been investigating the enzymes such as starch branching enzyme and starch synthesis which are member of the key starch biosynthetic enzymes. Starch debranching enzyme plays role in accumulation of starch in granule. It is, therefore, of our interest to understand the characteristics of this enzyme in cassava in order to be able to apply the knowledge to improve the quality of cassava.

The objectives of this thesis are:

1. To isolate and partially purify starch debranching enzyme from cassava tuber (*Manihot esculenta* Crantz).

2. To characterize the partially purified starch debranching enzyme.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant material

Cassava tubers (*Manihot esculenta* Crantz) of 5 minutes cultivar were purchased from Pakklong talard market.

2.2 Chemicals

Chemicals	Company
Acrylamide	Merck
Amylopectin, corn	Sigma
Amylose, potato	Sigma
Aquasorb	SM.lab
Coomassie blue R-250	Acros
Coomassie blue G-250	Fluka
DEAE – Sepharose	Amersham Bioscience
Dithiothreitol	Amersham Bioscience
Ethelenediaminetetraacetic acid	Carlo
Glutatione reduced form	Sigma
Glycine	Amersham Bioscience
Glycogen, Rabbit liver	Sigma
Hydrochloric acid	BDH
Iodoacetic acid	Sigma
Isoelectric focusing calibration kit pH 3-10	Amersham Bioscience
Iodine	Fluka

Chemicals	Company
Maltose	Sigma
Maltotriose	Sigma
β-Mercaptoethanol	Scharlau
Methanol	BDH
N,*N*,*N'*,*N'*-Tetrametylene ethylene diamine	Fluka
N,*N*-methyl-bis-acrylamide	Amersham Bioscience
N-Ethylmaleimide	Sigma
Potassium iodide	Fluka
Potato starch soluble	Kanto chemical
Sephadex G-150	Amersham Bioscience
Sodium chloride	BDH
Sodium citrate anhydrate	Carlo
Sodium laulyl sulphate	Sigma
Tris(hydroxymethyl) aminomethane	Amersham Bioscience
Polyethylene glycol 6000	Fluka

2.3 Equipments

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

2.4 Preparation of starch debranching enzyme from cassava tuber

Cassava tubers were peeled and the cortex was removed. The parenchyma was chopped and homogenized in a blender. The pH of the cassava juice was adjusted to pH 7.5 with 50 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol, 3 mM $CaCl_2$ and 0.5 mM PMSF. The homogenate was centrifuged at 10000 rpm for 1 hour at 4 °C to remove starch. The supernatant was collected as crude enzyme and kept at 4°C for further work.

2.5 Purification of DBE from cassava tubers

2.5.1 Ammonium sulfate precipitation

Solid ammonium sulfate was slowly added to cassava crude enzyme to reach 20%saturation. Afterward, the supernatant was collected by centrifugation at 10000 rpm for 1 hour at 4 °C. Solid ammonium sulfate was further added to the supernatant to give 50% saturation. The pellet was collected by centrifugation at 10000 rpm for 1 hour at 4 °C and dissolved in 50 mM Tris-HCl pH 7.5 containing 10 mM 2-mercaptoethanol and 3 mM $CaCl_2$ and dialyzed against 3 L. of 50 mM Tris-HCl pH 7.5 containing 10 mM 2-mercaptoethanol and 3 mM $CaCl_2$.

2.5.2 DEAE-Sepharose column chromatography

DEAE-Sepharose was washed with 0.5N sodium hydroxide followed with distilled water until the pH is neutral and packed into a glass column (2.5 x 30cm.). It was equilibrated with starting buffer (50 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol and 3 mM $CaCl_2$). The sample in section 2.5.1 was loaded

onto the column and eluted with linear gradient of 0-0.6 M sodium chloride in starting buffer at flow rate 12 ml/h. Fractions of 3 ml were collected using fraction collector. The eluted fractions were monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was detected by the method described in section 2.7. The fractions with enzyme activity were pooled and checked activity by native starch PAGE. The enzyme was concentrated by aquarsorb for next step.

2.5.3 Sephadex G-150 chromatography

The column (1.5 x 90cm.) was equilibrated with 50 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol, 3 mM $CaCl_2$ and 0.10M sodium chloride. Enzyme was concentrated and loaded on to the column, and eluted with the same buffer at flow rate 10 ml/h. Fractions of 2 ml were collected and detected for activity of enzyme.

2.6 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.

2.7 DBE activity assay

DBE activity was determined by the reaction mixture containing 1% (w/v) pullulan, 100 mM acetate buffer pH 6.0, and enzyme in total volume of 0.5 ml. After incubation for about 45 minutes at 37 °C, 0.5 ml dinitrosalicylate reagent

was added and then boiled for 10 minutes. The amount of reducing sugar present was measure at A_{540}. One unit of activity was defined as the release of 1 μmol of reducing sugar per minute.

2.8 Polyacrylamide gel electrophoresis (PAGE)

2.8.1 Non-denaturing starch-PAGE

Non-denaturing polyacrylamide gel was prepared as described in Appendix A, with addition of 0.4 % (w/v) soluble starch in the gel solution on slab gel (10x8x1.5) of 10% (v/v) separating gel and 4% (v/v) stacking gel. Cold Tris-glycine buffer pH 8.3 was used as electrode buffer. To preserve activity, 10 mM β-mercaptoethanol was added to electrode buffer. The electrophoresis was performed at a constant current of 16 mA. For DBE activity stain, gel strip was rinsed with distilled water and soaked in 50 mM acetate buffer pH 6.0 for 2 hour at 37 °C and incubated 15 minutes in iodine solution (Appendix C). The debranching enzyme activity appeared sharp blue band.

2.8.2 SDS - PAGE

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

2.9 Characterization of starch debranching enzyme

2.9.1 Determination of molecular weight

2.9.1.1 Sephadex G-150 chromatography

From the method described in section 2.5.3. The molecular weight was determined from calibration curve prepared from standard molecular weight marker proteins; namely catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa). Blue dextran and potassium dicromate were used to determine the void volume (V_o) and total volume (V_t), respectively. The partition coefficient (K_{av}) of the standard marker protein and SBE were calculated from the formula

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

2.9.1.2 SDS-PAGE

After electrophoresis, proteins in the gel were visualized by coomassie blue staining. The molecular weight of SBE subunits were determined form calibration curves obtained from R_f and molecular weight of standard proteins; namely phosphorylase b (97 kDa), bovine serum albumin (66 kDa), lvalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa).

2.9.2 Effect of pH on DBE

The partially purified starch debranching enzyme was used to study the effect of pH on its activity. The enzyme was assayed as described in section 2.7. The enzyme was incubated with substrate solutions prepared in universal buffer at various pH's. After incubation for 45 minutes the reaction was stopped by heating in boiling water for 5 minutes. The result was expressed as the percentage relative activity.

2.9.3 Effect of temperature on DBE

The partial purified enzyme was used to study effect of temperatures on its activity. The enzyme was assayed by incubation at 4, 25, 30, 37, 40, 45, 50, and 60 °C for 45 minutes. The activity was measured as described 2.7. The result was expressed as the percentage relative activity.

2.9.4 Temperature stability of DBE

The temperature stability of starch debranching enzyme was studied. The temperatures at which more than 60% DBE activity remaining after incubation for 45 minutes was used for stability study (4, 25, 30, 37, 40, 45, 50 and 60 °C). The enzyme was incubated at the selected temperature for 0, 6, 12, 24, 36 and 48 hours and collected for assay as described previously. The activity was measured as described in 2.7. The result was expressed as the percentage relative activity.

2.9.5 Effect of sulfhydryl reagents on DBE

The effect of the sulfhydryl group reagents on starch debranching enzyme activity were studied. Different concentrations (2 mM, 5mM, and 10 mM) of DTT, NEM, IAA,GSH and β-mercaptoethanol were added to different reaction mixtures. After incubation at 37°C for 45 minutes, the activity was measured as described in 2.7. The result was expressed as the percentage relative activity.

2.9.6 Effect of divalent metal ions on starch DBE

The effect of various divalent metal ions on starch debranching enzyme activity were studied. Various concentrations (1mM, 5mM and 10 mM) of $CuSO_4$, $CoCl_2$, $MnCl_2$, $HgCl_2$ and $NiCl_2$ were added to reaction mixtures and DBE activity was monitored as described section 2.7

2.9.7 Isoelectric focusing polyacrylamide gel electrophoresis

2.9.7.1 Preparation of gel support film

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

2.9.7.2 Preparation of the gel

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

2.9.7.3 Sample application and electrophoresis

The sample was loaded and the gel with the absorbed samples was turned upside-down and directly placed on top of the graphite electrode. Focusing is carried out constant voltage conditions in a stepwise fashion. The gel was firstly focused at 100 V 90 minutes, followed by an increase up to 200 V for 90 minutes and finally run at 450 V for 120 minutes. After completion of electro focusing, the gel was stained. Standard protein markers with known pI in the ranges 3-10 were run in parallel. The pI of the sample protein was determined by the standard curve constructed from the pI of standard proteins and their migration distance from cathode.

2.9.8 Determination of K_m and V_{max}

The partially purified DBE was used to study for K_m and V_{max} with pullulan as the substrate of this enzyme. The reaction mixture consisted of various concentrations of pullulan, 2, 4, 5, 6, 7, 8, 9, 10, 12.5, 15, 17.5 and 20.0 mg/ml. The reaction was stopped by adding DNS and boiled for 10 minutes, and the activity was measured as described in section 2.7.

2.9.9 Comparison of DBE activity with various substances

The partially purified enzyme was used to study substrate specificity for amylose, amylopectin, glycogen, pullulan, α-cyclodextrin, γ-cyclodextrin and soluble starch. The enzyme was assayed by incubation at 37 °C for 45 minutes. After the reaction was stopped by adding DNS and heated in boil water for 10 minutes, the activity was measured as described in 2.7. The result was expressed as the percentage relative activity.

CHAPTER III

RESULTS

3.1 Purification of starch debranching enzyme from cassava tubers

3.1.1 Preparation of crude enzyme

Crude starch debranching enzyme was prepared from 5 kilograms of parenchyma and cortex from cassava tubers as described in section 2.4. The starch debranching enzyme (DBE) activity was measured as described in 2.7. The amount of protein obtained from parenchyma was 1371 mg with 103.56 units of DBE activity and 28 mg with no DBE activity in cortex, when assayed at the same amount of protein.

3.1.2 Precipitation with ammonium sulfate

To get rid of other contaminating proteins and small starch granules, the crude enzyme from parenchyma was subjected to ammonium sulfate precipitation with 20-50% saturation as described in section 2.5.1. Significant amount of proteins was removed, leaving 781.5 mg protein in the precipitate. However, DBE activity dropped to 48.9 units of DBE yielding 1.1 folds of purification. No significant amount of DBE was detected in other fractions.

3.1.3 DEAE-Sepharose chromatography

The 20-50% precipitate from 2.5.1 was dissolved in 50 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol and 3 mM $CaCl_2$ and dialyzed to remove ammonium sulfate and was loaded on DEAE-Sepharose prepared as described in section 2.5.2. Unbound proteins were washed with the starting buffer and DBE was eluted with linear gradient of 0-0.6 M sodium chloride in starting buffer at flow rate 12 ml/h. Fractions of 3 ml were collected. The chromatographic

profile is shown in Figure 3.1. Peaks 2 and 3 showed isoamylase activity while, only peak2 showed pullulanase activity. The DBE activity peaks were pooled and concentrated using aquasorb and then dialyzed against 2 L of 50 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol and 3 mM $CaCl_2$.

To confirm the DBE activity (pullulanase and isoamylase) in each peak, the pooled peaks were subjected to electrophoresis on 10% native polyacrylamide gel containing 0.4% soluble starch and stained with I_2 with the techniques described in section 2.8.1. Lane 2 showed blue band of pullulanase activity (Figure3.2) while lanes 1 and 3 did not. Peak 3 which showed isoamylase activity did not show any isoamylase activity band (colorless) in native PAGE (Lane3). Since DBE was defined to be capable of both isoamylase and pullulanase activity only peak 2 will be used for further studies. This fraction contained 73.5 mg of protein and 27.75 units of DBE.

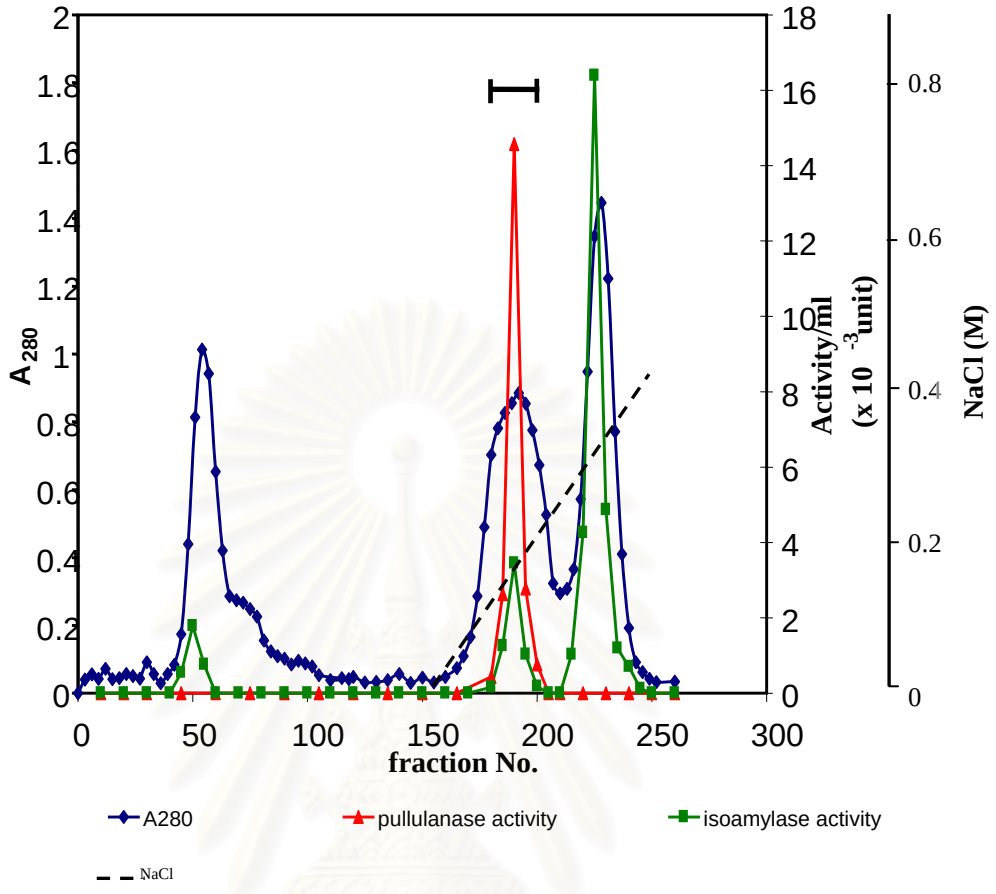


Figure 3.1 DEAE-sepharose chromatographic profile of DBE from parenchyma of cassava tubers in 20-50% Ammonium sulfate precipitated fraction that was eluted with 0-0.6 M NaCl in starting buffer. Fractions of 3 ml were collected and flow rate of 12 ml/h

1 2 3

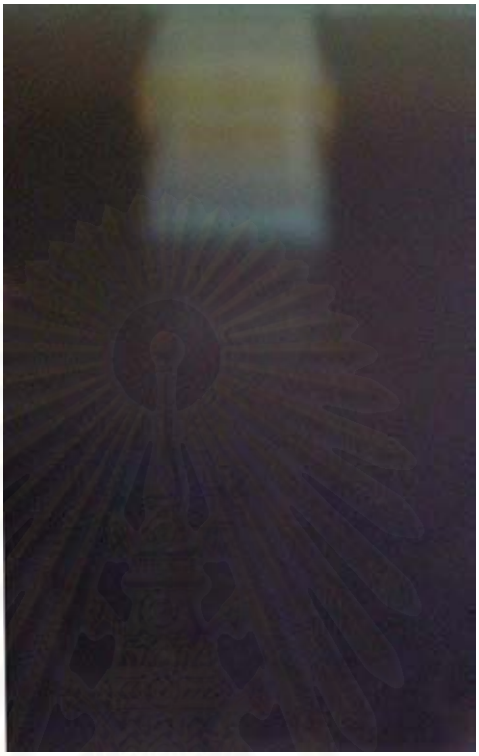


Figure 3.2 Activity staining of pooled fractions from DEAE-sepharose

Lane 1= peak1 from DEAE
2= peak2 from DEAE
3= peak3 from DEAE

3.1.4 Sephadex G-150 column chromatography

Fractions from peak 2 of DEAE-Sepharose column in 3.1.2 were pooled, concentrated, dialyzed against 50 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol, 3 mM $CaCl_2$ and loaded on Sephadex G-150 column, prepared as described in section 2.5.3. DBE activity was identified by pullulanase and isoamylase activity and found to be in fractions 110-145 (Figure 3.3).

Table 3.1 summarized the purification of DBE achieved at each step of purification. DBE was purified to 18 folds by Sephadex G-150. Figure 3.4 showed activity staining of cassava DBE on starch polyacrylamide gel electrophoresis from each step of purification.

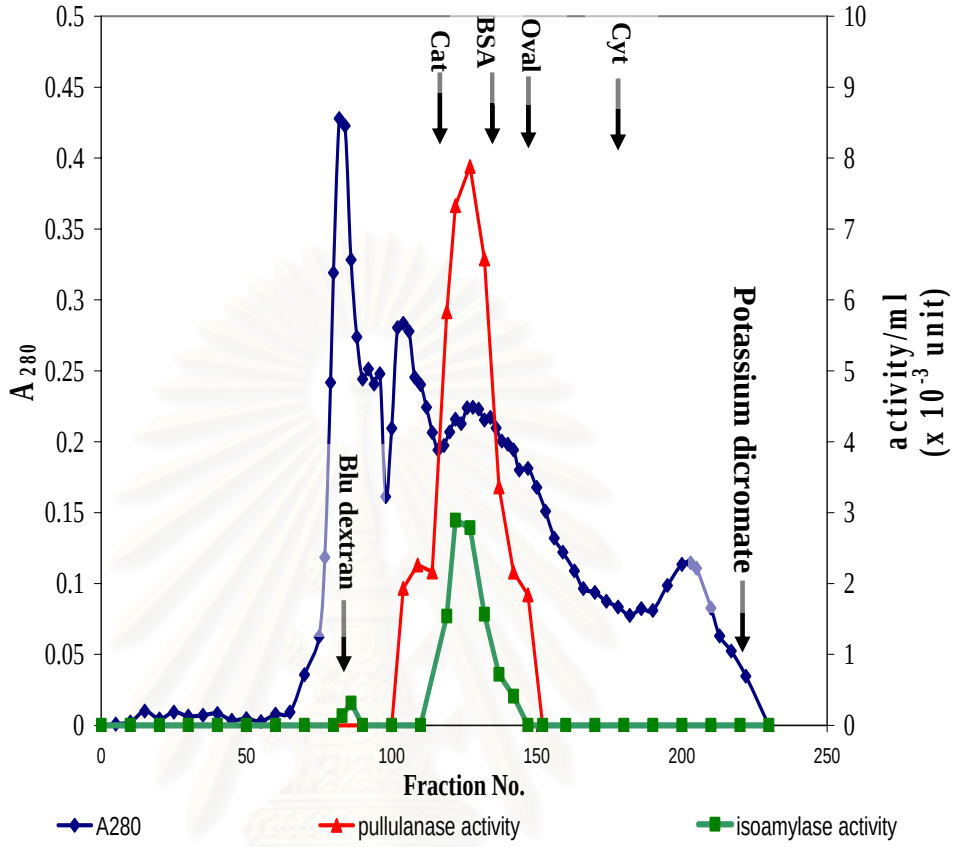


Figure 3.3 Sephadex G-150 chromatographic profiles of DBE

Cat = Catalase MW = 232,000 Da

BSA = Bovine serum albumin MW = 66,000 Da

Oval = Ovalbumin MW = 43,000 Da

Chymo = Chymotripsinogen A MW = 27,000 Da

Cyt = Cytochrom C MW = 11,700 Da

Table 3.1 Purification of debranching enzyme from cassava tuber.

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (10^{-2} units /mg protein)	Purification fold	Recovery %
Crude extract	1371.0	103.56	7.55	1.0	100
20-50% $(NH_4)_2SO_4$	589.5	48.90	8.30	1.1	47
DEAE-Sepharose	73.5	27.75	37.78	5.0	27
Sephadex G-150	5.1	6.94	136.31	18.0	7

1 2 3 4

Figure 3.4 Activity staining of cassava DBE on starch polyacrylamide gel electrophoresis.

Lane 1 Crude enzyme ($5x10^{-3}$ units)

Lane 2 20-50% saturated ammonium sulfate precipitation ($5x10^{-3}$ units)

Lane 3 DEAE- Sepharose ($1x10^{-3}$ units)

Lane 4 Sephadex G-150 ($1x10^{-3}$ units)

3.2 Characterizations of starch debranching enzyme

Since pullulanase activity lost very rapidly and characterization of pullulanase activity was very specific using pullulan, the partially purified DBE from DEAE-Sepharose column chromatography was used for the characterization studies.

3.2.1 Molecular weight determination

3.2.1.1 Native molecular weight

Native molecular weight of the DBE was determined by loading standard protein on same Sephadex G-150 used for purification of DBE in section 3.1.4. Molecular weight calibration curve was constructed (Figure 3.5) and native Standard proteins were loaded on the same column. DBE was eluted by starting buffer and the molecular weight determined from calibration curve was 103,000 Daltons.

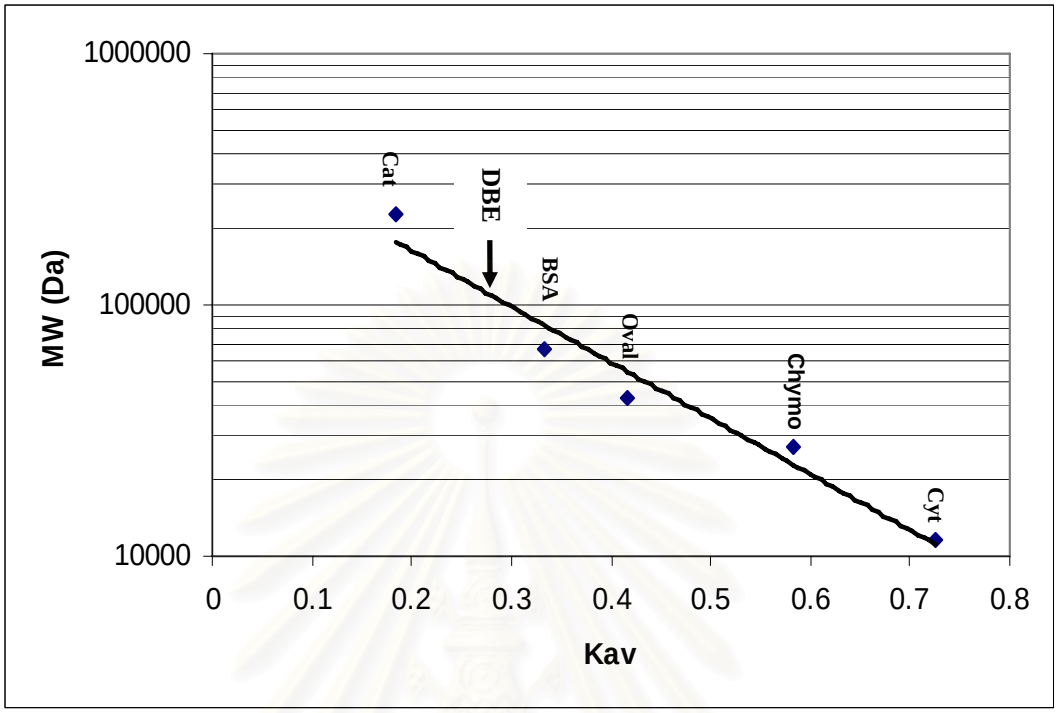


Figure 3.5 Calibration curve for native molecular weight determined by chromatography on Sephadex G-150 column.

Cat = Catalase	MW = 232,000 Da
BSA = Bovine serum albumin	MW = 66,000 Da
Oval = Ovalbumin	MW = 43,000 Da
Chymo = Chymotripsinogen A	MW = 27,000 Da
Cyt = Cytochrom C	MW = 11,700 Da

3.2.1.2 Molecular weight of DBE on SDS-PAGE

Since the DBE obtained was only partially purified, it can not be used for determining molecular weight on SDS PAGE. The DBE band on native PAGE identified by activity stain was cut and protein eluted and then used to determine molecular weight of DBE on 10% SDS PAGE as described in section 2.9.1.2. Standard proteins were run in parallel (Figure 3.6) and a standard curve was constructed from their molecular weight and relative mobilities (Figure 3.7). DBE showed one major band with molecular weight 35,000 Daltons as determined from the standard curve.

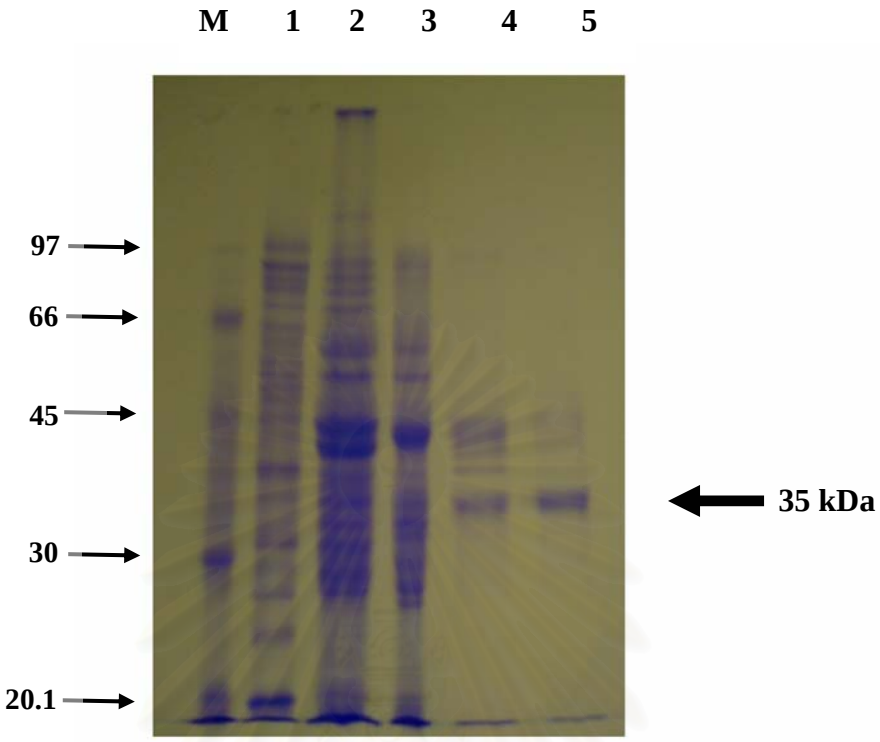


Figure3.6 SDS-PAGE pattern of cassava pullulanase

Lane M Standard molecular weight proteins
Phosphorylase b (97 kDa)
BSA (66 kDa)
Ovalbumin (45 kDa)
Carbonic anhydrase (30 kDa)
Soybean trypsin inhibitor (20.1 kDa)

Lane 1	Crude enzyme	20 μg
Lane 2	20-50% saturated ammonium sulfate precipitation	20 μg
Lane 3	DEAE-Sepharose	15 μg
Lane 4	Sephadex G-150	5 μg
Lane 5	eluted activity band from native gel electroferesis	5 μg

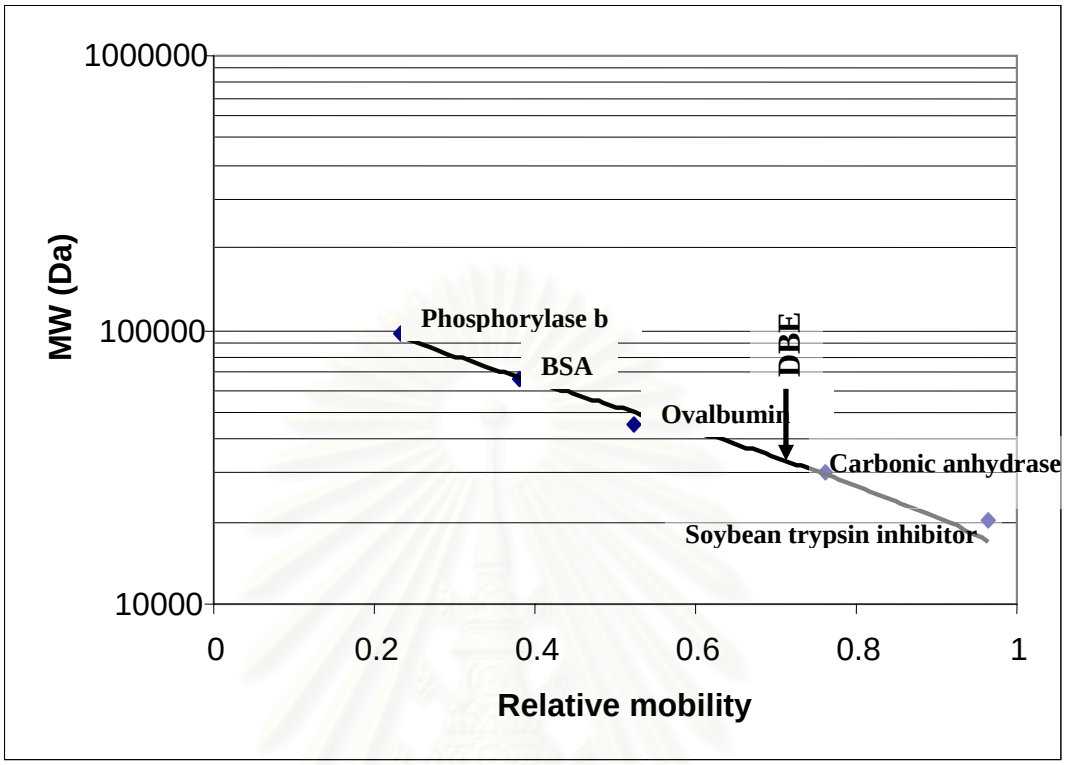


Figure 3.7 Calibration curve of molecular weight markers on SDS-PAGE. The molecular weight of the standard markers were plotted against their relative mobility on 10% SDS-PAGE.

Standard molecular weight proteins;

Phosphorylase b (97 kDa)

BSA (66 kDa)

Ovalbumin (45 kDa)

Carbonic anhydrase (30 kDa)

Soybean trypsin inhibitor (20.1 kDa)

3.2.2 Optimum pH for activity of DBE

DBE was assayed at various pH's as described in section 2.9.2. The results were shown in Figure 3.8. DBE activity was expressed as % relative activity with the highest activity referred as 100%. The DBE showed highest activity at pH 6.0. At pH 5.5-7.5, DBE activity was over 80% of that at pH 6.0. Below pH 5.0 and above 9.0, the activity was below 10% and completely lost at pH 4.5 and 10.0.

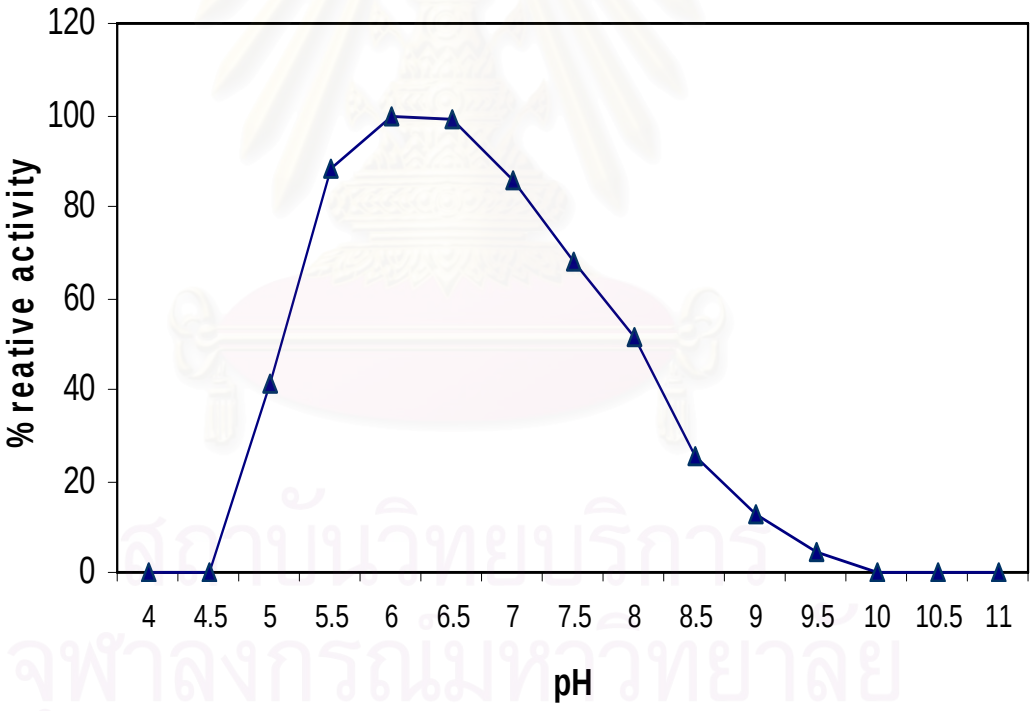


Figure 3.8 Effect of pH on activity of DBE.

3.2.3 Optimum temperature for activities of DBE

DBE was assayed at different temperatures as described in section 2.9.2. The result is shown in Figure 3.9, with DBE activity at 37 °C and more than 90% of activities retained at 20-40 °C, the DBE activities decreased to less than 50% at 55°C and absolute loss of activities occurred at 70 °C.

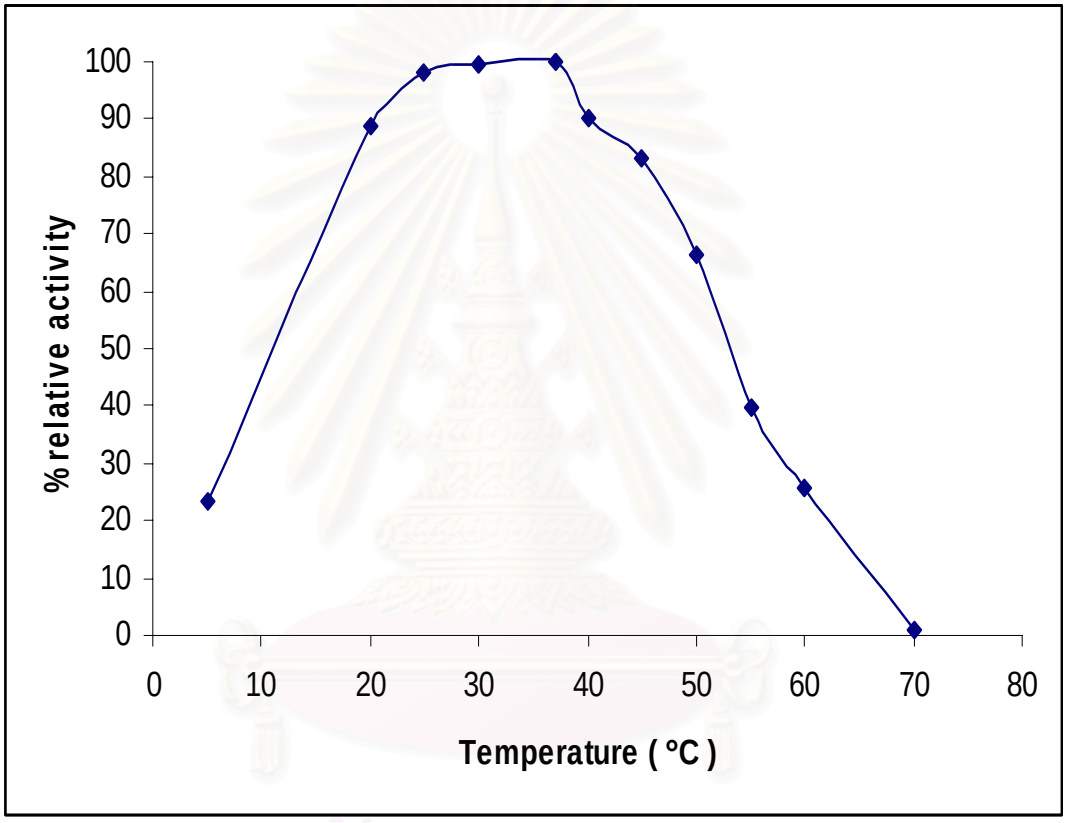


Figure 3.9 Effect of temperature on activity of DBE

3.2.4 Temperature stability of activities of DBE

DBE (10 munit) was tested for its stability at various temperatures according to the method described in section 2.9.3. It was found to be quite stable at 4-40 °C at with more than 80% of the activities retained after incubation for 48 hours. At 45 °C, more than 90% of activities was still retained within 6 hours. At 50 and 60°C, activity was completely lost after 6 hours (Figure 3.10).

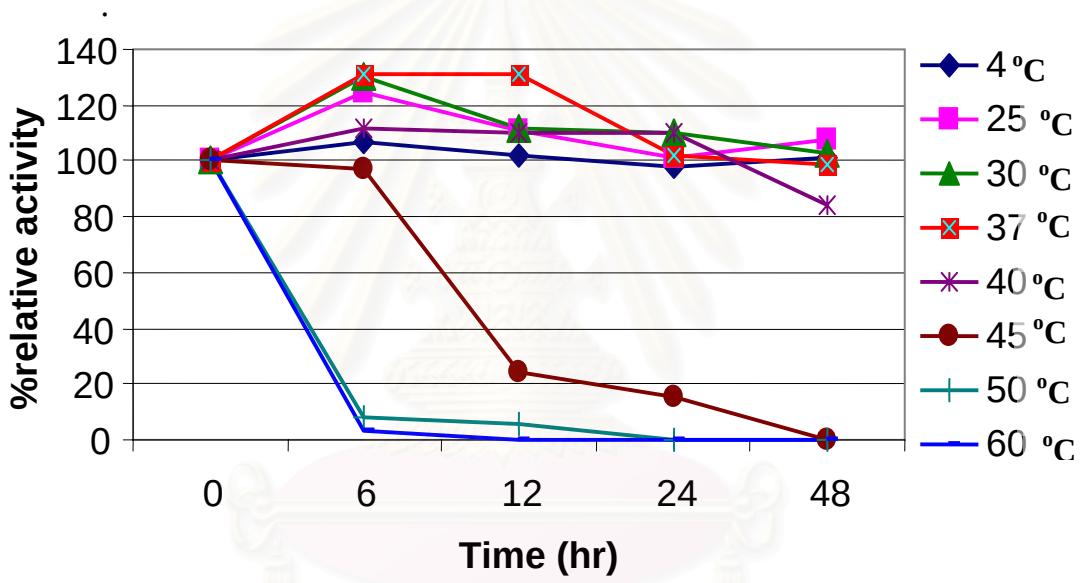


Figure 3.10 Temperature stability of DBE activities

3.2.5 Effect of sulfhydryl reagents on DBE activity

DBE (10 munit) was assayed with added sulfhydryl reagents to determine the effect of SH- group on its activity as described in 2.9.5. The results are shown in Figure 3.11. Control was the reaction mixture without adding sulfhydryl reagent**.** Increasing DTT and β-mercaptoethanol concentrations caused increase activity, 10 mM DTT caused the highest increase about 3 folds of control. On the other hand, NEM, IAA and GSH caused decrease in activity of DBE, at 5 mM IAA completely inhibited the activity of the enzyme. The effect of GSH and 2-mercaptoethanol in the range 2-10 mM were relatively small.

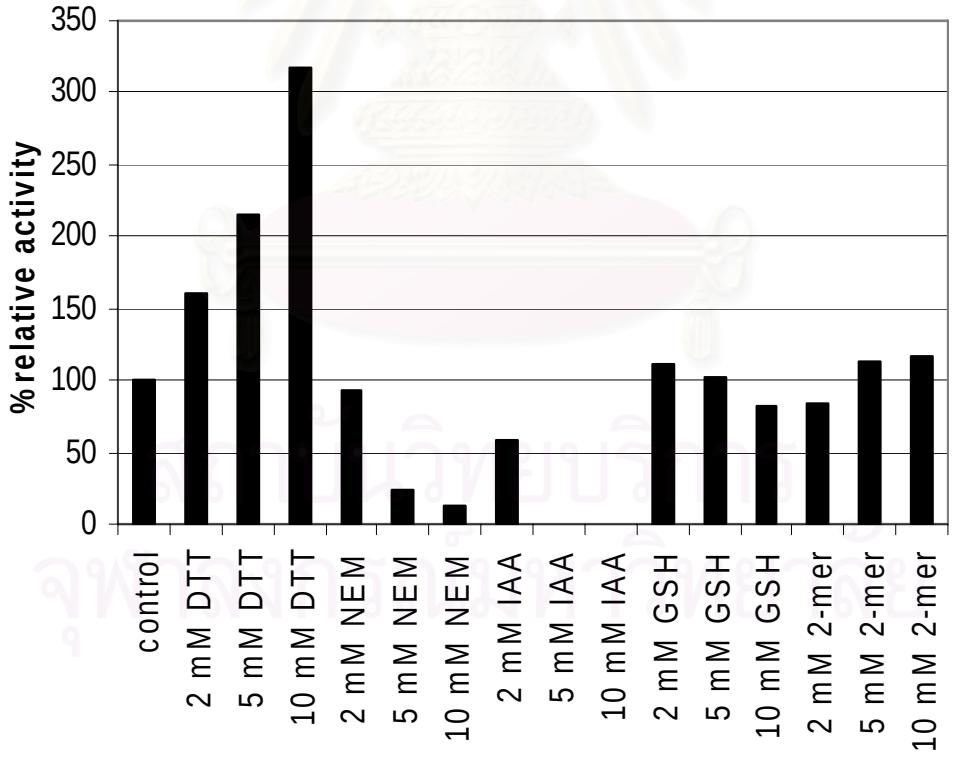


Figure 3.11 Effect of sulfhydryl reagent on DBE activity

3.2.6 Effect of divalent metal ions on DBE activity

DBE (10 munit) was assayed with added divalent metal ions at 1, 5 and 10 mM to determine the effect of divalent ions on its activity as described in 2.9.6. Control was reaction mixture without adding metal ion and represent as 100% activity. Hg^{2+} and Cu^{2+} were found to be inhibitory on pullulanase activity, with complete inhibition at 5mM. Ni^{2+} was a less potent inhibitor of the enzyme with 58% in inhibition at 10 mM. Mn^{2+} and Co^{2+} activated the enzyme at 1 mM but the activation effect decreased about 10-15% at 5 and 10 mM. (Table 3.2).

Table 3.2 Effect of divalent metal ions on DBE

Metal ion	**Relative activity (%)**		
	1 mM	**5 mM**	**10 mM**
Ni^{2+}	86	65	42
Mn^{2+}	130	118	113
Hg^{2+}	86	0	0
Cu^{2+}	88	0	0
Co^{2+}	131	121	108

3.2.7 Determination of pI of DBE

Eluted activity band from native gel electroferesis and standard pI marker proteins were subjected to electrofocusing gel according to the method described in section 2.9.7. The result is shown in Figure 3.12. A pI standard curve was constructed from the pI's and distance migrated from cathode of the standard proteins (Figure 3.13). From the standard curve, pullulanase was found to have the pI of 4.75.

3.2.8 K_m and V_{max} of DBE with pullulan as substrate

Cassava DBE(10 munit) was assayed with varying concentrations of pullulan as substrate at 37 °C for 45 minutes as described in 2.9.8. Figure 3.9 showed the saturation curves and Lineweaver-Burk plot of DBE. From Lineweaver-Burk plot, K_m for pullulan was calculated at 0.88 mg/ml and the V_{max} was 24.81 nmol maltose/min.

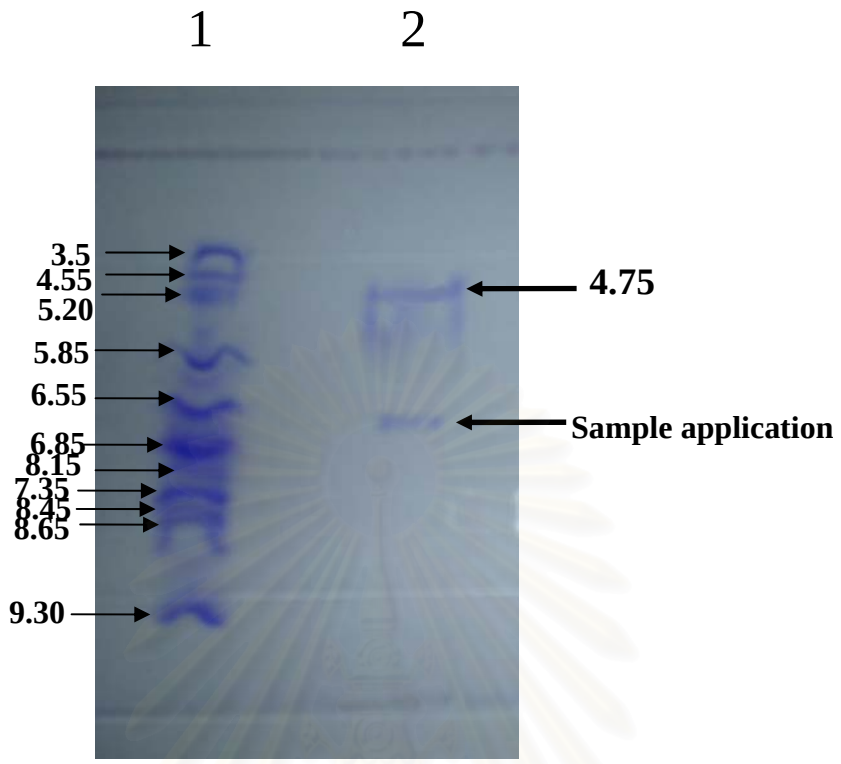


Figure 3.12 Isofocusing polyacrylamide gel electrophoresis of DBE.

Lane 1 standard pI marker :

Amyloglucosidase	pI 3.5
Soybean trypsin inhibitor	pI 4.55
β-lactoglobulin	pI 5.20
Bovine carbonic anhydrase B	pI 5.85
Human carbonic anhydrae B	pI 6.55
Myoglobin-acidic	pI 6.85
Myoglobin-basic	pI 7.35
Lentil lectin-acidic	pI 8.15
Lentil lectin-middle	pI 8.45
Lentil lectin-basic	pI 8.65
Trypsinogen	pI 9.30

Lane 2 eluted activity band from gel electroferasis 5 μg

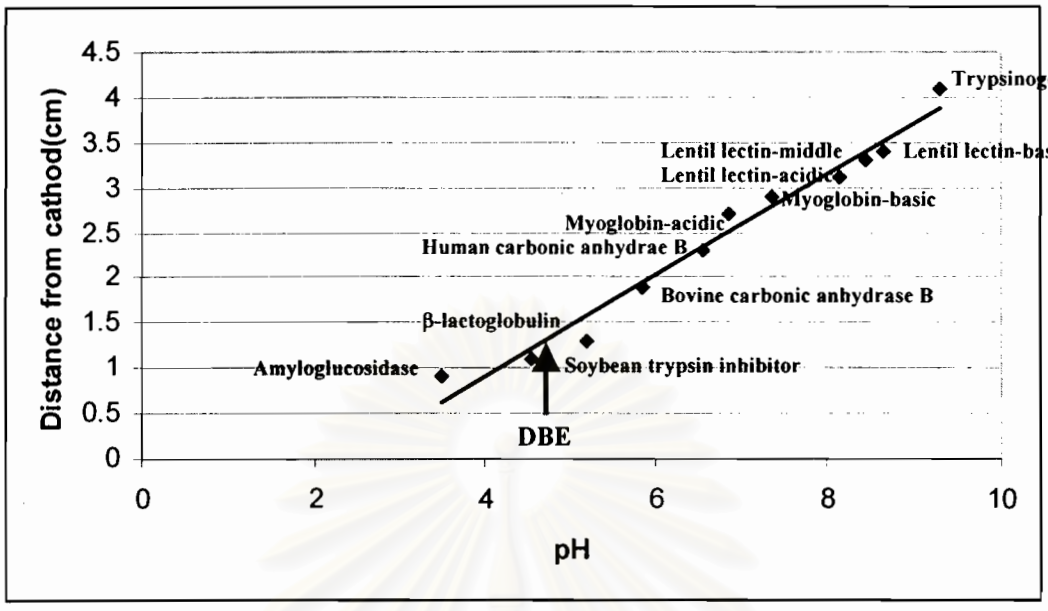
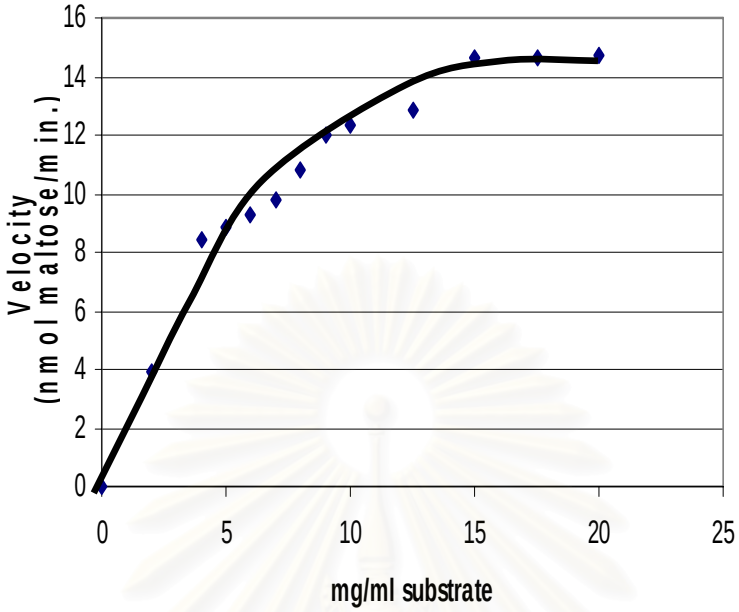
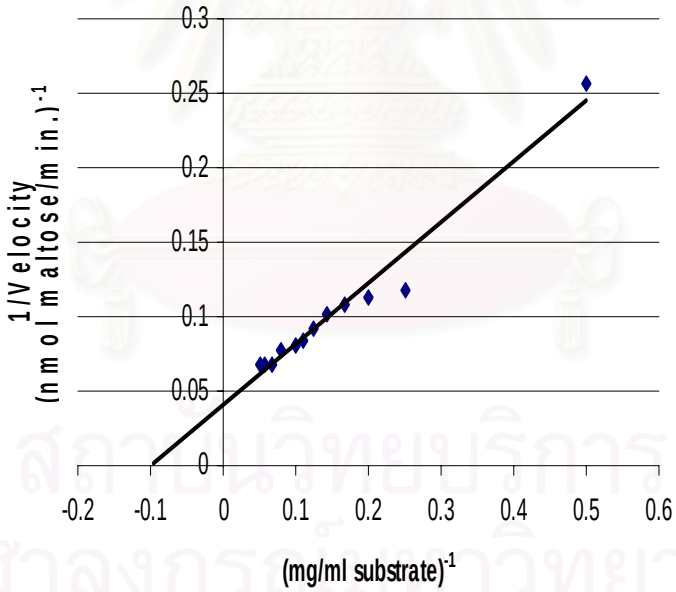


Figure 3.13 Calibration curve of standard pI markers.

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



A.



B.

Figure 3.14 Kinetic studies of DBE activity with pullulan as substrate

A) Saturation curve

B) Lineweaver-Burk plot

3.2.9 Comparison of DBE activity with various substrate

To compare the substrate specificity, amylose, amylopectin, glycogen, pullulan, α-cyclodextrin, γ-cyclodextrin and soluble starch at 1%(w/v) were used as substrates and assayed as described in 2.9.9. The results were shown in Table.3.3. The enzymes was able to debranch amylopectin and soluble starch, but to a lesser extent as compared with pullulan. Pullulanase did not react significantly with glycogen, amylase, α-cyclodextrin and γ- cyclodextrin.

Table.3.3 Comparison of DBE activity with various substrates 1%(w/v)

Substrate	Relative activity (%)
Pullulan	100
Amylose	0
Amylopectin	37
Glycogen	0
Soluble starch	20
α-cyclodextrin	0
γ- cyclodextrin	0

CHAPTER IV

DISCUSSIONS

Cassava is one of the world's most important, and under-exploited staple food crops and source of income. The problem of cassava trade in Thailand are the falling price. However, cassava needs improvement in qualities and quantities of starch to add more value to its products. Debranching enzyme plays in starch synthesis via its important role in determining the fine structure of starch granule. This thesis concentrated on the understanding of DBE in cassava tuber which is one of the enzymes can improve starch quality of cassava.

4.1 Assay methods for DBE

The activity of starch debranching enzyme can be assayed by measuring the amount of reducing sugar with dinitrosalicylate reagents [68]. In case of pullulanase activity, pullulan is highly specific substrate, we can measured the true activity without interference from other enzyme activities. In contrast to pullulanase activity, ISA activity was detected using amylopectin which, is also substrate for several enzymes. It is difficult to measure isoamylase activity without interference from other amylolytic enzyme activities, such as amylase or SBE. Pullulanase which is highly specific to pullulan can also hydrolyze amylopectin. Therefore, DBE was always monitored by both pullulanase and ISA activity in pararel.

4.2 Activity of DBE in cassava tuber

Debranching enzymes (DBE) which hydrolyze α-1,6-glucosidic linkages of α -polyglucans are classified into two types, namely a direct debranching enzyme

and an indirect debranching enzyme [69]. The direct debranching enzymes which are present in plants and bacteria can directly hydrolyze α-1,6-branches while the indirect debranching enzyme in animals and yeasts can remove α -1,6-linkages by the combined actions of α-aglucanotransferase (EC 2.4.1.25) and amylo-1,6- glucosidase (EC 3.2.1.33). The direct debranching enzymes are further divided into the two types, namely the R-enzyme (RE) or pullulanase type (EC 3.2.1.41) and the isoamylase type (ISA) (EC 3.2.1.68).

More informations on characterization and presence of debranching enzymes is needed to understand their precise roles in amylopectin biosynthesis in cassava. It is rather surprising to note that the presence and distribution of pullulanase and ISA seem to differ greatly among plant species and plant organs, although currently available information is limited. The presence of ISA has been detected only in sweet corn kernel [70], potato tuber [71,72] and maize kernels [73]. As shown in Table 4.1

Table 4.1 Basic kinetic and physico-chemical parameters of isoamylase in plants [10].

Plant	Tissues	Substrate specificity	pH	MW(kDa)(method)
Maize	Developing endosperm	Ap>βLd>>Pg>Pu=0	6.0-7.5	141 (GPC; Ultrogel AcA44)
Pototo	Tuber	Ap=Pg=Gc=βLd>> Pu=0	5.5-6.0	520(GPC; Sephadex G-200)

aLd, a-limit dextrin; BLd, B-limit dextrin; Ap, amylopectin; Gc, glycogen; Pu, pullulan; Pg, phytoglycogen; and GPC, gel permeation chromatography.

In our experiment, we found that debranching enzyme activity, both pullulanase and ISA, were detected only in the parenchyma of the tuber but not in the cortex. This agreed with studies on cassava branching enzyme [73] and starch synthase (unpublished data) in our laboratory. Therefore, it may be proposed that starch biosynthesis in cassava occurred in the parenchyma of tubers. However, identification of the DBE on native PAGE showed only the blue color band of pullulanase activity but not the colorless band of ISA on the gel. This may be due to the ISA activity was in the same band as pullulanase or the ISA activity detected was the activity of other amylolytic activity. On the other hand, the ISA activity might not be stable and present in minimal quantity which was below sensitivity of the activity stain.

4.3 Isolation and purification of DBE

A debranching enzyme was first described in yeast by Nishimura [75], who detected the enzyme activity by increased iodine staining of amylopectin, and hence designated the enzyme as ‘amylosynthease’. The enzyme activity was subsequently observed in potato tuber and rice endosperm [76]. Later, Maruo *et al*. [77] showed that amylosynthease did not synthesize starch, but produced linear glucans by debranching amylopectin.

Several steps were employed to purify DBE from cassava tuber. The first step of purification involved the use of ammonium sulfate to precipitate other contaminating proteins and trace of reducing sugars in crude enzyme. In our experiment, 20-50% saturated ammonium sulfate can remove 781.5 mg proteins, but total activity of 54.66 unit DBE was lost. Specific activity of DBE activity was

shown to increase by 1.1% after this step (Table 3.1). Although, this step did not give high purification but is was an essential step because of the removal of other starch metabolizing enzymes.

In preliminary experiment which DBE was chromatographed on DEAE-Sepharose and eluted with NaCl gradient, the activity peak showed activity of pullulanase in peak2 and isoamylase activity when use amylopectin as substrate in peaks 1,2 and 3(Figure 3.1). Each peak was pooled and checked activity by native starch PAGE. We found blue band of pullulanase activity in peak2 but did not found isoamylase activity colorless band in peaks 1 and 3 (Figure 3.2). It implied either that isoamylase did not exist in cassava tuber and what was detected with reaction on amylopectin was other amylolytic activity, or isoamylase existed in the tuber but was very unstable. Ion exchange chromatographies on DEAE-Sepharose was performed in Tris-HCl buffer pH 7.5 and pullulanase activity bound to the column, indicating that its pI was lower than 7.5. The pooled DBE fraction was further purified on Sephadex G-150 column up to 18 folds, but the yield was very small. Since pullulanase activity was very specific to pullulan, the assay method should detect only the pullulanase not other amylase. Therefore the fraction from DEAE-Sepharose was used for characterize studies.

4.4 Characterization of DBE

4.4.1 Molecular weight and pI determination

Native molecular weight of pullulanase was 103 kDa determined by gel filtration on sephadex G-150 column (Figures 3.3 and 3.5). Since pullulanase eluted from DEAE-Sepharose or G-150 was not pure enough for determination of molecular weight on SDS-PAGE, eluted pullulanase activity band from native

starch PAGE was used. It showed major band at 35 kDa (Figure 3.6). Since the molecular weight of DBE on sephadex G-150 was around 103 kDa and molecular weight of DBE on SDS-PAGE was 35 kDa, it may be postulated that the cassava DBE consisted of 3 identical 35 kDa subunits. Many DBE reported in other plants were from the endosperms with molecular weight in the range 90-110 kDa on gel filtration column with a few DBE in maize and rice reported at 58-70 kDa (Table 4.2). In potato tuber, which is storage root similar to cassava, DBE was also reported around 110 kDa. However, its molecular weight on SDS-PAGE was not determined. Therefore, native molecular weight of DBE from cassava tuber was in the same range with most reported DBE. Table 4.2 summarized the reported molecular weights and other properties of pullulanase in other plants. The pI of DBE determined by IEF gel was 4.75 which was belowed pH 7.5 as speculated from the result of DEAE-Sepharose column.

Table 4.2 Basic kinetic and physico-chemical parameters of pullulanase in plants.

Plant	Tissues	Substrate specificity	K_m(mg/ml)	pH	M_w(kDa)(method)	Reference
Maize	Mature endosperm	βLd=αLd>Pu>>Ap>Gc			110(GPC;Sephadex G-200)	[77]
		Pu> βLd>>Ap>Pg		5.5	68(GPC;Ultrogel AcA 44)	[73]
Rice	Developing endo-sperm	Pu> αLd> βLd>>Ap >Pg=0		5.6		[78]
		Pu> βLd> αLd>>Ap	0.22(Pu),3.1(βLd), 4.0(αLd),5.0(Ap)			[79]
					100(SDS-PAGE)	[80]
					100(SDS-PAGE)	[81]
	Mature endosperm	βLd>Pu>>Ap	6.0(Ap)	5.6	70(GPC;Sephadex G-100)	[82]
	Germinating endo-sperm	Pu>>Gc=Pg		5.5	58(GPC;Bio-Gel P-150)	[83]
Sorghum	Mature endosperm	βLd>Pu>>Ap	0.2(Pu),2.5(βLd)			[84]
		βLd>Pu>Ap		5.0-5.4	90(GPC;Sephadex G-150)	[85]
Oat	Mature endosperm	βLd>Pu>>Ap	10(Ap)			[84]
		Pu> βLd >>Ap	0.23(Pu),3.0(βLd), 1.0(Ap)		85(GPC;Sephadex G-100)	[86]

Plant	Tissues	Substrate specificity	K_m(mg/ml)	pH	MW(kDa)(method)	Reference
Broad bean	Mature embryo	βLd>Pu>>Ap	1.0(βLd),1.2(Ap)	6.4-6.8	80(GPC;Sephadex G-150)	[87]
Potato	Tuber	Pu= βLd= αLd>>Ap				[88]
Sugar beet	Leaf	Pu> βLd>>Ap		5.5	110(GPC;Sephadex G-150)	[89]
					105(SDS-PAGE)	
Spinach	Leaf	αLd> Pu > βLd>Ap		6.0-7.0		[90]
		>Gc=0				
		Pu>>Ap>>Gc=0	0.78 (Pu),7.0(Ap),	5.0-5.5	110(GPC;TSKgel G3000SW)	[91]
					102(SDS-PAGE)	

βLd, β -limit dextrin; αLd. α -limit dextrin: Ap, amylopectin; Gc. glycogen; Pu. pullulan; Pg. phytoglycogen; and GPC, gel permeation chromatography

4.4.2 Effect of pH and temperature on DBE activity

Partially purified pullulanase was incubated in various pH's and its activity measured. It was found that pullulanase was most active at pH 6.0, and the activity decrease about 50% at pH lower than 5.25 and higher than 8.0. It was active in range of pH 4.5-10.0. This result also complied with those reported for other plants DBE (Table 4.2) which showed optimum pH for their activity around 5.5-6.8. When the enzyme was incubated at various temperatures, the activity showed the highest temperature at 37°C, after which the enzyme activity decreased about 50% at temperature higher than 50°C and lost all activity at temperature higher than 70°C . When stability of pullulanase was studied by incubation up to 48 hours at 4-60°C, it was found that pullulanase still maintained more than 80% of its activity in the temperature range 4-40°C. At 45°C, activity of enzyme steadily declined after 6 hour then completely lost activity at 48 hours. At 50 and 60°C, activity of enzyme steadily declined before 6 hours then completely lost activity at 24 hours. Therefore, pullulanase should be kept around 4-40 °C when carry out experiments. The assay should be performed at pH 5.5-7.0 and 20-40°C.

4.4.3 Effect of sulfhydryl reagents and divalent ions on activity of DBE.

Sulfhydryl reagents such as dithiothreitol and β-mercaptoethanol are effective not only for stabilizing pullulanase but also for enhancing its higher catalytic capacity [90]. In our experiment, 10 mM DTT activated pullulanase activity up to 300% while the same concentration of β-mercaptoethanol gave only

120% activation. Table 3.11 showed comparison of the result from our studies with that reported on spinach leaves. Li, B., Servaites, J.C. and Geiger, D.R. (1992) also reported greater stimulation effect by DTT than β-mercaptoethanol at the same concentration. They speculated that DTT may have other effect such as on the conformation of the enzyme rather than maintaining –SH group at the enzyme active site [92]. DTT is a preferred reagent for reducing disulphide bonds because of its low redox potential which ensures rapid and complete reaction [93]. It contain two reactive –SH groups in a molecule and more stable compared to β-mercaptoethanol which contain only one –SH group and less stable. NEM, IAA and GSH, as sulfhydryl modifying reagents, severely inhibits pullulanase. NEM and IAA are a penetrating, irreversibly sulfhydryl modifying reagents, gave highly inhibition while GSH showed less effect. These results indicate that sulfhydryl groups are involved in the reaction of pullulanase.

Metal ion have been reported to affect enzyme activity by either activating or inhibiting the activity. In our experiment, Hg^{2+}, Cu^{2+}, and Ni^{2+} were potent inhibitors of the pululanase activity, while Mn^{2+} and Co^{2+} had activating effect. In other plants, Hg^{2+} at 1 mM is a potent inhibitor of the pullulanase prepared from sweet corn endosperm [94] and rice endosperm [95]. Other cations such as Cu^{2+}, Zn^{2+}, Ag^{+} and Cd^{2+} at concentrations of 1 mM are also inhibitory to the rice endosperm enzyme [95].

4.4.4 Kinetic constants of DBE and substrate specificity

Pullulanase employed pullulan as its main and specific substrate. Experiments were carried out to investigate the K_m and V_{max} for pullulan. (Figures 3.14). The K_m constant was 0.88 mg/ml and V_{max} was 24.81 nmol maltose/min.

Table 4.1 showed K_m constant of pullulanase from several plant tissues. The K_m of pullulanase for pullulan is about 0.2 mg/ml for enzymes of rice endosperm, sorghum endosperm and oat endosperm, but was found to be 0.78 mg/ml for the spinach leaf enzyme. The K_m for pullulanase was not reported in potato tuber. It may be speculated that in tissue such as endosperm which acted as energy source for developing embryo, pullulanase activity hydrolyze the storage starch which serves as transient nutrient to yield short chain polysaccharides for energy production. In such case, lower K_m for the substrate would be beneficial for the utilization of the substrate. On the other hand, tissues such as leaf or storage tubers required pullulanase activity for trimming or modifying structure of amylopectin to facilitate organization of starch granule, milder pullulanase activity may be better for retaining amount of storage starch while sufficient for the trimming action. In the latter case, pullulanase with higher K_m for the starch would serve the purpose. The K_m for pullulan of DBE in cassava tubers reported in our study was in the same range reported for spinach leaves, supporting our hypothesis. When the activity of the partially purified DBE was tested on several carbohydrates, it was found to be able to hydrolyze only pullulan, amylopectin and soluble starch with activity on amylopectin was 37% of pullulan and activity on soluble starch which contained amylopectin was 20% of pullulan. The enzyme cannot act on other kinds of carbohydrates, confirming the high specificity of the enzyme for pullulan. Glycogen is the storage form carbohydrate in animals. Although the chemical composition of glycogen is the same as that of amylopectin, their three-dimensional structure and physico-chemical properties are distinctly different.

The average chain length of amylopectin is larger than that of glycogen, and the frequency of branching is lower in amylopectin as compared with glycogen. The most remarkable structural feature of amylopectin is its refined multiple cluster structure, while the whole glycogen molecule is reported to form a tree-like or bushlike structure (Table 1.2). The structure of glycogen may be unsuitable for interaction with the enzyme active site, hence no hydrolytic activity of pullulanase on glycogen.

CHAPTER V

CONCLUSIONS

1. DBE activity was detected in parenchyma, but not in cortex of cassava tuber.
2. Crude DBE from parenchyma of cassava tubers was partially purified by 20- 50% saturated ammonium sulfate precipitation.
3. The pullulanase activity was isolated by DEAE-sepharose column was eluted at 0.15 M NaCl. Further purification by sephadex G-150 yielded 18 folds purified DBE with 7% yield.
4. The native molecular weight of pullulanase was estimated to be 103 kDa by gel filtration on Sephadex G-150 and SDS –PAGE to be 35 kDa.
5. Activity stain of pullulanase on native gel electrophoresis showed characteristics blue band on iodine stain.
6. The optimum pH for pullulanase was 6.0 and optimum temperature was 37 °C. The enzyme was stable at 4-40 ^{o}C.
7. The isoelectric point (pI) estimated by isoelectricfocusing gel was 4.75.
8. The sulfhydryl reagents, namely NEM and IAA, showed inhibitory effect on activities of pullulanase. On the other hand, DTT and β-mercaptoethanol showed activating effect on the enzyme.
9. Divalent ions, Hg^{2+}, Ni^{2+} and Cu^{2+}, strongly inhibited pullulanase activities while Mn^{2+} and Co^{2+} , at low concentration, activated pullulanase activities.
10. The K_m for pullulan was 0.88 mg/ml and V_{max} was 24.81 nmol maltose/min.
11. Pullulanase was highly specific to pullulan and can hydrolyze some branch of amylopectin and soluble starch but no activity with amylose, glycogen, α-cyclodextrin and γ- cyclodextrin as substrate.

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APPENDICES

APPENDIX A

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

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

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

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

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

Solution B (SDS PAGE)

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

Solution C (SDS PAGE)

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

2. Non- denaturing PAGE

10.0 % Seperating gel

30 % Acrylamideml solution	2.5	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	2.39	ml
10 % $(NH_4)_2S_2O_8$	50	µl
TEMED	10	µl

5X Sample buffer

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

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

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

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g

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

3. SDS-PAGE

10 % Separating gel

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

4.0 % Stacking gel

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

5X Sample buffer

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

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

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

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

APPENDIX B

Preparation for isoelectric focusing gel electrophoresis

Stock solution for isoelectric focusing gel electrophoresis

24.25% Acrylamide; 0.75% bis-acrylamide

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

Adjusted volume to 100 ml with distilled water

0.1% Riboflavin

Riboflavin	50 mg
Distilled water	40 ml

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

Monomer-ampholyte solution

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

This solution was degassed

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

Fixative Solution, 100 ml

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

Immerse gels in this solution for 30 minutes

Staining Solution, 100 ml

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

Dissolve the $CuSO_4$ in water before adding the methanol. Either dissolve the dye in

alcohol and filtrate or add it to the solution at the end.

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

Destaining Solution

First destaining solution

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

Dissolve the $CuSO_4$ in water before adding the ethanol. Immerse the gel in two or three

change of this solutiomunit the background nearly clear.

Second destaining solution

Ethanol	25	ml
Acetic acid	7	ml
Distilled water	68	gm

Immerse the gel in this solution removethe last traces of the $CuSO_4$

APPENDIX C

Iodine's Solution

Iodine solution

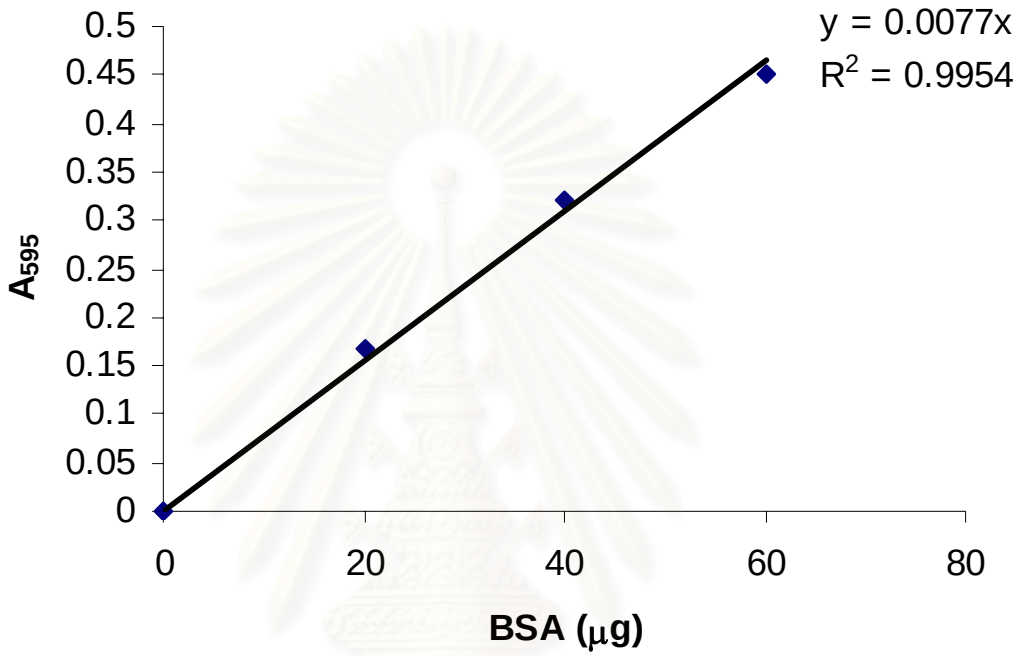
1% Potassium iodide; 0.1% Iodine

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

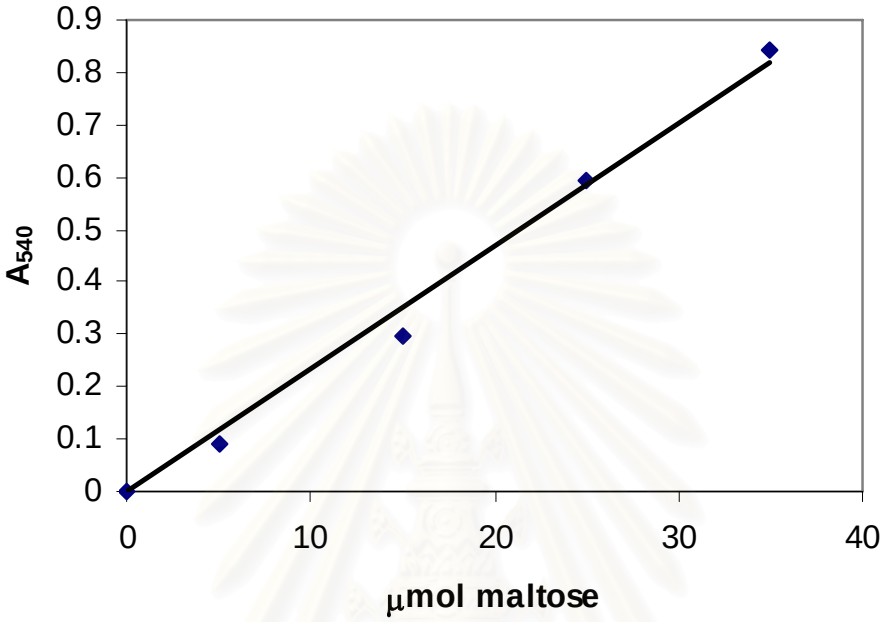
APPENDIX D

Calibration curves

1. Calibration curve of protein concentration



2. Calibration curve of maltose



APPENDIX E

Universal buffer

Universal buffer, 1 L.

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

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

BIOGRAPHY

Mr. Wasin Sinlapawisut was born in June 10th, 1979 in Nakhomsrithammarach. He finished Mattayom 6 at Princess Chulaporn collage, Nakhomsrithammarach and enrolled in the Faculty of Science Chulalongkorn University in 1997and graduated with B.Sc. in Biochemistry in 2001. He continued for M.Sc. in Biochemistry later that year.