CHAPTER I

INTRODUCTION

1.1 Cassava

Cassava (*Manihot esculenta* Crantz) is a root crop belonging to section fructicosae of family Euphorbiaceae, Dicotyledonae (Jos, 1969). Cassava (*Manihot esculenta*), also called manioc, tapioca or yuca, is one of the most important food crops in the humid tropics, being particularly suited to conditions of low nutrient availability and able to survive drought (Burrell, 2003). The plant grows to a height of 1 to 3 m and several roots may be found on each plant. Although cassava leaves are sometimes consumed, the major harvested organ is the tuber, which is actually a swollen root. 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 (720.1 x 1012 kJ day^{-1}) ranking fourth after rice, sugarcane and maize, and sixth among crops in global production. A typical composition of the cassava root is moisture (70%), starch (24%), fiber (2%), protein (1%) and other substances including minerals (3%).

Cassava is a high starch producer with levels between 73.7 and 84.9% of its total storage root dry weight (Figure 1.1). This attribute together with the unique properties of its starch creates demand for particular food and nonfood applications. For example, cassava starch readily gelatinises 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.

Cassava starch can be converted to maltotriose, maltose, and glucose as well as to other modified sugars and organic acids (Tan et al., 1984). Starch from cassava can be used to make fructose syrups (Vuilleumier, 1993) and formulate gelatin capsules (Nduele et al., 1993). The use of cassava as a source of ethanol for fuel is already being exploited and very promising. Recently, Roble et al., 2003 demonstrated the production L-Lactic acid from raw cassava starch in a bioreactor using *Aspergillus awamori* (fungus) and *Lactococcus lactis* spp. *lactis* (bacteria). Furthermore, cassava dreg as byproduct from cassava starch processing could be employed for phytase production after the addition of a nitrogen source and mineral salts (Hong et al., 2001), while activated carbons prepared from waste cassava peel (Rajeshwarisivaraj et al., 2001) are efficient as adsorbents for dyes and metal ions.

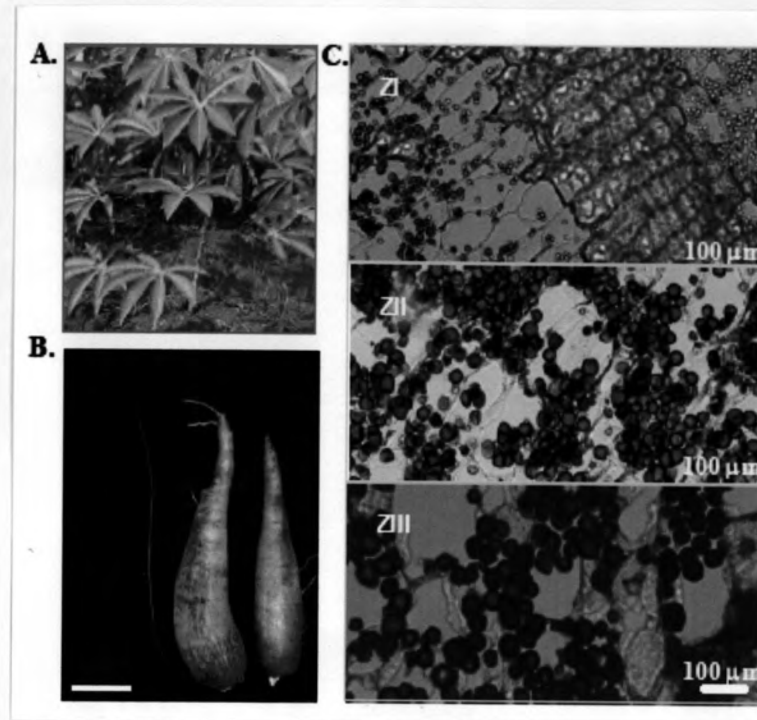

Figure 1.1 Display of a cassava plant and its starch granule architecture (Baguma et al., 2004). **A**. Cassava plant **B**. Cassava storage roots. **C**. Iodine stained map showing cellular distribution of amylose (blue-black stained) and amylopectin (reddish-brown stained fractions). Abbreviations: ZI, periderm region; ZII, cortical region; ZIII, parenchyma region

1.2 Starch

Starch is the most significant form of carbon reserve in plants in terms of the amount made, the universality of its distribution among different plant species, and its commercial importance (Martin and Smith, 1995). It consists of different glucose polymers arranged into a three dimensional, semicrystalline structure called the starch granule.

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 cap cells, but its major site of accumulation is in storage organs, including seeds, fruits, tubers, and storage roots. Starch can be chemically fractionated into two types of glucan polymer: amylose and amylopectin.

1.2.1 Amylose

Amylose consists of predominantly linear chains of $\alpha(1\text{-}4)$ linked glucose residues, up to1000 residues long (Figure 1.2). Amylose is usually branched at a low level (approximately one branch per 1000 residues) by $\alpha(1\text{-}6)$ linkages and makes up ~30% of starch (Table 1.1). This proportion, however, may vary considerably with the plant species (a range of 11 to 35% was found in a survey of 51 species; Detherage et al., 1955) and variety (a range of 20 to 36% was found in a survey of 399 maize varieties; Detherage et al., 1955) and also with the plant organs, the developmental age of that organ, and, to some extent, the growth conditions of the plant (Shannon and Garwood, 1984). Once extracted from plants and in solution, amylose forms hydrogen bonds between molecules, resulting in rigid gels. However, depending on

the concentration, degree of polymerization, and temperature, it may crystallize and shrink (retrogradation) after heating (Shewmaker and Stalker, 1992).

1.2.2 Amylopectin

Amylopectin, which consists of highly branched glucan chains, makes up ~70% of starch. Chains of roughly 20 α(l-4) linked glucose residues are joined by α(1-6) linkages to other branches (Figure 1.3). The branches themselves form an organized structure (Figure 1.4 A). Some are not substituted on the six positions and are called A chains. These chains are α(1-6) linked to inner branches (B chains), which may be branched at one or several points. A single chain per amylopectin molecule has a free reducing end (the C chain). The branches are not randomly arranged but are clustered at 7 to 10 nm intervals. An average amylopectin molecule is 200 to 400 nm long (20 to 40 clusters) and 15 nm wide (Kainuma, 1988; Smith and Martin, 1993). After extraction, amylopectin has more limited hydrogen bonding than amylose in solution and is more stable, remaining fluid and giving high viscosity and elasticity to pastes and thickeners. Some starch, most notably that from potato tuber, is also phosphorylated. Potato tuber amylopectin carries up to one phosphate per 300 glucan residues (Takeda and Hizukuri, 1982).

Within the starch granule, which may vary in size from 1 pm to 100 pm, the amylopectin molecules are arranged radially, and adjacent branches within the branch clusters may form double helices that can be packed regularly, giving a crystallinity to the starch granule (Figure 1.4 C). The degree of crystallinity is determined in part by the branch lengths in the amylopectin (Smith and Martin, 1993). The degree of branching and consequently the crystallinity of starch granules may vary considerably,

even between different organs of the same plant. The starch granule is not uniformly crystalline and contains relatively amorphous regions. Amylose molecules form single helical structures and are thought to be packed into these amorphous regions, which are present throughout the granule. Granules from storage organs and leaves have rather different macrostructures. Starch granules from storage organs show internal semicrystalline growth rings that are differentially sensitive to chemical and enzymatic attack (Figure 1.4 B). 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 highly branched glucan polymers (Steup et al., 1983; Beck, 1985).

1.3 Pullulan

Pullulan is a linear homopolysaccharide of glucose that often is described as an $\alpha(1\text{-}6)$ linked polymer of maltotriose subunits (Leathers, 2004). This unique linkage pattern endows pullulan with distinctive physical traits. Pullulan has adhesive properties and can be used to form fibers, compression molding, and strong, oxygen-impermeable films. Pullulan is derivatized easily to control its solubility or provide reactive group. Consequently, pullulan and its derivatives have numerous potential food, pharmaceutical and industrial applications. Pullulan is essentially a linear glucan containing $\alpha(1\text{-}4)$ and $\alpha(1\text{-}6)$ linkages in a ratio of 2: 1 (Figure 1.5)

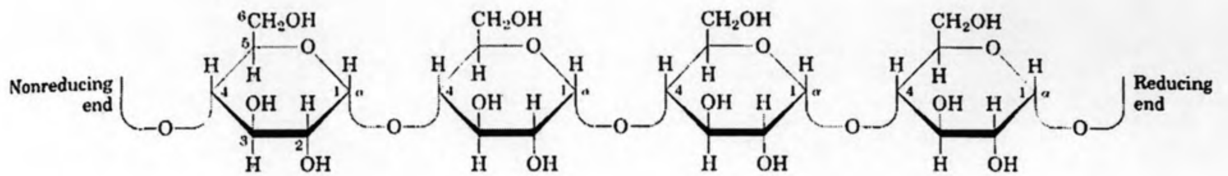

Figure 1.2 Short segment of amylose, a linear polymer of D-glucose residues in α(1-4) linkage. A single chain can contain several thousand glucose residues. (Nelson and Cox, 2003)

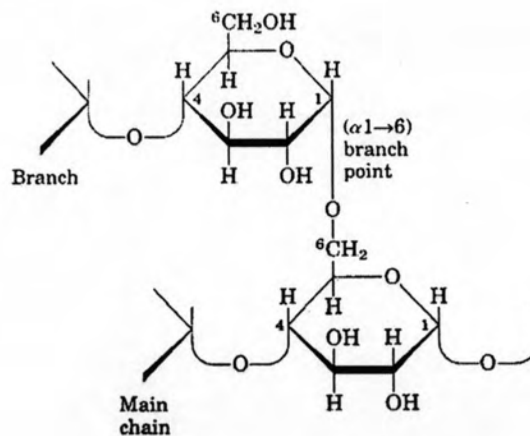

Figure 1.3 An α(1-6) branch point of amylopectin (Nelson and Cox, 2003)

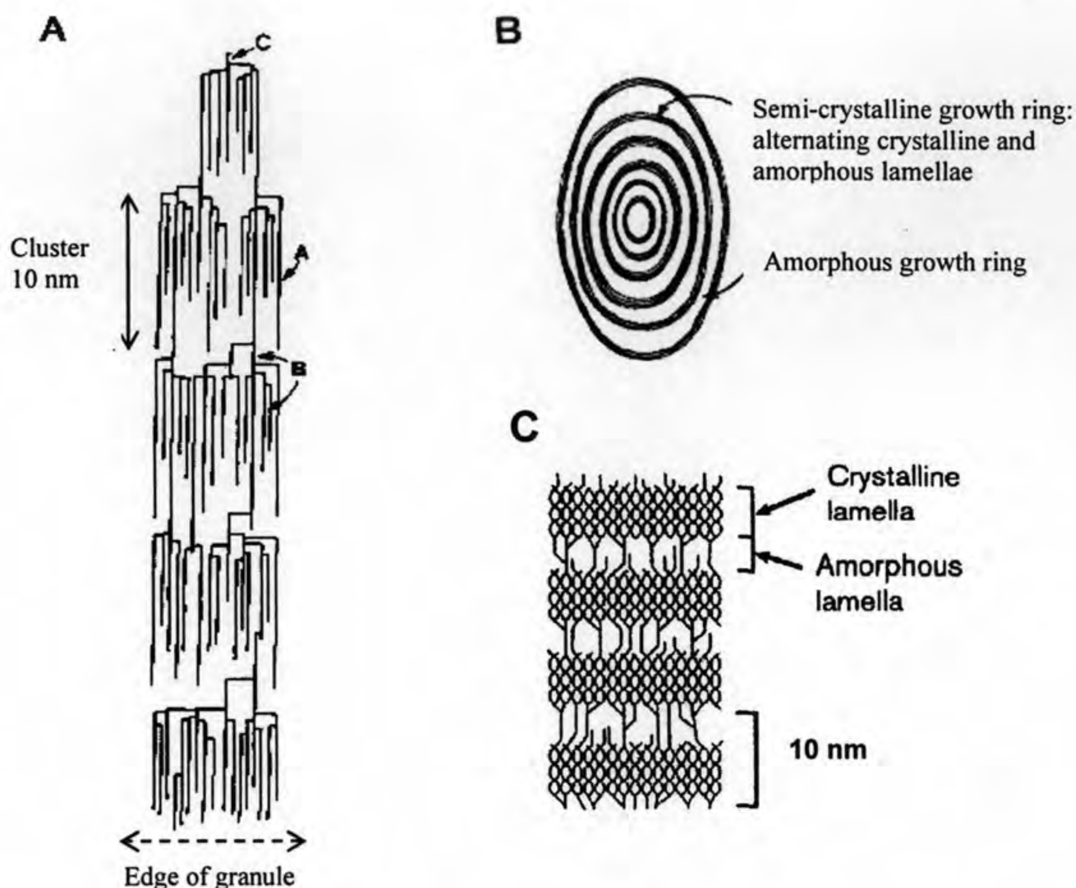

Figure 1.4 Amylopectin Structure and Diagrammatic presentation of Starch Granule (Martin and Smith, 1995)

A. Diagrammatic representation of an amylopectin molecule. α(1-4) linked glucans are attached by α(1-6) linkages to form a highly branched structure. Short glucan chains (A chains) are unbranched but linked to multiple branched B chains. There is a single reducing end to the C chain glucan. The branches are arranged in clusters ~10 nm long, with a few longer chains linking more highly branched areas.

B. Diagrammatic representation of a starch granule from storage tissue showing alternating semicrystalline and amorphous growth rings. The semicrystalline regions are thought to consist of alternating crystalline and amorphous lamellae.

C. Arrangement of cluster to form alternating crystalline and amorphous lamellae. The crystalline lamellae are produced by the packing of double helices in order arrays.

Table 1.1 Approximate amylose and amylopectin content of common food starches (Fennema, 1985)

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

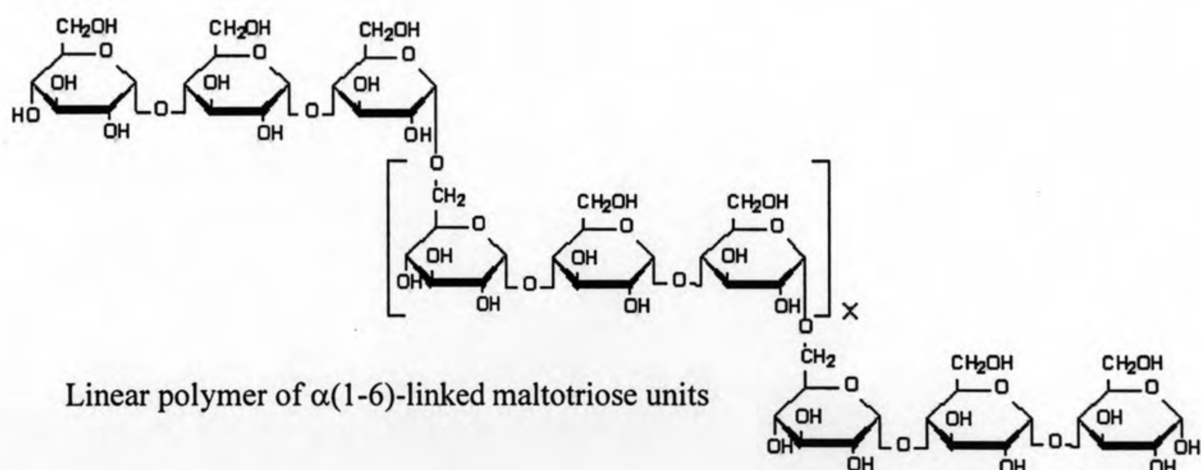

Figure 1.5 Structure of pullulan (Leathers, 2004)

1.4 Starch biosynthesis and the enzymes involved

Starch, the major reserve carbohydrate in plant, is found in both photosynthetic and non-photosynthetic tissues. Starch found in the chloroplasts of leaves and other photosynthetic cells is termed 'transitory starch' owing to the diurnal rise and fall of its level in these tissues. Long-term storage of starch occurs in non-photosynthetic storage organs as tubers, roots and seeds. Because of its greater availability, almost all end uses of plant starch are of the reserve type.

Transitory starch is synthesized in chloroplast by the process of carbon fixation during photosynthesis. During the night, transitory starch is degraded and transported as sucrose to the amyloplasts of storage organs, where it is incorporated into storage starch. Therefore, the pathway of starch synthesis in non-photosynthetic tissues involves the conversion of sucrose into ADP-glucose, and the subsequent conversion of ADP-glucose into starch. Sucrose synthase (SuSy) is responsible for the conversion of sucrose to starch. The enzyme exists in both cytosolic and membrane-bound forms. Activity of the cytosolic form is correlated with the production of storage products such as starch, while the membrane-bound form is believed to be involved in cellulose synthesis. The UDP-glucose and fructose formed by SuSy are converted to hexose phosphates. The resulting hexose phosphates may be imported to amyloplasts for use in the formation of ADP-glucose, or they may be directly converted to ADP-glucose in the cytosol prior to uptake activated sugar. Hexose phosphates and ADP-glucose were transported to the amyloplast via hexose phosphate transporters and ADP-glucose transporters, respectively (Figure 1.6).

The first committed step in plant starch synthesis is the synthesis of ADP-glucose from Glc-1-P and ATP by ADP-glucose pyrophosphorylase (AGPase; EC

2.7.7.23). The next step, the ADP-glucose is transferred by starch synthase (SS; EC 2.4.1.21) to the non-reducing end of an α-1,4 glucan resulting into the generation of linear α-1,4 glucans. Following this, the linear α-1,4 glucans are used as substrates by starch branching enzyme (SBE or Q-enzyme; EC 2.4.1.18) to introduce α-1,6 interlinear chain linkages resulting into 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) (Figure1.7) (Ball et al., 1996; Colleoni et al., 1999; James et al., 1995; Smith et al., 1997; Sun et al., 1997).

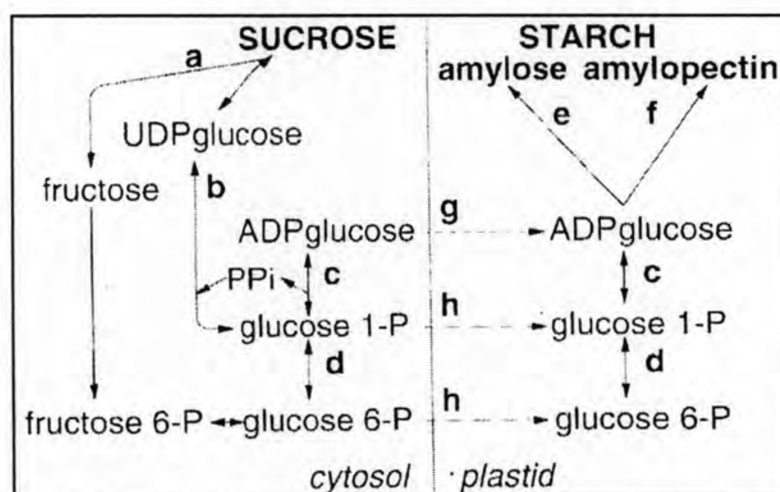

Figure 1.6 The major metabolites and enzymes involved in the conversion of sucrose to starch in storage organs. Carbon is shown entering the plastid either as a hexose phostohate (Smith et al., 1995) or as ADP-glucose. Enzyme are: a, sucrose synthase; b, UDPglucose pyrophosphorylase; c, ADPglucose pyrophosphorylase; d, phosphoglucomutase; e, starch synthase (GBSSI); f, starch synthase and starch branching enzyme; g, ADPglucose transporter; h, hexose transporter. PPi: inorganic pyrophosphate.

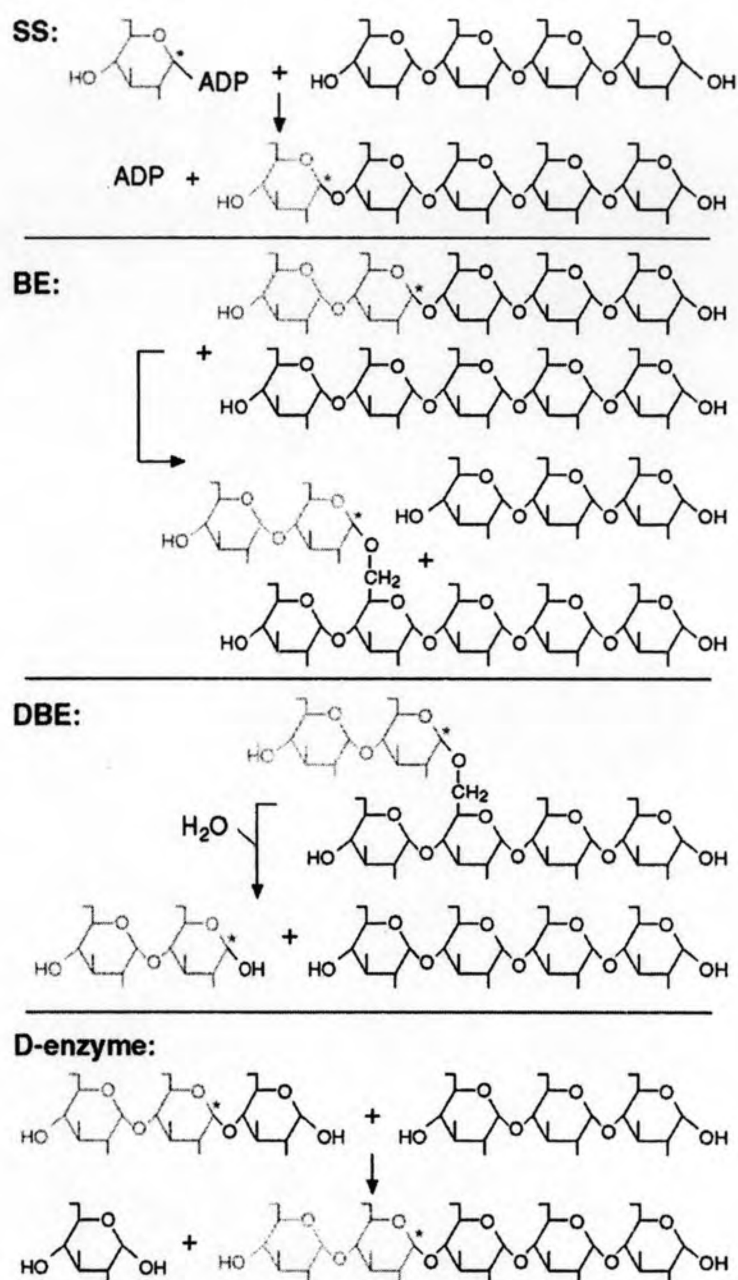

Figure1.7 Reaction of enzymes involved in amylopectin synthsis (Myers, 2000).

1.4.1 The synthesis of ADP-glucose through ADP-Glucose pyrophosphorylase (AGPase)

AGPase catalyses a rate limiting reaction in prokaryotic glycogen and plant starch synthesis (Smith et al., 1995). AGPase from bacterial is a homotetrameric enzyme encoded by a single gene, whereas the higher plant AGPase is a heterotetrameric enzyme ($\alpha\beta_2$) composed of a pair of large subunits and a pair of small subunits, encoded by different genes (Salamone et al., 2002). 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, AGPase from blue-green algae and higher plants are activated by 3-phosphoglyceric acid (3-PGA) and inhibited by Pi, key intermediates in CO_2 assimilation by the C_3 pathway. Structure function analysis of AGPase indicates that the 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 (Smith-White and Preiss, 1992), 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.

Recently, it has been shown through phylogenetic studies that plant AGPase's are distinctly localised and subdivided into leaf, stem, roots and endosperm types. At plastid level, AGPase are further classified into two groups based on subcellular localisation, i.e. plastidial and cytosolic forms (Denyer et al., 1996; Okita, 1992; Smith, 1988; Thorbjornsen et al., 1996).

The role of cytosolic and plastidial AGPase in starch synthesis has been elucidated through studies involving mutants of maize, *Shrunken-2* and *Brittle-2* (Denyer et al., 1996; Giroux, 1996; Hannah andNelson, 1976) and barley, *Risø 16* (Johnson et al., 2003). *Risø 16* lacks cytosolic AGPase activity but has unaffected plastidial activity. *Risø 16* exhibits reduced starch content indicating that a cytosolic AGPase is required to achieve the normal rate of starch synthesis. In barley, the plastidial activity by itself is sufficient for normal starch synthesis, albeit reduced rate of accumulation. In contrast, maize plastidial activity alone is not sufficient for normal rate of starch accumulation (Thorbjornsen et al., 1996). More work is required to completely understand the physiological function of the various AGPase isoforms.

1.4.2 The role of starch synthase

Starch synthase exist in many forms and were classified into three distinctly localized 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.

1.4.2.1The synthesis of amylose

The synthesis of amylose was attributed to the granule-bound starch synthase (GBSS) that includes GBSSI and GBSSII. GBSSI is encoded by the *Waxy* locus in cereals, functioning specifically to elongate amylose (de Fekete et al., 1960; Nelson and Rines, 1962) and is found, essentially, completely within the granule matrix. The expression of GBSSI appears to be mostly confined to storage tissues. The second

form of GBSS is GBSSII, which is encoded by a separate gene, is thought to be responsible for amylose synthesis in leaves and other non-storage tissues which accumulate transient starch (Nakamura et al., 1998; Fujita and Taira, 1998; Vrinten and Nakamura, 2000). The discovery of GBSS involved amylose synthesis 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 (Murata et al., 1965), maize (Weatherwax, 1922), wheat (Nakamura et al., 1995), barley (Ishikawa etal., 1994), *amf* potato (Hovenkamp-Hermelink, 1987) and *lam* pea (Denyer et al., 1995). 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 (Visser, 1991). 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 GBSSII also controls amylose production.

1.4.2.2 The synthesis of amylopectin

Various studies have demonstrated that SSI, SSII and SSIII are involved in amylopectin synthesis. While no mutants for SSI have been reported, biochemical evidence suggests that SSI is primarily responsible for the synthesis of the shortest glucan chains, i.e. those with a DP of 10 glucosyl units or less (Commuri and Keeling, 2001), and further extension of longer chains is achieved by the activities of SSII and SSIII isoforms, each of which act on progressively longer glucan chains. In pea, studies of *rug5* (Craig et al., 1998), 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) (Craig et al., 1998;

Fontaine et al., 1993). In potato tubers, antisense inhibition of SSII and SSIII singly (Edwards et al., 1999; Lloyd, 1999) or in combination (Lloyd, 1999) 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 (Yamamori et al., 2000). Recently discovery of *sugary–2-like* phenotype in maize mutants defective in SSIIa, demonstrated that SSIIa is involved in starch synthesis. Present evidence indicates 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* (Gao et al., 1998), *Chlamydomonas reinhardtii* mutant, *STA3* (Fontaine et al., 1993) and transgenic potato carrying an antisense SSIII construct (Edwards et al., 1999) that SSIII contributes to amylopectin branch-length distribution. In our laboratory, three isoforms of SS (SSI, SSII and SSIII) were isolated from cassava tubers and characterized. These isoforms were different in their biochemical characteristics which may reflect different roles in the starch biosynthetic process (Pao-in, 2006).

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

Starch branching enzymes are involved in amylopectin synthesis. They catalyse 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 C6 of a glucosyl moiety of an α-1,4 glucan template. They belong to the α-amylase family characterised by a catalytic $(\beta/\alpha)_8$-barrel domain (Jespersen et al., 1993; Svenson, 1994). The domain encompasses specific active sites that arise from the inter-connecting 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 (Burton et al., 1995). 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 (Jobling et al., 1999). Type I SBE, has been identified in maize (Baba et al., 1991), rice (Kawasaki et al., 1993), pea (Burton et al., 1995), cassava (Salehuzzaman et al., 1992), and wheat (Morell et al., 1997). 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 tuber, whereas SBEI is the major isoform in the tuber (Jobling et al., 1999). In our laboratory, SBE from cassava tuber was isolated into 3 isoforms. Isoforms 1 and 2 were more specific to amylose and grouped as SBEII. Isoform3 were more specific to amylopectin and grouped as SBEI (Yaiyen, 2003).

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 (Ball and Morell, 2003; Blauth et al., 2002; Satoh et al., 2003; Seo et al., 2002). However, it has been shown that loss of BEI protein in rice resulted in significant changes in the fine

structure of amylopectin and physicochemical properties of the resulting starch in the rice endosperm (Satoh et al., 2003). As for SBEII, exclusive elimination of SBEII in potato led to increased levels of amylose (Jobling et al., 1999), although combined suppression of both SBEII and SBEI markedly increased the amylose content of the resulting starch phenotype (Schwall et al., 2000). 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 (Ball and Morell, 2003). In spite of these efforts, the question of function for the different isoforms remains not clearly resolved.

1.4.4 The role of debranching enzymes (DBE)

Starch debranching enzymes belong to the α-amylase super family (Jespersen et al., 1993). 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) (Doehlert and Knutson, 1991; Ishizaki et al., 1983). The defining difference is their substrate specificity in which pullulanases debranch pullulan and amylopectin but not glycogen, whereas isoamylase debranch both glycogen and amylopectin (Nakamura et al., 1996). 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 (Burton et al., 1995). Conversely, in potato, two distinct subunits define an equally large heteromultimeric enzyme (Ishizaki et al., 1983). In our laboratory, DBE from cassava tuber cv.5 minute was studied and pullulanase was detected (Sinlapawisut, 2004).

Many ideas and researches in the synthesis of amylopectin lead to several models that have been proposed to explain the involvement of DBE in amylopectin synthesis in which not only SS and SBE. The 'preamylopectin trimming model' (Ball et al., 1996) is the general idea that DBE is involved in amylopectin synthesis. This model arises from the observation that the endosperm of *sugary1* (*su1*) mutants of maize and rice, which are deficient in DBE activity, synthesise not only amylopectin but also a highly-branched, soluble glucan call phytoglycogen (Doehlert et al., 1993; James et al., 1995; Nakamura et al., 1996 and Nakamura et al., 1997). Further support for an involvement of DBE in amylopectin synthesis has recently been provided by the discovery of DBE-deficient, phytoglycogen-accumulating mutants of *Chlamydomonas* (*sta7*) (Mouille et al., 1996) and *Arabidopsis* (*dbe1*) (Zeeman et al., 1998), by the demonstration that DBE are probably of widespread occurrence in starch synthetic organs (Zhu et al., 1998) and by the discovery that a gene at the *su1* locus of maize actually encodes a DBE of the isoamylase class (James, 1995). The preamylopectin trimming model proposes a specific, sequential course of events in which a highly-branched structure synthesised by SS and SBE at the surface of granule is trimmed by DBE to provide the substrate for chain elongation by SS (Figure 1.8). The model provides an explanation of the clustering of chains of amylopectin and hence, potentially, of the semicrylstalline ordering of the granule. This idea is tempting in that it integrates the synthesis of the amylopectin polymer

with its organization to form the granule, and provides an explanation of the accumulation of phytoglycogen in mutants deficient in DBE. However, proof that the trimming mechanism actually operates *in vivo* is still lacking. An alternative explanation for the accumulation of phytoglyciogen in DBE-deficient cell has arisen from study of the *dbe1* mutant of *Arabidopsis*. Chloroplasts in the mutant accumulate both phytoglycogen and apparently normal amylopectin in the same time (Zeeman et al., 1998). This phenotype led Zeeman and colleagues to propose the 'soluble glucan recycling model' which DBE is not directly involved in amylopectin synthesis. They suggested that DBE, together with other starch-metabolizing enzymes, plays scavenging role in the stroma, degrading any soluble glucans produced by the action of SS and SBE on malto-oligosaccharides. In the absence of DBE, SS and SBE will be able to elaborate branched, soluble glucans, resulting in phytoglycogen accumulation and reducing the amount of these enzymes available for the synthesis of amylopectin at the granule surface (Figure 1.9). Both of these models for the involvement of DBE in starch synthesis are extremely difficult to test in rigorous and direct way, and at present the question of whether DBE is directly involved in amylopectin synthesis remains open.

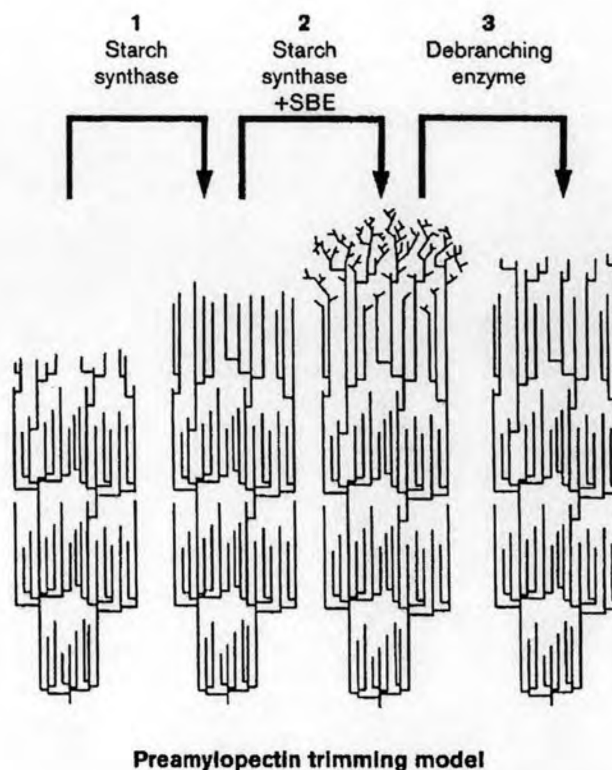

Figure 1.8 Models to explain the involvement of DBE in starch synthesis. The preamylopectin trimming model (Ball et al., 1996) proposes a direct involvement of DBE in amylopectin synthesis. It is envisaged that a sequence of synthetic events at the surface of the granule creates a cluster within an amylopectin molecule, as follows: (1) short chains are elongated by SS; (2) when chains reach the sufficient length to become substrates for SBE, a highly branched preamylopectin is formed; (3) selective trimming of this structure by DBE creates a bed of short chains from which the next round of synthesis can occur. When DBE activity is reduced or eliminated, preamylopectin accumulates. This highly-branched, soluble material is the phytogylcogen seen in the *su1*, *sta7* and *dbe1* mutants.

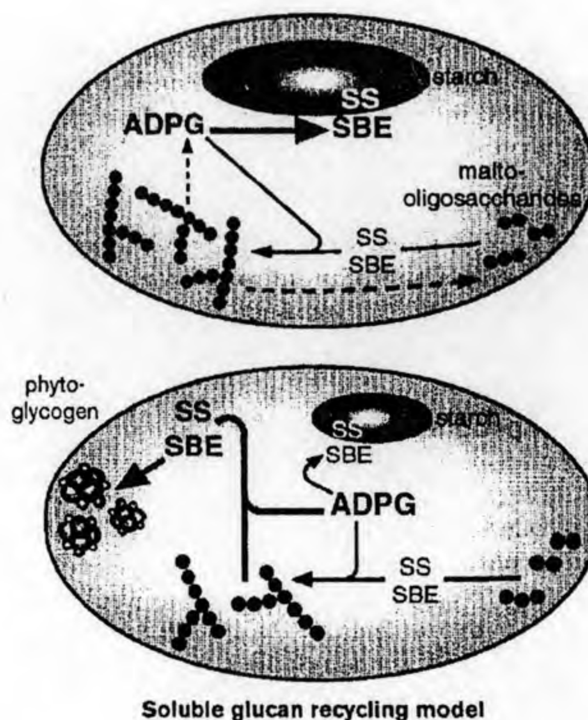

Figure 1.9 Models to explain the involvement of DBE in starch synthesis. The soluble glucan recycling model (Zeeman et al., 1998) proposes that DBE is only indirectly involved in starch synthesis. Amylopectin synthesis required only SS and SBE. In a normal plastid (top), small soluble malto-oligosaccharides in the stroma may be elongated by SS and then branched by SBE. Any glucans thus synthesized will be degraded by a suit of enzymes including DBE (dashed lines), preventing the accumulation of such products. In a plastid in which DBE activity is reduced or eliminated (bottom), this degradative mechanism is incomplete. Soluble branched glucans formed by SS and SBE from malto-oligosaccharides can be further elaborated, providing more substrates for SS and SBE, and leading to both accumulation of phytoglycogen and a reduction in the rate of starch synthesis.

1.4.5 The function of disproportionating enzyme (D-enzyme)

D-enzyme was first reported in potato tuber. Later, it was detected in beans, carrot, peas, spinach, tomato and Arabidopsis (Lin and Preiss, 1988; Manners and Rowe, 1969; Okita et al., 1979). The enzyme disproportionate soluble oligosaccharides of at least three glucose residues or amylopectin into maltooligosaccharides (Colleoni et al., 1999; Lin and Preiss, 1988; Takaha et al., 1996). In vitro analysis of potato D-enzyme showed that the protein was capable of transferring branched glucans and or producing cyclic glucans (Colleoni et al., 1999; Takaha et al., 1998). An attempt to understand its function in starch biosynthesis through mutation studies in Chlamydomonas and Arabidopsis showed conflicting roles. In Chlamydomonas, the mutants showed significant reductions in total glucopolysaccharide, abnormal starch granule, altered component proportions and atypical amylopectin chain-length distribution relative to the wild type. Conversely, Arabidopsis mutants overproduced starch (Critchley et al., 2001) while transgenic potato tubers transformed with antisense D-enzyme constructs showed no effect on starch synthesis and its fine structure (Takaha et al., 1998). These inconclusive findings suggest additional research is essential to clearly establish the function of D-enzymes in starch synthesis.

1.4.6 Other factors and evidence implicating their involvement

RI protein has been reported to be involved in phosphorylation of tuber starch (Lorberth et al., 1998). Recently, ADP-glucose pyrophosphatase, an enzyme likely with dual role in controlling levels of ADPG linked to starch synthesis and other metabolic pathways has been added to the list of enzymes involved in starch biosynthesis (Kleczkowski, 2001). Also, starch phosphorylase (P-enzyme) has been

shown to be involved in the catalysis of reversible phosphorylytic cleavage of starch and it seems very likely that P-enzyme is a part of the complex degrading enzymes involved in starch breakdown (Larsson et al., 1996; Lin and Preiss, 1988). It has also been shown that several enzymes involved in starch degradation e.g. α-amylase, β-amylase, D-enzymes, α-glucosidase (maltase), glucan water dikinase (GWD) and α-glucan phosphorylase are equally important players in starch biosynthesis. Most significantly, is the fact that glucan water dikinase (GWD) appears to control the overall rate of starch breakdown with a central rate limiting role in starch breakdown machinery and downstream starch synthesis. A detailed account on the roles, subcellular localisation and regulation of these enzymes has been recently reviewed (Zeeman et al., 2004). In the same review, the authors present a model for starch breakdown and its connection to starch synthesis. Lastly, sucrose invertase (SI), sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) are crucial in sucrose metabolism and contribute to sink strength in divergent crop species (Hajirezael et al., 2003; Roitsch et al., 2003).

1.5 Objectives

Cassava starch is composed of different glucan chain, amylose and amylopectin. The relative amounts of amylose and amylopectin are what give starch their unique physical and chemical properties. Now, the industrial applications of starch are limited because it is used mainly in its unmodified form. Therefore, it might be of value to produce cassava starch with novel uses and high quality which is benefit for industry and can be increase the commercial price of cassava. These can be achieved by modifying the levels and properties of the starch biosynthetic enzymes. To be able to manipulate starch biosynthesis, it is important to study all enzymes involved starch biosynthesis in cassava tuber such as starch synthase, starch branching enzyme and starch debranching enzyme. In this research we purify and characterize starch debranching enzyme in cassava tuber cultivar KU50, a high starch strain, for understanding the characteristic of this enzyme in order to get more information about its role in cassava starch biosynthesis.

Objectives of this research

1. to purify DBE from cassava tuber cultivar KU50
2. to characterize the properties of DBE from cassava tuber cultivar KU50