CHAPTER I

INTRODUCTION

1.1 Cassava

Cassava (*Manihot esculenta* Crantz) is a tropical crop grown mostly for its starchy thickened roots in the lowlands of Asia, Africa and South America. It is also known by other common names such as Ubi kettella in Indonesia, manioca, rumu or yucca in Spanish America, mandioca or aipim in Brazil, manioc in Madagascar and French-speaking Africa, tapioca in India and Malaysia, and cassava or cassada in English-speaking regions of Africa, Thailand, and Sri Lanka (Corbishley, 1984). It is an important crop for 500 million people (Cooke, 1989). Cassava belongs to the family Euphorbiaceae, which includes Para rubber (*Hevea brasilensis*) and caster bean (*Ricinus communis*). It shows extreme variations of shrub branch, root shape, coloration of petiole or of external and internal parts of root, indicating large degrees of hybridization (Cock, 1985).

Cassava can be grown throughout the year because it has no critical period of propagation and harvesting. The propagation is easy by using seeds, tubers or stem cuttings. The plant requires minimal attention during growth and it can be propagated on almost any soil types and moisture conditions, including sandy soil, sandy-loam soil, poor and exhausted land. It is able to tolerate either extremely acidic or basic pH ranges. Furthermore, cassava can also withstand a number of pests and diseases.

Cassava has entered the modern market economy and there is a growing demand for its use in processed foods and feed products. Increasingly, cassava starches and starch derivatives such as dextrin, glucose, and high fructose syrup have become the main products of cassava

agro-industry. Cassava starch has also been used in the paper and textile industry as well as in the manufacture of alcohol and adhesives (Munyikwa, 1997).

Cassava was brought to Thailand, firstly in the South from Malaysia, and it was later introduced to the Eastern provinces and finally brought to the Northeast, the West and the upper central region. Today the major areas for cultivation of cassava are in the East and the Northeast, which represent around 90 percent of the total cassava production.

Figure 1.1 Cassava tree and its underground tuberous roots.

The chemical components of cassava root is dependent on species, soil types, climate and harvesting ages (Corbishiay, 1984).

Table1.1 A typical cassava root analysis (Corbishlay, 1984).

Composition	Percent (%)
Moistures	70
Starch	24
Fiber	2
Protein	1
Fat, mineral, sugar	3

Root tissues were divided into peel (secondary phloem with the thin cork tissue layer removed) and up to three parenchymal tissue age group. Up to 40 % of the starch concentration can be deposited in root parenchymal tissue. Root starch is permanently removed from parenchymal tissue to older roots, probably in association with new foliage growth at the start of a new growing season (O' Hair, 1989).

1.2 Starch components

Starch is the principal reserve carbohydrate in plants. It is localized in the plastids, both the chloroplast in leaves and the amyloplast in non-photosynthetic tissues. The starch produced in nonmutant endosperms or other storage organ consists of two major components, a linear and branched polysaccharides, amylose and amylopectin, respectively.

In amylose, D-glucose units are linked together by α–1,4 bonds. The average chain length varied between 100-10,000 glucose molecules. The iodine affinity was 20.1 g/100 g with an absorption maximum (λ-max) of

644 nm, following reaction with iodine. Under standard conditions, amylose binds approximately 20 % of its own weight of iodine, whereas amylopectin binds none. Although amylose is usually described as a linear α–1,4 glucan, some of the molecules are sparsely branched (Nelson, 1995).

Amylopectin consists of α–1,4 linked D-glucose units, which are cross-linked by α–1,6 bonds. Amylopectin is structurally similar to glycogen but has fewer branch point than glycogen. The iodine affinity was 1.05-1.25 g/100 g with λ-max between 544 and 556 nm, following reaction with iodine. The average chain length varied between 20–30 glucose molecules (Nelson, 1995).

Both the amylose and amylopectin components can be completely degraded by the combined action of β-amylase and pullulanase, indicated that the branched points result from α–1,6 linkages. With β-amylase alone, the amylose samples are about 82 % hydrolyzed, and the amylopectin samples are 59 % hydrolyzed (Nelson, 1995). The properties of amylose and amylopectin are summarized in Table 1.2.

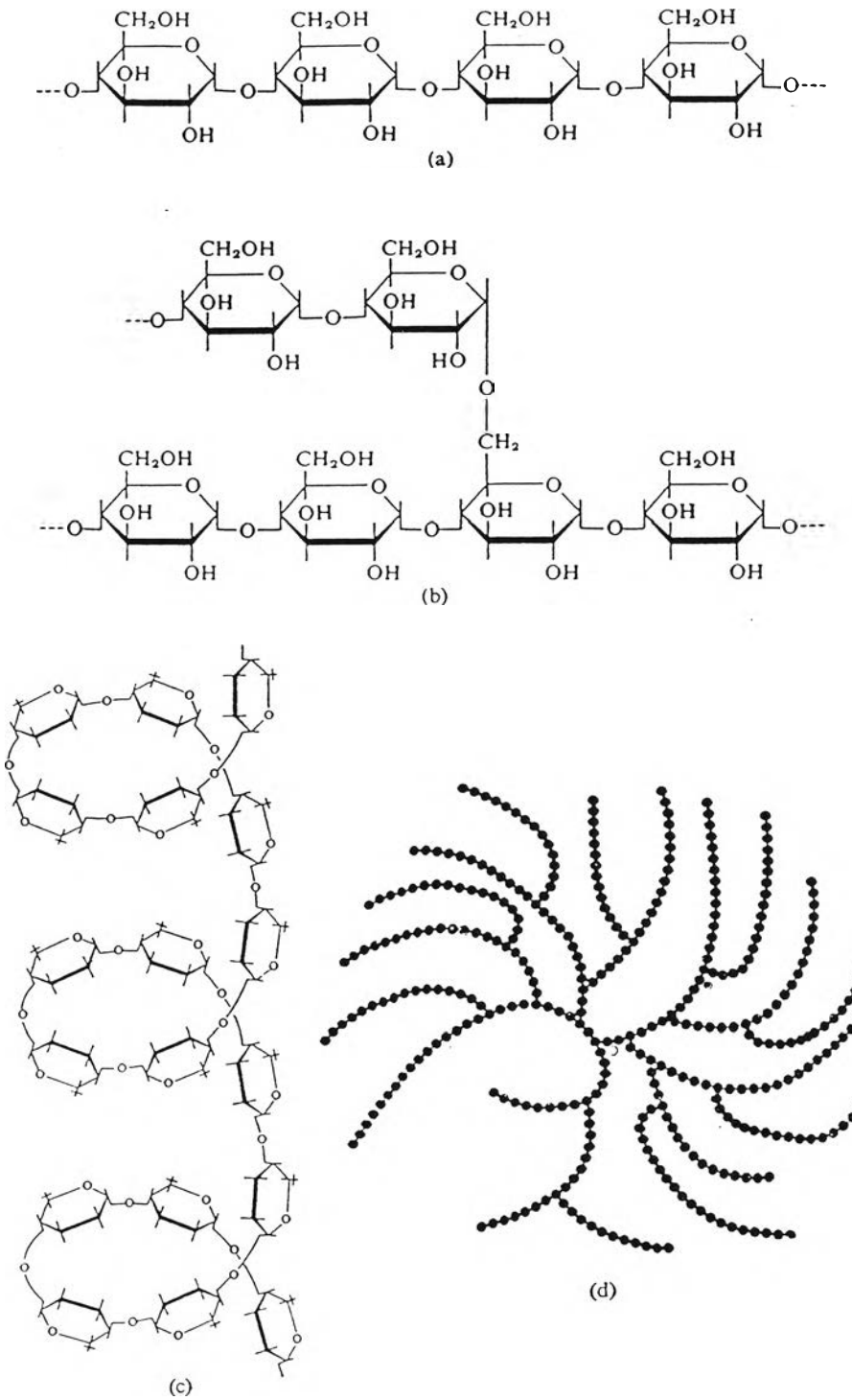


Figure1.2 Structures of amylose and amylopectin

(a) Amylose, showing the mode of linkage of the chain

(b) Amylopectin, showing the branching point

(c) The conformation of the chain in amylose

(d) The branched structure of the amylopectin and glycogen type of molecule (John, 1992 and Dey, 1997).

Table1.2 Properties of the amylose and amylopectin components of starch (Shannon and Garwood, 1983, Rickard, 1991 and Nelson, 1995).

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

The amount of amylose and amylopectin of a number of starches is shown in Table 1.3. Starches from different sources, such as wrinkle pea, corn and waxy maize, contain widely different amounts of amylose. Most native starches contain 20–30 % by weight of amylose and 70–80 % of amylopectin (Salehuzzaman, 1992).

Table1.3 Percent of amylose and amylopectin in reserve plant starch (Young, 1984).

Starch	**Amylose** (%)	**Amylopectin** (%)
Rice	18.5	81.5
Waxy	0	100
Wheat	28	72
Barley	22	78
Waxy	0	100
Oat	27	73
Corn (*Zea mays*)	28	72
Waxy	0.8	99.2
Tapioca	16.7	83.3
Potato	20	80
Sweet potato	17.8	82.2
Pea		
Smooth	35	65
Wrinkled	66	34

1.3 Biosynthesis of starch

Starch is stored in the form of water-insoluble granules in amyloplasts and chloroplasts. The starch biosynthesis is very complex process. There is more to starch synthesis than the polymerization of glucose units. The ratio of two polymeric components, amylose and amylopectin is fixed in each reserve starch plants (Preiss, 1980). Although there is considerable information on the starch synthesizing enzymes, very little is known about the initiation or elongation of their amylose and amylopectin or about the way in which these polymers assemble into starch granules (Ponstein, 1990). The photomicrograph of starch granules in Figure 1.3 shows the different structure of each reserve starch plants (Fitt, 1984).

However, the pathway of starch synthesis has been proposed. It is generally held that starch is synthesized from sucrose. The cytosolic sucrose is converted to hexose phosphate and ADP-glucose, which are then transported to the amyloplasts via hexose phosphate transporters and ADP-glucose transporters, respectively (Figure 1.4). And at least three kinds of enzymes are involved in the biosynthesis of starch;

1. ADP-glucose pyrophosphorylase (AGPase)
   (ATP: G1P-adenyltransferase, EC 2.7.7.27)

2. Starch synthase (SS)
   (ADP-glucose: α–1,4–glucan-4–glucosyltransferase, EC 2.4.1.21)
   - 2.1 Soluble starch synthase (SSS)
   - 2.2 Granule bound starch synthase (GBSS)

3. Starch branching enzyme (SBE)
   (α–1,4–glucan: α–1,4–glucan-6-glucosyltransferase, EC 2.4.1.18)
   (Munyikwa, 1997).

It is generally accepted that the enzyme ADP-glucose pyrophosphorylase (AGPase) is responsible in all plant organs for the synthesis of ADP-glucose from ATP and glucose-1-phosphate (Okita, 1992). ADP-glucose is the primed glucose molecule which functions as the glucosyl donor for glucan synthesis by Starch synthase (SS) and Starch branching enzyme (SBE) (Ponstein, 1990). The enzyme AGPase consists of large and small subunits, which show considerable similarities but can be distinguished by features of their primary amino acid sequences. The sequences of the small subunits are highly conserved between species, whereas those of the large subunits are more divergent (Smith-White, 1992).

The starch synthase (SS), mainly granule bound starch synthase (GBSS), catalyze the conversion of ADP-glucose into amylose through α-1,4-linkage of a ADP glucose to a pre-exiting glucan chain. *In vitro* SS are able to utilize both amylose and amylopectin as substrates. Amylose is produced by SS whereas amylopectin is produced by the combined action of both SS and SBE (Vos-Scheperkeuter, 1989). The synthesis of amylose is not a prerequisite for the formation of amylopectin, therefore, the two pathways are independent (Kram, 1993).

Amylopectin is thought to be formed mainly from the action of Starch branching enzyme (SBE) and Soluble starch synthase (SSS). SBE introduces branch points in the amylopectin molecules by removing chain segment from α–1,4-glucan donor and attached it to an acceptor chain by α–1,6-linkage. The activity of SBE is important for starch quality and quantity. The wrinkled seed pea and the amylose extender are the pea mutant which lack the activity of one of the isoforms of SBE. Consequently the plants are characterized by less branched amylopectin, reduced starch levels, increase amount of sugars, as well as deeply fissured starch grains (Bhattacharyya et al, 1990).

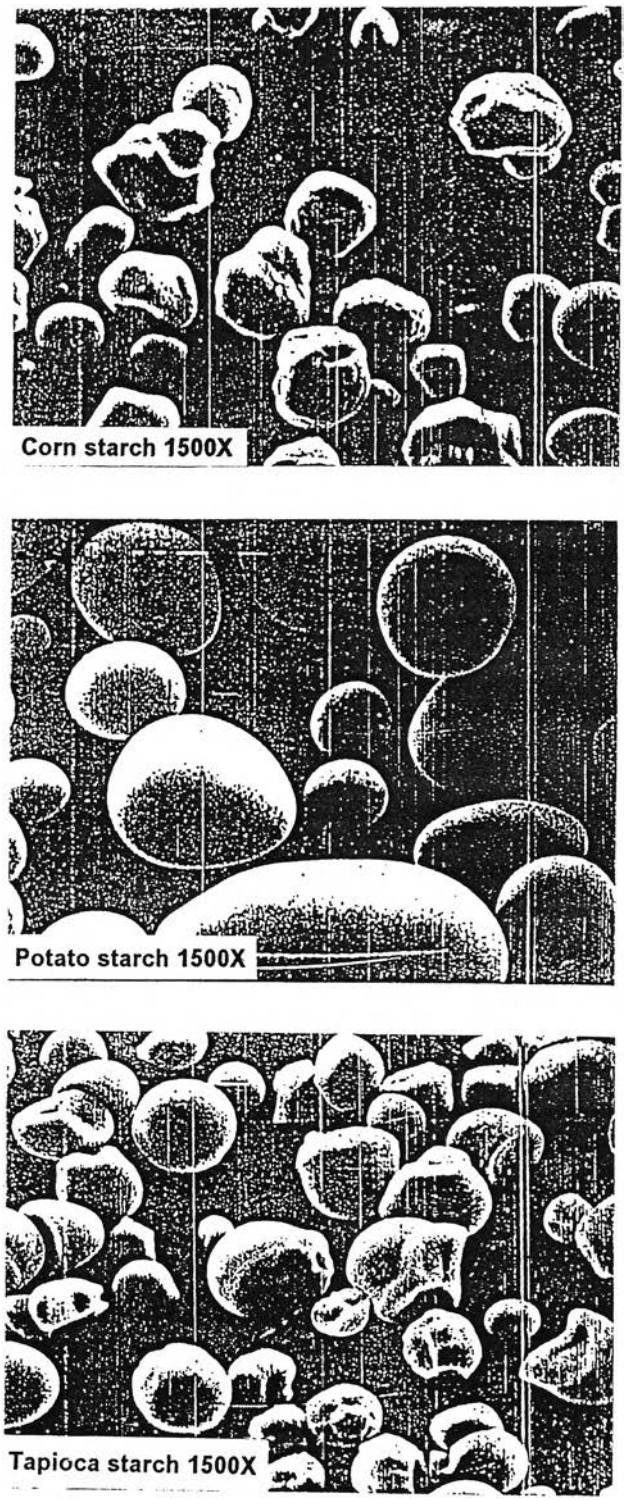


Figure 1.3 Photomicrograph of starch granules from scanning electron microscope (Fitt, 1984).

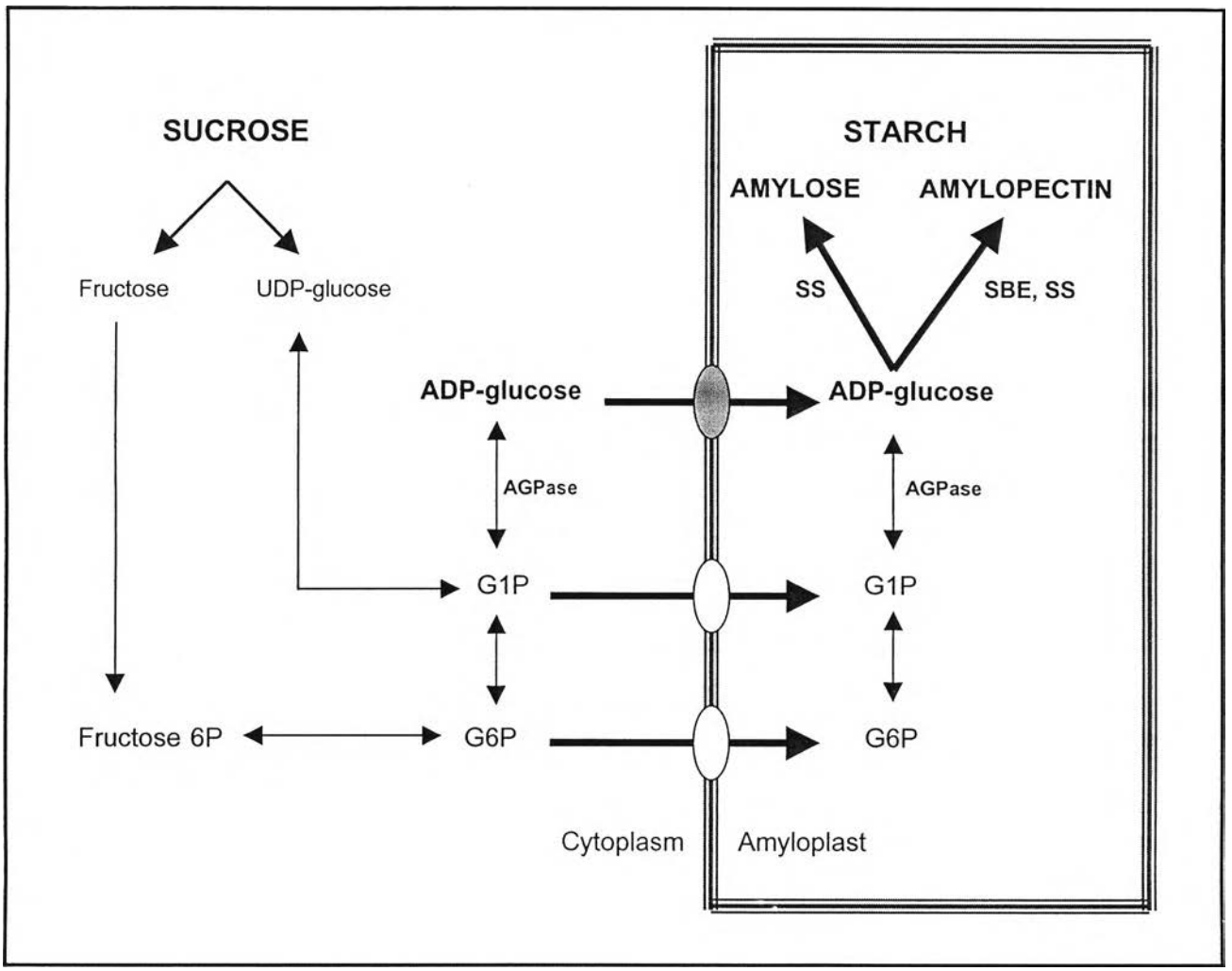


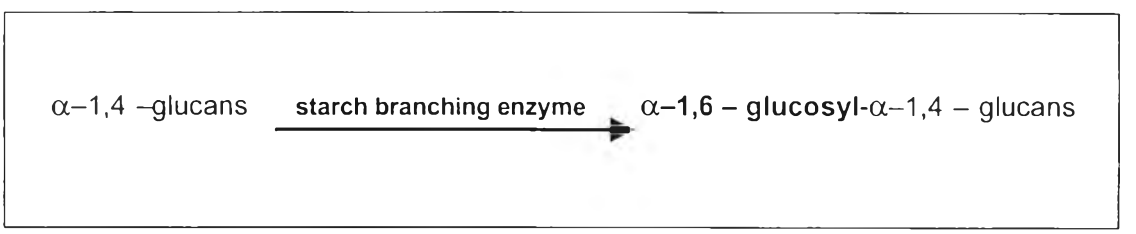
Figure 1.4 Biosynthetic pathways of starch (Adapted from Smith, 1997).

ADP-glucose transporter

Hexose-phosphate transporter

1.4 Starch branching enzyme (SBE)

Starch branching enzyme also called Q-enzyme (α–1,4–glucan: α–1,4–glucan-6-glucosyltransferase, EC 2.4.1.18) introduces branch points in the amylopectin molecules by hydrolysis of the α–1,4–glucan chains at 15-20 units from the non-reducing end. It then catalyses the formation of an α–1,6 cross linkage between the reducing end of the cleaved chain and another glucose residue (Munyikwa, 1997).



Plant branching enzyme (Q-enzyme) was first identified in potato. *In vitro,* the branching enzyme converts amylose into amylopectin-like polysaccharides, which corresponds to native amylopectin in its average chain length, solubility, iodine staining ability and its extent of degradation by β-amylase (Drummond, 1972).

SBE was extracted from spinach leaf and separated on DEAE-cellulose chromatography, resulting in multiple peaks of branching enzyme activity. One of the peaks coincided with one of the starch synthase activity peaks. The molecular weight of the spinach leaf SBE is about 80 kD (Hawker, 1974).

In 1975 Borovsky *et al* purified the SBE from poiato. It was a monomer with a molecular weight of 85 kD. They postulated two possible models for catalytic mechanism of the branching enzyme. In the first model the branching enzyme reacts with the amylose molecule to form a covalent bond with a fragment from the donor chain. The branching enzyme-oligosaccharide

complex then reacts with an acceptor amylose chain to form the branch linkage. In the second model, the two amylose chains form a double helix first, which facilitates the branching enzyme action of transferring a portion of the oligosaccharide chain from one amylose molecule to another. They believed that the chain length requirement of 40 saccharide units for the action of branching enzyme could be explained by the need for a double helix formation (Borovsky, 1975 and 1976).

In 1978 Boyer and Preiss purified the Q-enzyme from developing maize (*Zea mays*) kernels that separated with two major forms after DEAE-cellulose chromatography, BE-I eluted with the column wash and was unassociated with starch synthase activity. BE-II was bound to DEAE-cellulose and wash co-eluted with both primed and unprimed starch synthase activities. Both enzymes were primarily monomeric with a native molecular weight at 70-90 kD. BE-II was separated into two fractions (II-a and II-b) by chromatography on 4-aminobutyl-sepharose. The fractions differed only in the branching of amylopectin, fraction II-b being more active than II-a.

In 1984 Boyer and Fisher purified starch synthase (SS) and branching enzyme (BE) from developing seeds of maize and teosinte. Two fractions of SS and three fractions of BE were obtained by DEAE-cellulose chromatography. BE-I was eluted before the start of KCl gradient, followed by BE-IIb which was co-eluted with SS-I, and BE-IIa co-eluted with SS-II. The BE-I fraction was very active in the branching of amylose, while BE-IIa and BE-IIb were less active. During the course of reactions containing amylose and BE-I, the absorbance maxima of the amylose-iodine complex decreased from 660 nm to 540 nm in 120 minutes, They found that BE's from teosinte and maize showed similar kinetic and chromatographic properties. In addition, antibodies prepared against maize BEs cross-reacted with the teosinte enzyme.

In 1985 Singh and Priess characterized SBE from maize kernel by using polyclonal and monoclonal antibodies. They found no difference between BE-IIa and BE-IIb but a significant difference from BE-I. They suggested that only two forms of starch branching enzyme may be present in maize kernels.

In sorghum, four fractions of branching enzymes were separated from DEAE-cellulose chromatography. One fraction eluted in the buffer wash, the other three co-eluted with the starch synthase (Boyer, 1985).

Activities of SBE from rice (*Oryza sativa* L.) endosperm were observed. Two or three peaks of enzyme activity were resolved by ion-exchange chromatography. Rice branching enzyme had an apparent molecular weight of 40 kD as estimated by gel permeation chromatography (Smyth, 1988).

Purification of the SBE from potato (*Solanum tuberosum*) consists of three steps, precipitation of bulk protein with polyethyleneglycol (Figure 1.5) followed by batch-wise adsorption, desorption on DEAE-cellulose and finally precipitation with ammonium sulphate. These preparations consist of mono and dimeric form of the starch branching enzyme with monomeric molecular weight ranging from 86-103 kD. The k_m value of the enzyme was determined to be 0.02 mg/ml and K_{cat} was in the order of 1000 sec^{-1} using potato amylose as the substrate (Blennow, 1991).

In developing rice endosperm, starch branching enzyme (Q-enzyme) has been purified by ion-exchange chromatography. Two isoforms, QE-I and QE-II have molecular weight of about 80 and 85 kD, respectively. The isoforms are distinct proteins encoded by different genes because antibodies against QE-I showed no immunological cross-reaction with the QE-II protein and anti-QE-II serum did not react with the QE-I protein (Nakamura, 1992).

In rice endosperm, after electrophoresis on a native polyacrylamide gel and followed by activity staining, the QE-II fraction was found to be composed of two isoforms, QE-IIa and QE-IIb. And QE-IIa was detected only in the extract of endosperm, whereas QE-IIb was present in extracts of all tissues examined (Yamanouchi, 1992).

A cDNA coding for cassava (*Manihot esculenta* Crantz) branching enzyme was cloned from a λgt11 cDNA library using a potato cDNA probe. Southern analysis suggested that there is a single gene for this particular branching enzyme in the cassava genome and Northern hybridization showed that the gene is highly expressed in tubers. The transcript is detectable in the stem and petiole, but not in the leaves. The expression levels at different stages of tuber growth are similar with exception of very young tubers in which it is relatively low. And there is a difference in the level of branching enzyme expression between cassava genotypes (Salehuzzaman, 1992).

Three forms of SBE in maize endosperm have been identified and characterized in term of their level of branching activity on amylose and amylopectin and by immunological properties. And the second form of SBE has cloned, which the deduced molecular mass of the mature protein is 84,772 D that larger than size estimates of 80,000 D based upon SDS-PAGE analysis (Fisher, 1993).

BE isoforms from maize (*Zea mays*) endosperm properties were characterized by three distinct assays, phosphorylase a stimulation assay, BE linkage assay, and iodine stain assay. This study presents the first evidence that the BE isoforms differ in their action on amylopectin. BE-I showed the highest activity in branching amylose, but its rate of branching amylopectin was less than 5% of that of branching amylose. Conversely, BE-II isoforms had lower rate in branching amylose and had higher rates of branching amylopectin than BE-I. (Guan, 1993)

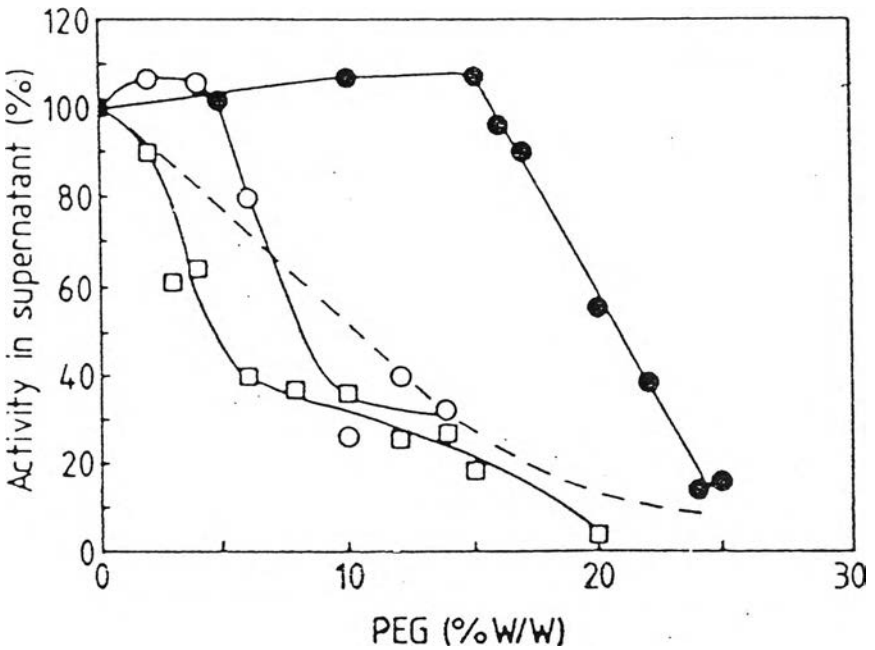


Figure 1.5 Precipitation curves of proteins in potato juice with polyethyleneglycol (Blennow, 1991).
-----Protein; ---●--- Starch branching enzyme;
---□--- Starch synthase; ---o--- α-amylase.

In conventional assays based on stimulation of unprimed phosphorylase activity, the specific activities of maize were BE-IIb > BE-IIa > BE-I. However from the assay method with reduced amylose as the substrate which is based on determination of reducing power of the chains transferred by BE after they are released from the branched products with isoamylase, the specific activity found to be the highest for BE-I and the lowest for BE-IIb. The optimum pH of the three enzymes is 7.5, the optimum temperatures of BE-I, BE-IIa and BE-IIb are 33, 25, and 15-20 ^{o}C respectively. BE-I has a lower k_m (2 μM of the nonreducing terminal) for the reduced amylose of average chain-length (cl) 405 than BE-IIa (10 μM) and BE-IIb (11 μM), and BE-I preferentially transfers longer chains than BE-IIa and IIb (Takeda, 1993).

The two isoforms of starch branching enzyme (SBEI and SBEII) have been isolated from pea embryos. The amylopectin products of the enzymes from these two families are qualitatively different. Pea SBEI and SBEII are differentially expressed during embryo development. SBEI is relatively highly expressed in young embryos whilst maximum expression of SBEII occurs in older embryos (Burton, 1995).

In 1996 Larsson *et al* extracted proteins from tuber starch of potato and separated by SDS gel electrophoresis. For the specific protein bands were digested to produced peptides and then separated on reversed phase chromatography and finally sequencing. The data showed that three isoforms of starch synthase and two isoforms of branching enzyme were present in the starch of potato tuber.

Maize branching enzyme (BE) was significantly inactivated by phenylglyoxal (PGO) in triethanolamine buffer pH 8.5. The inactivation followed a time- and concentration-dependent manner and showed pseudo first-order kinetics. The loss of BE activity results from as few as one arginine residue modified by PGO. And BE inactivation was positively correlated with [^{14} C] PGO incorporation into BE protein and was considerably protected by amylose and/or amylopectin. The modified arginine residue may be involved in substrate binding or located near the substrate-binding sites of maize branching enzymes I and II (Cao, 1996).

In maize, mutations affecting the filling of the grain are easily recognized by the altered appearance of the grain. The particular destination of the mutant variety indicates it phenotypic appearance, for example *brittle* and *dull*. The mutant variety *waxy* acquired its name from the shiny appearance of the cut surface of the endosperm. It looked as though it contained wax, although of course the appearance was due not to wax but to an altered composition of the starch. The high amylose varieties, which set

out to increase the proportion of the linear polymer, like other natural straight-chain polymers such as cellulose, could form films and fibers with potential industrial applications. Usually the endosperm of the mutant varieties is smaller than normal and contains less starch. *Shrunken-1* has a lower proportion of amylose, and *amylose extender*, a greater proportion of amylose than normal. *Waxy* has no amylose at all. In *amylose extender* the mutation affects the level of one of the isoforms of the branching enzyme. The other forms of the branching enzyme and the starch synthase are unaffected. This is consistent with the observed low percentage of amylopectin and the high average chain length of the amylopectin that is present. In many of the mutant endosperms enzymes deficiencies that lead to the particular altered condition have been identified (Figure 1.6) (John, 1992).

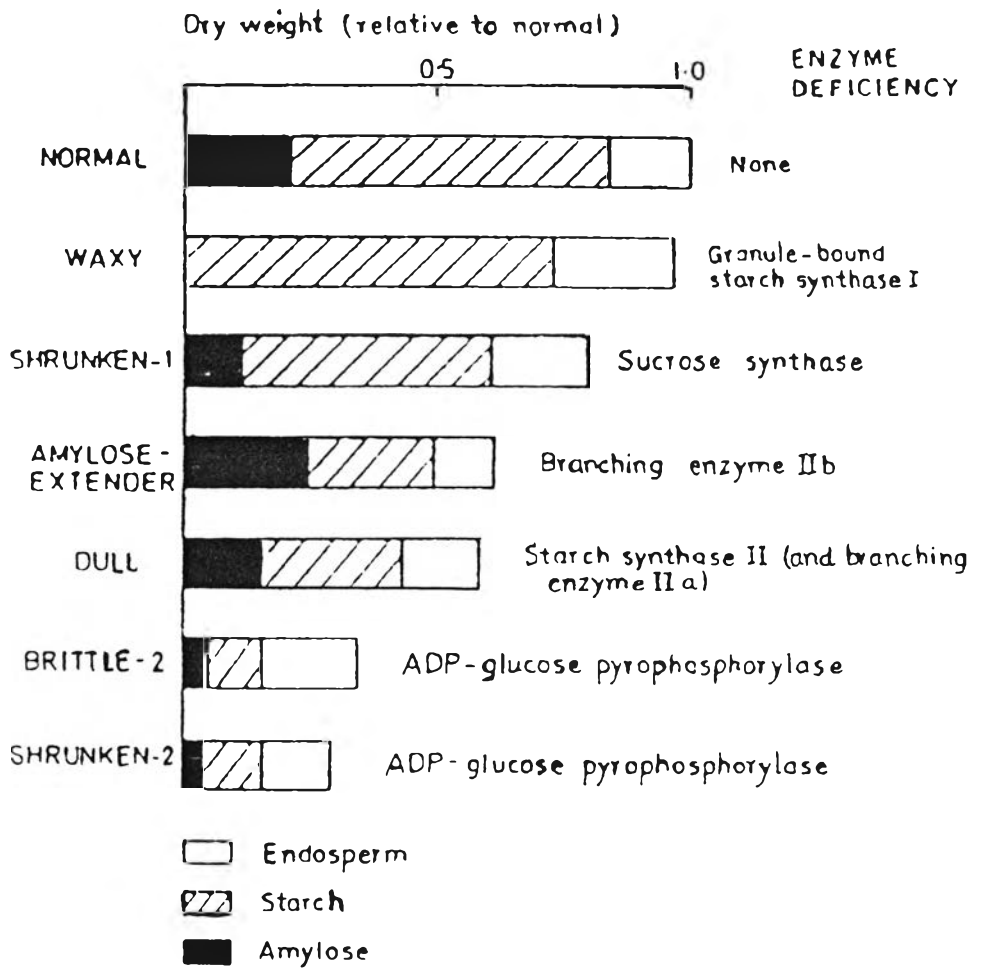


Figure 1.6 Relative sizes, starch and amylose compositions of the mature endosperms of various genotypes of maize (John, 1992).

1.5 Aim of thesis

Although cassava has entered the modern market economy, being used as food, feed products and other industrial products, its market price has been very low. It is, therefore, essential to add value to the crops by improving the quality of its major product i.e. cassava starch. The quality of starch is the result of interaction between starch synthase and starch branching enzyme, since the characteristics of the starch, in term of texture, viscosity, stability are all strongly influenced by the relative proportion and molecular size of amylose and amylopectin fractions. It is, therefore, of our interest to understand the characteristics of these enzymes in cassava in order to be able to apply the knowledge to improve the quality of cassava.

This thesis is, therefore, aiming at purification of starch branching enzyme from cassava tuber and characterize some of its properties.