CHAPTER IV

DISCUSSION

Starch is the major component of the products of many economically important crop plants. It is also important raw material for many industrial processes. Its applications depend on starch quality especially its compositions of amylose and amylopectin. Genetic manipulation of these plants has been successful in many cases to increase yield and provide novel raw materials by alteration of starch synthesis pathway (Miller-Rober, 1994 and Wasseman, 1995). Improved understanding of the enzyme involved in starch synthesis would provide regulation of the process resulting in production of starch raw material of the desired properties. Several studies on the enzymes involving in starch synthesis such as ADP pyrophosphorylase, starch synthase and starch branching enzyme have been performed in many crop plants but little has been done in cassava. Our group aims at achieving a better understanding of the starch synthesizing enzymes in cassava. This research focusses on the starch branching enzyme.

4.1 Assay method for SBE

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

Method I : Decrease of absorption of the amylose-iodine complex

Mixture in a total volume of 200 µl containing 100 mM sodium citrate, pH 7.0, 0.1 % of amylose and branching enzyme. The reaction was incubated at 30 ^{O}C for 15 min, the reaction was stopped by mixed with 1.0 ml of iodine reagent. The absorbance was determined at 680 nm. One unit of enzyme activity is defined as the decrease of one absorbance unit at 680 nm per min at 30 ^{O}C (Borovsky,1975).

Method II : Stimulation of an α-glucan synthesis from α-D-glucose-1-phosphate catalyzed by rabbit-muscle phosphorylase *a*

The reaction was conducted in a mixture (0.2 ml) containing 0.1 M sodium citrate, pH 7.0, 50 mM α-D-[^{14}C]-glucose-1-phosphate, 1 mM AMP, 10 μg of rabbit-muscle phosphorylase a and an appreciate amount of enzyme. After incubation at 30 ^{O}C for 60 min, the mixture was boiled for 3 min to terminate the enzyme reaction. To the mixture, 20 μl of glycogen solution (10 mg/ml) was added to co-precipitate the newly formed glucan with methanol. The amount of the radioactive, methanol-insoluble material was measured using a liquid scintillation counter. One unit of enzyme activity was defined as 1 mmol of D-[^{14}C]-glucose incorporation from α-D-[^{14}C]-glucose-1-phosphate into the methanol-insoluble material per min under the condition used (Mizuno, 1993).

From preliminary test comparing of the above methods, it was concluded that radioactive assay was more suitable. Although it was more tedious and more expensive. This method was more sensitive and more direct than the I_2 method decrease in absorbance of amylose remaining may give false result from other starch hydrolyzing enzyme and the measurement of the products was indirect measurement.

4.2 Purification of SBE

Several steps were employed to purify SBE from cassava tuber. The first step of purification involved the use of polyethyleneglycol 6000 (PEG_{6000}) to precipitate other contaminating proteins especially amylase and starch synthase. Blennow used the PEG precipitation in purifying SBE from potato tuber. The SBE was purified five times with full activity in a single step by addition of PEG to 15 % (w/w) precipitating other proteins. In this step most of the α-amylase and starch synthase were removed (Blennow, 1991).

In our preliminary experiment, it was found that 10 % PEG removed significant amount of other proteins (80 %) while SBE was retained. And the total SBE activity was shown to increase by 20 % after this step (Table 3.1). This may be due to partial removal of the amylase, which may interfere by hydrolyzing the products of SBE on the assay of crude enzyme, or the removal of some SBE inhibitory factors in the crude enzyme.

Two successive column chromatography steps running on ion exchange resin, DEAE-cellulose and Q-Sepharose, produced SBE which was 25.4 folds purer but a loss of 60 % of SBE activity. Pooled fractions from the DEAE-cellulose column gave a large in volume; therefore, it was concentrated by 80 % ammonium sulfate precipitation and redissolved to the desired volume. Pooled fractions from Q-Sepharose gave a smaller volume; therefore, concentration was performed using aguasorb, which removed water from the sample.

Sephadex G-200 seemed to be most efficient in all purification process employed in term of purity of SBE but a great amount of enzyme was lost. Analysis of the purified SBE from Sephadex G-200 showed a single band on non-denaturing or SDS polyacrylamide gel electrophoresis. However activity stain on non-denaturing starch-PAGE with I_2 showed a smear of yellowish stain on top of the gel especially in the preparation from Q-Sepharose column. This may be caused by the amylolytic activity of the SBE itself, which can cut α-1,4 polysaccharide chain before attaching it to an acceptor as a branching point with α-1,6 bonds. The activity may occur primarily during the electrophoretic run, during which movement of the protein was too fast to allow the α-1,6 bonding to occur. However, this kind of activity should appear as continuous smear. The SBE band itself appeared as reddish brown band when incubated for further 4 hours which allowed complete branching activity (formation of α-1,6 linkage) to occur. On the other hand, it may be the activity

of contaminating amylase which was not completely removed by 10 % PEG_{6000}. However, this may only partially contribute to the effect since protein bands were not detectable at the position where the yellowish stain appeared.

4.3 Characterization of SBE

The purified SBE was further characterized. Molecular weight determination of the enzyme by Sephadex G-200 showed its molecular weight of 160 kD. However, SDS-PAGE analysis showed SBE molecular weight to be 80 kD. Since, SDS-gel produced only single band of SBE from the Sephadex G-200 preparation, it may be concluded that SBE in cassava tuber consists of 2 identical subunits with molecular weight of 80 kD.

Determination of isoelectric point of SBE on IEF gel in comparison of pI standard proteins revealed one sharp band of protein with faint smear band closed to the acidic zone. This may be caused by the aggregation of proteins resulted from excessive concentrations of the sample before application to IEF gel. No pI value has been reported for other starch branching enzymes from other sources studied so far.

The enzyme activity was maximum when assayed at pH 7.0 and the most suitable temperature for assay SBE activity is 37 ^{O}C (Figures 3.8 and 3.9). However, the enzyme has been assayed at 30 ^{O}C from the start of this work and its activity was not much lower than at 37 ^{O}C (about 90 % of 37 ^{O}C). Therefore the enzyme was assayed at 30 ^{O}C in all other experiments.

Since SBE functions by removing chain segment from α–1,4-glucan donor and attached it to an acceptor chain by α–1,6-linkage. In our assay system the donor chain and/or acceptor chain of α–1,4-glucan was produced

by phosphorylase a with its substrate, labeled glucose-1-phosphate which was added in the substrate mixture for SBE assay method (Figure 4.1).

It was speculated that building labeled α–1,4-glucans from ^{14}C G1P by the action of phosphorylase a may take time and provide limited amount of substrate for SBE. Providing α–1,4- polysaccharides of suitable chain length may enhance SBE activity. Experiments adding glycans of different sizes and structures into the reaction mixture of SBE show that the presence of large molecule carbohydrates like starch and glycogen greatly enhanced SBE activity (Table 3.2). Smaller branched polysaccharides like amylopectin and dextrin also enhanced SBE activity but to a smaller extent. Short chain oligosaccharides like pentose and maltose or cyclic oligosaccharides like α or β cyclodextrins showed no effect. Long linear polysaccharide like amylose also showed little effect. These observations may be explained as follow :-

1. The added glycans immediately acted as polysaccharide chains of suitable chain length for phosphorylase a to add ^{14}C glucose unit from ^{14}C G1P which subsequently acted as substrate for SBE.

2. The glycans help in stabilizing the labeled products of phosphorylase a, preventing them from reverse reactions of phosphorylase a, since reverse reaction of phosphorylase a would reduce the amount of labeled polysaccharide for SBE branching reaction.

3. The branching activity of SBE preferred pre-exiting branching in the substrates.

The first two hypotheses were supported by the finding that similar experiments where glycans were added but SBE were omitted resulted in

increase labeled precipitates in the same pattern as when SBE were included but to a lesser extent (Table 4.1, column 1)

The third hypotheses was supported by the further in increase in radioactive products when SBE was included. Therefore, it seemed that all three proposed hypotheses may all play roles in effect of the glycans on the increase in SBE activity.

Table 4.1 Effects of different Glycans on phosphorylase a and SBE activities.

Glycans	1 phosphorylase a (CPM)	2 phosphorylase a + SBE (CPM)	3 2-1 (CPM)
G1P (control)	566	4,667	4,101
G1P + Maltose	742	3,594	2,852
G1P + Pentose	519	4,107	3,588
G1P + α-Cyclodextrin	342	4,415	4,073
G1P + β-Cyclodextrin	368	4,364	3,996
G1P + Amylose	3,337	7,848	4,511
G1P + Dextrin	3,822	11,755	7,933
G1P + Amylopectin	3,998	12,230	8,232
G1P + Glycogen	11,162	20,995	9,833
G1P + Starch	12,843	36,013	23,170

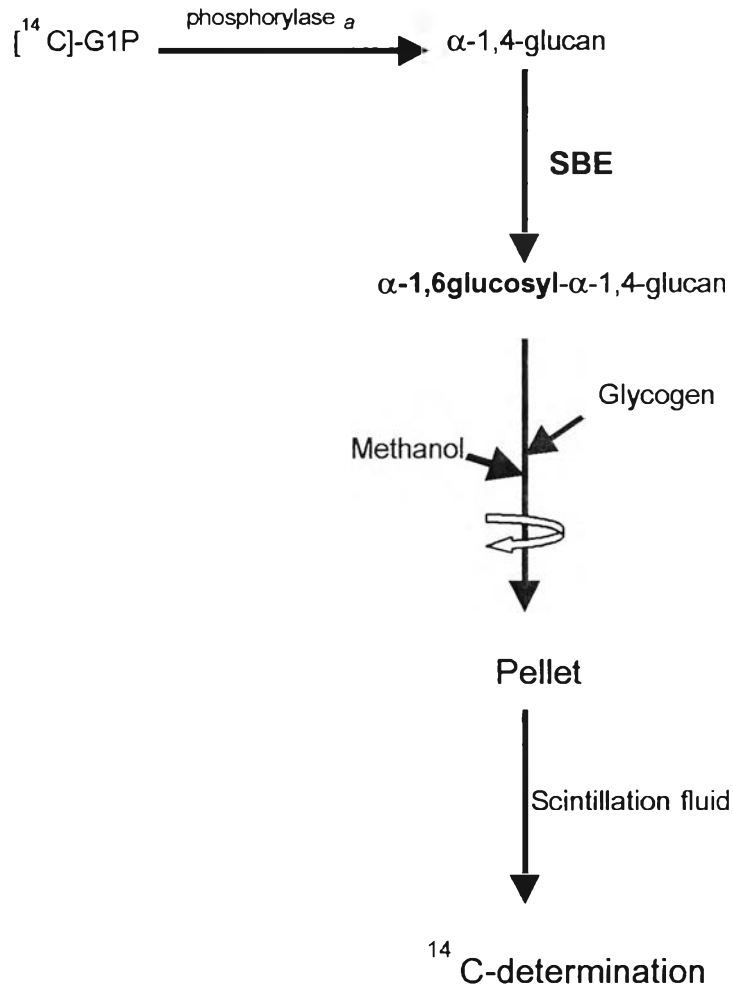

Figure 4.1 Assay of SBE activity.

On the other hand, it may be speculated that the presence of these glycans in the reaction mixture may facilitate the precipitation of radioactive products by methanol after the reaction was stopped. As mentioned in section 2.13, glycogen (10 mg/ml) was added after SBE reaction was stopped to act as carrier for the radioactive products, facilitating precipitation of the products by methanol (Figure 4.1). An experiment was carried out adding the series of glycans that produced effect on SBE activity into the reaction mixture after the SBE activity was stopped to use these glycans as carrier in place of glycogen. It was found that adding these glycans after the SBE activity was stopped (Table 3.3) did not result in higher radioactive products in the precipitate. Therefore it can be concluded that the effect of the glycans added at the start of the reaction was due to their effects on the SBE reaction itself. The precipitation only required any branched glycans to be the carrier for product precipitation regardless of their sizes or structures.

Characteristics of SBE from cassava tuber are compared with SBE purified from other sources as summarized in Table 4.2. All of SBE from other plants were reported to have isoforms of SBE while our study on cassava tuber has only one form. Most isoforms in different plants have their molecular weight determined by SDS-PAGE within the same range (70-90 kD). It was speculated that isoforms of SBE may function to create chain of different lengths or branch points at different frequencies (Smith *et al*, 1997). However, there is no strong supporting evidence for such hypothesis. In our finding, only one form of branching enzyme was found by the purification and monitoring methods employed. However, there were no reports on the existence of dimers in any SBE studied so far. When cassava SBE were chromatographed on Sephadex G-200, its native molecular weight was twice that determined by denaturing SDS-PAGE, indicating existence of SBE as dimer of 2 identical subunits.

Table 4.2 Comparative properties of starch branching enzymes from various starch reserve plants.

Properties	Rice	Wheat	Maize	Potato	Cassava
Isoforms	QE-I QE-II	WBE-I WBE-II	BE-I BE-IIa BE-IIb	SBE-I SBE-II	SBE
Mw (kD)*	QE-I 40 QE-II 85	WBE-I 88 WBE-II 80	70-90	SBE-I 95 SBE-II -	80
pI	-	-	-	-	5.4
pH optimum	-	-	-	7.5	7.0
Optimum temperature (^{o}C)	-	-	BE-I 33 ^{o}C BE-IIa 25 ^{o}C BE-IIb 15-20 ^{o}C	-	37 ^{o}C
References	Nakamura 1992	Morell 1997	Takeda 1993	Blennow 1991	-

* The value determined by SDS-PAGE

4.4 Further study of cassava SBE

More detailed characterization of cassava SBE should be carried out including kinetic study, peptide sequencing, probing for the gene coding for SBE. These information together with data of other starch synthesizing enzymes including starch synthase of cassava tuber would be useful in the regulation of the starch synthesis pathway to produce cassava plant with improved starch quality.