CHAPTER IV

DISCUSSION

DBE is one of the enzymes that is involved in cassava starch biosynthesis. The properties of DBE in many plants were studied and its role in process of starch biosynthesis was proposed. The important role of DBE is defined as involvement in determining the fine structure of granule and removal of soluble glucans produced by SS or SBE to decrease substrate that can be reduce the activity of SS and SBE in starch biosynthesis (Tetlow et al., 2004). In cassava, the most important food crop, the information of DBE was still lacking. For more information and the understanding of cassava starch biosynthesis, this thesis was concentrated on the characterization of DBE.

4.1 Assay methods for DBE

There are two groups of DBE exist in plant: isoamylase and pullulanase which efficiently hydrolyze α(1-6) linkages in different substrates. Pullulanase debranches pullulan and amylopectin but not glycogen, whereas isoamylase debranches both amylopectin and glycogen but not pullulan. Pullulanase generates maltosyl groups, while isoamylase releases maltotriosyl and large oligosaccharides. Therefore, the activity of DBE can be assayed by measuring the amount of reducing sugar that was released from the reaction with dinitrosalicylic acid reagent (Bernfeld, 1995). Pullulan was used as substrate for assay pullulanase activity because pullulan is very specific substrate that no other enzyme can hydrolyze. For ISA assay, amylopectin was use as substrate although it can be substrate of other enzymes such as SBE or amylase because in several studies of isoamylase, amylopectin was used as substrate such as in

maize, chlamydomonas, potato and cassava (Beatty et al., 1999; Dauvillee et al., 2001; Hassain et al., 2003; Rahman et al., 1998; Sinlapawisut, 2004). Both types of DBE can be purified and separated from cassava tuber cv. KU50. Their activities were confirmed again by running on native PAGE containing soluble starch or amylopectin. The activity band of isoamylase and pullulanase appeared as colorless and blue band, respectively (Dinges et al., 2003; Zeeman et al., 1998). The band pattern of DBE that appeared on soluble starch and amylopectin native PAGE was the same because isoamylase and pullulanase can hydrolyze both starch and amylopectin as shown in the results from the study of substrate specificity (Table 3.5). However, sharper and higher intensity of activity band was observed in amylopectin native PAGE because isoamylase and pullulanase were more specific towards amylopectin more than soluble starch. On soluble starch native PAGE, other amylolytic enzymes which can hydrolyze starch also showed colorless minor bands.

4.2 Preparation of crude extraction

Parenchyma of cassava tubers harvested at Rayong Field Crop was used to extract DBE. DBE and other starch synthesizing enzymes such as SS, SBE and D-enzyme were detected in this part of tubers but not detected in the cortex (Sinlapawisut, 2004; Yaiyen, 2003; Pao-in, 2006). DBE from 2 cultivars, Rayong1and KU50, were compared for the activity during development. Rayong1, the original cultivar, showed lower DBE activity than KU50, a developed strain which produces more starch. When DBE activity was monitored between 3-12 months, DBE highest activity (both isoamylase and pullulanase) was attended 9 months which correlated with SS and SBE from the same cultivar (Poa-in, 2006 and Yaiyen, 2003). However,

significant increase in pullulanase was observed during 3-12 months while isoamylase was observed from the beginning and the change in enzyme activity during 3-12 months was not drastic. During the first six months, cassava tubers were in the stage of development. Energy essential for metabolism was provided by the starch being synthesized in the tubers. Isoamylase contributes more in hydrolyzing starch to small sugar molecules for use in metabolism because its specificity towards carbohydrates was wider. After six months development, cassava development may have reached near mature stage where starch storage may become more active. Pullulanase has a limited specificity towards pullulan-type structure and is more important in trimming of branches in amylopectin for starch granule formation. This may explain the more drastic increase in pullulanase between 6-9 months in Rayong1. In KU50, the high starch cultivar, pullulanase was observed from early development. The observed developmental pattern of pullulanase was well correlated to starch synthase and starch branching enzyme, the other two enzymes involved in starch biosynthesis (Yaiyen, 2003 and Pao-in, 2006). Sinlapawisut (2004) reported pullulanase in cassava tuber in five minutes cultivar. Isoamylase was also detected but in the same peak as pullulanase in the chromatographic profiles on DEAE-Sepharose and Sephadex G-150. No separate peak or activity band on native PAGE was observed. Hussian and colleague (2003) described this hypothesis on isoamylase like protein (*ISA1*, *ISA2* and *ISA3*) of *Arabidopsis*, *ISA1* and *ISA2* were essential in starch synthesis and *ISA3* presumably might be important in starch degradation during metabolizing of transitory starch. The specific activity of isoamylase was higher than pullulanase. Part of the enzyme activity detected may be contributed by other amylolytic enzymes that hydrolyze amylopectin present in the crude extract of the tuber, including pullulanase.

That is why isoamylase activity was also present in the chromatographic profile of pullulanase. Assay of pullulanase was not interfered by the presence of other amylolytic enzyme.

4.3 Purification of DBE

From about 1.5 kilograms of parenchyma of 9 months old cassava tuber, 250 ml crude extract and 358 mg protein were obtained. In the assay of DBE in crude enzyme, there were many contaminating enzymes and reducing sugars in enzyme solution. The total isoamylase activity obtained was 67.5 units and pullulanase activity was 15 units. Ammonium sulfate precipitation in the next step eliminated many other enzymes and some starch or reducing sugars. After precipitation proteins were dissolved in starting buffer, about 60% of proteins was lost in this step. The specific activity of isoamylase and pullulanase decreased to 0.2 and 0.5 fold, respectively. It was possible that ammonium sulfate removed other contaminated hydrolytic enzymes which could also act on the substrate or product of the reactions (Beatty et al., 1999). Enzyme preparation from this step was run on soluble starch and amylopectin native PAGE. There were two bands of DBE activity, the blue band of pullulanase and colorless band of isoamylase. The red brown band that appeared below blue band in land 1 of crude enzyme was starch branching enzyme activity band.

Enzyme was loaded on DEAE-Sepharose to separate isoamylase and pullulanase by ionic charges on proteins. We eluted bound protein which contained isoamylase and pullulanase activity by linear gradient of 0-0.3 M NaCl in starting buffer. The remaining proteins bound to the column was eluted by 0.5M NaCl in starting buffer. This step eliminated significant amount of proteins. DBE should have

pI less than 7.5 because in buffer pH 7.5 these enzymes show negative charges and bound to DEAE-Sepharose. Both pooled fractions were run on native PAGE, isoamylase showed one colorless band while pullulanse showed blue band and three colorless minor bands, indicated some contamination of isoamylase and other amylolytic enzymes.

Each pooled peak was separately loaded on gel filtration column. From chromatograms of isoamylase and pullulanase showed the protein was different by size and activities. Although the specific activity of both enzymes increased, the amount of proteins remained were very small. On native PAGE, pullulanase showd one blue band and ISA showed one colorless band.

4.4 Characterization of DBE

4.4.1 Molecular weight of DBE

Native molecular weight of isoamylase was 98 kDa determined by gel filtration on Sephacryl S-200 column and its calibration curve (Figure 3.7). On SDS-PAGE, isoamylase showed 2 major protein bands at 41 and 34 kDa, the 34 kDa band with higher intensity. Therefore, the isoamylase may consist of one 41 kDa and two 34 kDa subunits. Molecular weight of pullulanase determined from gel filtration was 175 kDa, whereas on SDS-PAGE showed 3 major bands of 54, 46 and 41 kDa. From the band intensity observed on SDS-PAGE, it may be speculated that pullulanase may contain 4 subunits, one each of 54, 46 kDa and two of 41 kDa. The molecular weight determination of DBE by different method gave the different results. The molecular weight of DBE reported in other plants were quite different (Tables 4.1 and 4.2). Molecular weight of pullulanase from endosperms were in the range 90-110 kDa on

gel filtration while molecular weight of pullulanase in rice and maize were in range of 58-70 kDa. Molecular weight of isoamylases reported were in range 75-520 kDa. However, in those reports, there were no mention on subunit compositions. Sinlapawisut (2004) reported the molecular weight of pullulanase from cassava cultivar five minutes to be 103 kDa, with 3 subunits of 35 kDa.

4.4.2 Effects of pH and temperature on DBE

Isoamylase and pullulanase were incubated at various pH and assayed for their activities. Both enzymes were most active at pH 6.0. The activity of pullulanase decreased about 50 % at pH higher than 8.0 and lower than 5.0. Pullulanase was active in range of pH 5.0-8.0. Isoamylase activity was active in the pH range of 4.5-9.0 and its activity decreased at pH higher than 9.0. Similar optimum pH's of DBE were reported in other plants DBE (Tables 4.1 and 4.2).

When DBE was assayed by incubation in various temperatures, ISA showed its highest activity at 70°C while pullulanase showed optimum temperature at 50°C. When activity stain of both enzyme were performed on native PAGE at the observed optimum temperatures, activity bands were more intense than incubation at 37°C as usually performed in other reports (Zhu et al., 1998; Li et al., 1992 and Ludwig, 1984). However, activity staining of the gel was performed only at 50°C because pullulanase activity was lost at 70°C. When DBE's thermal stability was studied by incubation up to 48 hr at 4-70°C, isoamylase still maintained 80% of its activity at temperature range 4-37°C. At optimum temperature, its activity was completely lost in 12 hr. Pullulanase was not stable at temperature higher than 37°C. It lost activity

completely in 12 hr at temperature higher than 50°C. Therefore, DBE should be kept at 4-37°C and performed at optimum temperature.

The optimum temperatures of both isoamylase and pullulanase from cassava tubers in this report were higher than other plants which were be in range 30-37°C (Rahman, 1998 and Sinlapawisut, 2004). This may result from the effect of Ca^{2+} in buffer on properties of enzyme as described by Li and colleague (1992). They found that 1 mM concentration Ca^{2+} can enhance endoamylase activity and the ability of endoamylase to degrade starch grains persisted even after it was heated at 70°C for 5 minutes.

4.4.3 Effects of sulfhydryl reagents and divalent metal ions on DBE

DTT, GSH and β-mercaptoethanol activated pullulalnase to increase activity 4-5 folds while NEM and IAA caused a decrease in pullulanase activity. In this experiment, 10 mM DTT can activate activity of pullulanase up to 546% while the same concentration of GSH and β-mercaptoethanol resulted only 236 and 440% activation. Ludwig and colleague (1984) also reported greater stimulation effect on spinach leaf debranching enzyme by dithiols more than monothiols. DTT is a preferred reagent for reducing disulphide bonds because of its low redox potential which ensures rapid and complete reaction (Dawson et al., 1986). It contains 2 reactive –SH groups in molecule and more stable compare to β-mercaptoethanol and GSH which contain one –SH group and less stable. NEM and IAA, are penetrating, irreversibly sulfhydryl modifying reagents, showed strong in inhibitory effect. The effect of these sulfhydryl reagents on isoamylase activity was similar to pullulanase but GSH activated ISA more than DTT. In addition, NEM and IAA inhibited ISA activity less than pullulanase. These results suggested that pullulanase contained

sulfhydryl groups that are necessary for enhancing its higher activity. Isoamylase may contain sulfhydryl groups which did not directly effect its activity because dithiols and monothiols increase its activity while IAA and NEM had small effect. Rahman and colleague (1998) reported that sulfhydryl reagents did not require for activity of SU1 (isoamylase from maize gene *sugary1*) but the maintenance of enzyme stability required the addition of 5 mM DTT.

Metal ions have been reported to effect activities of many enzymes by either activating or inhibiting the activities. In our experiment, Mn^{2+} and Co^{2+} were potent activator for pullulanase while Cu^{2+} and Ni^{2+} were strong inhibitor. On the other hand, only Co^{2+} activated isoamylase. Cu^{2+} also inhibited isoamylase activity like in pullulanase. Cu^{2+}, Zn^{2+}, Ag^{2+} and Cd^{2+} at concentration of 1 mM were also inhibitory to rice endosperm debranching enzyme (Iwaki et al., 1981). Rahman and colleague (1998) reported that cations were not required for activity of SU1. Enzymatic reactions of SU1 were conducted routinely in buffers devoid of divalent cations, the addition of 10 mM calcium or magnesium ions to the buffer did not alter its activity. The pullulanase from cassava tuber cultivar five minutes also was activated by Mn^{2+} and Co^{2+} whereas Cu^{2+} and Ni^{2+} were potent inhibitors of pullulanase activity (Sinlapawisut, 2004).

4.4.4 Kinetic constants of DBE and substrate specificity.

When partially purified pullulanase and isoamylase from Sephacryl S-200 column were used to study the substrate specificity towards various substrates, pullulanase was found to be very specific on pullulan but can also hydrolyze α-1,6 branched glucan in amylopectin, soluble starch and amylose. Soluble starch contains amylopectin and amylose. Amylose was little hydrolyzed by pullulanase, due to its

unbranched structure. The chemical position of glycogen is similar to that of amylopectin although their structures and physical-chemical properties are distinctly different. The structure of glycogen may be more compact than amylopectin resulting in less access of pullulanase to branched glucan in glycogen. Isoamylase can hydrolyze all polysaccharides with α-1,6 branched glucan. Isoamylase can hydrolyze amylopectin better than other polysaccharide, suggesting that its structure was more suitable for interaction with isoamylase. Amylose also has small amount of α-1,6 branched so isoamylase can hydrolyze and gave reducing sugars. The high isoamylase activity towards amylase may be partially contributed from the contamination of starch degrading enzymes in the enzyme preparation.

Pullulan and amylopectin were employed as substrates for kinetic studies of pullulanase and isoamylase. In case of pullulanase, the K_m for pullulan of pullulanase determined from Lineweaver-Burk plot to be 39.49 mg/ml and the V_{max} was 35.31 nmol maltose/min. For isoamylase, the K_m for amylopectin was 21.14 mg/ml and the V_{max} was 52.10 nmol maltose/min. Tables 4.1 and 4.2 showed K_m of DBE reported in several plant tissues. The K_m of pullulanase for pullulan is about 0.2 mg/ml for enzyme of rice endosperm, sorghum endosperm and oat endosperm, but was found to be 0.78 mg/ml for the spinach leaf enzyme. It may be speculated that in tissue such as endosperm which acted as energy source for developing embryo, pullulanase activity hydrolyze the storage starch for energy production (Sinlapawisut, 2004). 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 DBE activity for trimming or modifying structure of amylopectin to facilitate organization of starch granule, milder DBE activity may be better for retaining level of storage starch while

sufficient for the trimming action. In the latter case, DBE with higher K_m for the starch would serve the purpose. The K_m of isoamylase and pullulanase for cassava tuber reported in our experiment were high, supporting the hypothesis. From the observation, K_m of DBE in cassava was higher than observed from another plants, this value may be different *in vivo*. The five minute cultivar is a cultivar suitable for human consumption. The lower K_m for pullulanase observed by Sinlapawisut (2004) may be according to its characteristics. The pullulanase in this cultivar at very mature stage may also function in degrading starch to add sweet taste to the tuber. Harvesting, of the five minutes tubers were not monitored in exact ages as KU50. They were purchased from fresh market and may be at the very mature age than 9 months old. There have been no previous report on kinetic study on DBE of storage root such as potato or cassava. From the data in Table 4.2, it seemed that K_m towards pullulan or amylopectin were much lower than our finding. To elaborate more on the difference, kinetic data of DBE in other storage organs of mature plants are needed.

From our studies, it can be concluded that DBE, both isoamylase and pullulanase, were found in cassava tubers cultivar KU50 and their level increased with age, especially pullulanase. Partially purified pullulanase and isoamylase showed distinct molecular weights and kinetic properties compared to those previously reported. However, their developmental patterns were well correlated with similar studies in our laboratory on soluble starch synthase and starch branching enzymes. The present results together with previous studies on other starch synthesizing enzymes and their expression patterns would help in understanding starch synthesis in cassava tubers.

Table 4.1 Basic physico-chemical parameters of isoamylase in plants

Plant	Tissues	Substrate specificity	pH	MW (kDa) (method)	Reference
Maize	Kernel	Ap>βLd>Pg =Gc >Pu =0	6.0	75 (SDS-PAGE)	Rahman et al.,1998
Maize	Developing endosperm	Ap>βLd>>Pg>Pu = 0	6.0-7.5	141 (GPC; Ultrogel AcA44)	Doehlert and Knutson, 1991
Potato	Tuber	Ap = Pg = Gc =βLd >>Pu = 0	5.5-6.0	520 (GPC; Sephadex G-200) 95, 83 (SDS-PAGE)	Ishizaki et al., 1983

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

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

Plant	Tissues	Substrate specificity	Km (mg/ml)	pH	MW (kDa) (method)	Reference
Maize	Mature endosperm	βLd=αLd>Pu>>Ap>Gc=0			110 (GPC;Sephadex G-200)	Lee et al.,1971
		Pu>βLd>>Ap>Pg		5.5	68 (GPC;Ultrogel AcA 44)	Doehlert and Knutson,1991
Rice	Developing endosperm	Pu>αLd>βLd>>Ap>Pg=0		5.6		Yamada and Izawa, 1979
		Pu>βLd>αLd>>Ap	0.22(Pu),3.1(βLd), 4.0(αLd),5.0(Ap)			Yamada and Kojima, 1981
					100 (SDS-PAGE)	Toguri, 1991
					100 (SDS-PAGE)	Nakamura et al., 1996
	Mature endosperm	βLd>Pu>>Ap	6(Ap)	5.6	70 (GPC;Sephadex G-100)	Yamada, 1981
						Dunn et al.,1973
	Germinating endosperm	Pu>>Gc=Pg		5.5	58 (GPC;Bio-Gel P-150)	Iwaki and Fuwa, 1981
					100 (SDS-PAGE)	
Sorghum	Mature endosperm	βLd>Pu>>Ap	0.2(Pu),2.5(βLd)			Dunn et al., 1973
		βLd>Pu>Ap		5.0-5.4	90 (GPC;Sephadex G-150)	Hardie et al.,1976

Table 4.2 (continue) Basic kinetic and physico-chemical parameters of pullulanase in plants

Plant	Tissues	Substrate specificity	Km (mg/ml)	pH	MW (kDa) (method)	Reference
Oat	Mature endosperm	βLd>Pu>>Ap	10(Ap)			Dunn et al., 1973
		Pu>βLd> >Ap	0.23(Pu),3.0(βLd), 1.0(Ap)		85 (GPC;Sephadex G-100)	Yamada,1981
Broad bean	Mature embryo	βLd>Pu>>Ap	1.0(βLd),1.2(Ap)	6.4 6.8	80 (GPC;Sephadex G-150)	Gordon et al.,1975
Potato	Tuber	Pu=αLd=βLd>>Ap				Drummond et al.,1970
Sugar beet	Leaf	Pu>βLd >>Ap		5.5	110 (GPC;Sephadex G-150) 105 (SDS-PAGE)	Li et al.,1992
Spinach	Leaf	αLd>Pu>βLd>Ap>Gc=0		6.0-7.0		Okita and Preiss,1980
		Pu>>Ap>>Gc=0	0.78(Pu),7.0(Ap)	5.0 5.5	110(GPC;TSKgclG3000SW) 102 (SDS-PAGE)	Ludwig et ai.,1984
Cassava	Tuber	Pu>>Ap>>Gc=0	0.88(Pu)	6	103 (GPC;Sephadex G-150) 35 (SDS-PAGE)	Sinlapawisut, 2004

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