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Isolation and Characterization of Branching and Debranching Enzymes from Cassava *Manihot esculenta Crantz* Tubers

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Abstract

Cassava starch is one of the major raw materials used in many industries. Starch is composed of the polysaccharide, amylose and amylopectin which were synthesized by many enzymes including starch branching enzyme (SBE) and debranching enzyme (DBE). Understanding the functions of the enzymes involved, would lead to the possible manipulation of the enzymes to produce starch with desired properties. In this study, starch branching enzyme and debranching enzyme were isolated from tubers of cassava *Manihot esculenta* Crantz. Starch branching enzyme from KU 50 was separated into 3 isoforms, with molecular weight of 60, 65and 65 by Sephadex G150. The molecular weight determined by SDS-PAGE were 108 and 57 kDa for isoform1 , 57 and 60 kDa for isoforms 2 and 3 . The pI of the three isoforms was 4.9, 4.9 and 5.0, respectively. Optimum pH's were 8.0, 6.5 and 7.5 and the optimum temperatures of isoforms 1 and 2 were 37°C and 30°C for isoform 3. The *Km* for amylose of the three forms were 1.12, 1.37 and 2.17 mg/ml, respectively. Isoforms 1 and 2 were more active than isoform 3 towards amylose as substrate, where as isoform 3 was more active than isoforms 1 and 2 with amylopectin as substrate. Debranching enzyme can show either pullanase or isoamylase activity or both. Debranching enzyme with both activities was isolated from tubers of 5 minutes cultivar by 20-50 % saturated ammonium sulfate precipitation followed by chromatographies on DEAE-Sepharose and Sephadex G-150. The activity peak showed molecular weight of 103 kDa on Sephadex G-150 and 35 kDa on SDS-PAGE. The debranching enzyme had optimum pH of 6.0 and optimum temperature of 37°C. It can be activated by DTT but inhibited by NEM and IAA, indicating the involvement of SH-group on pullulanase activity.

Introduction

Starch is composed of linear and branched chains of α-D-glucose residues. The branch synthesis involved starch branching enzyme and starch debranching enzyme. 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 chain then catalyzes the formation of α-1,6-cross linkage between the reducing end of the cleaved chain and another glucose residue (1).

That plant almost invariably contains the multiple isoforms of starch branching enzyme (SBE) raises the possibility that different forms create chains with different length or branches point at different frequencies. A and B isoforms of starch branching enzyme differ both in their substrate affinities and the length of branches they preferentially create (2,3). Multiple forms of SBE, have been reported and purified from a variety of plant tissues: maize endosperm (4), pea embryo (5), potato tuber (6,7) and kidney bean (8,9).

Debranching enzyme hydrolyses the α-1,6 glucan branches of amylopectin and is subdivided into two classes. The direct DBE, which involves in the hydrolysis of α-1,6- linkages of α-polyglucans and the indirect DBE, which is engaged in hydrolysis of α-1,6- branches by its 4-α -glucanotransferase and amylo-1,6-glucosidase activities. Direct DBE is 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 specificities in which pullulanase debranch pullulan and amylopectin but not glycogen, whereas isoamylases debranch both glycogen and amylopectin. Moreover, pullulanase 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 (10). Conversely, in potato, two distinct subunits define an equally large heteromultimeric enzyme (11). There is good accumulated evidence that DBEs plays a crucial role in starch biosynthesis. This paper reports the isolation and characterization of SBE and DBE from cassava tubers.

Materials and methods

Plant materials

Nine months old cassava tuber (*Manihot esculenta* Crantz) of cultivar KU50 from Rayong field crops Research Center at Rayong province, Thailand, was used as source of branching enzyme and tubers of five-minute cultivar for debranching enzyme.

Purification of enzymes

Extraction of crude enzyme

Cassava tubers were cleaned and cortex removed. The parenchyma was homogenized and volume adjusted using either 0.05 M Tris-HCl pH 7.5 contained 2 mM EDTA, 1 mM DTT and 1 mM benzamidine (Buffer A) or 0.05 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol, 3 mM $CaCl_2$ and 0.5 mM PMSF (Buffer B) for starch branching enzyme and starch debranching enzyme, respectively. Each homogenate was centrifuged at 15,000 x g for 1 hour at 4 °C. The supernatant was collected as crude enzyme and kept at 4 °C until used.

Purification of starch branching enzyme

DEAE-Toyopearl column chromatography

Solid polyethylene glycol 6000 was add to the crude enzyme to make 10% (w/v) solution with continuous stirring for 1 hour on ice bath to partially remove some protein SBE which was retained in the supernatant was collected by centrifugation 10,000 x g for 1 hour at 4 °C.

The supernatant was loaded onto a DEAE-Toyopearl (2X15 cm, Tosoh cooperation) equilibrated with buffer A at 1 ml/min. The column was eluted with 0.15 M NaCl in buffer A and fractions with SBE activity were collected, pooled and concentrated by Vivaflow 50 (molecular weight cutoff 30,000 daltons, Vivascience) and dialyzed against buffer A.

The enzyme pooled was loaded onto second DEAE-Toyopearl (1x13 cm) and eluted step-wise with 0.04, 0.07 and 0.1 M. NaCl in buffer A, respectively at flow rate 45 ml/hour. Fractions of 6 ml were collected and fractions with SBE activity were pooled.

Hitrap Q column chromatography

Each SBE isoform was concentrated and separately loaded on Hitrap Q column equilibrated with buffer A. Each column was eluted with linear gradient of 0-0.5 M NaCl at flowrate 1 ml/min. Fractions of 3 ml were collected and fractions with SBE activity were pooled.

Sephadex G-150 column chromatography

Each isoform of SBE was applied to Sephadex G-150 column (1.5x70 cm)and eluted with buffer A containing 0.1 M NaCl at flow rate 15ml/hour. Fractions of 1 ml were collected and detected for SBE activity. The column was also calibrated with standard proteins with known molecular weight.

Starch branching enzyme activity assay

Assay A SBE activity was followed by the incorporation of ^{14}C-glucose into α-D-glucose synthesized by stimulation with rabbit-muscle phosphorylase a modified from the method described by Mizuno (12)

Assay B The spectrophotometric method (13) measuring the decrease in the absorbance at 660 nm of the starch-iodine complex was used in kinetic study.

Characterization of starch branching enzyme

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out in 4% stacking gel and 10% separating gel, using a vertical slab gel apparatus with continuous system of Laemmi and stained with Coomassie brilliant blue R-250. The molecular markers used were low molecular weight markers from Amersham, Sweden.

Nondenaturing PAGE was performed in 4% stacking gel and 7% separating gel containing 3% soluble starch (Kanto-chemical, Japan). For SBE activity stain, gel strip was rinsed with distilled water and soaked in 50 mM MOPS-NaOH pH 7.0 for 4 hour at 30 °C followed by incubation in iodine solution (1% KI/0.1% I_2) for 15 minutes. SBE band appeared as red-brown band on the purple-blue background.

Isoelectric focusing electrophoresis

Isoelectricfocusing (IEF) was performed using Ampholine pH 3.5-10.0 (Amersham, Sweden). The pI standard proteins were the calibration kit (pH 3.0-10.0) from Amersham, Sweden.

Kinetic study of starch branching enzyme

The purified enzyme was used to study for kinetic constant (V_{max} and K_m) with amylose as the substrate of this enzyme. The reaction mixture consisted of various concentrations of amylose, reactions were performed using *assay B*.

Specificity of SBE isoforms towards amylose and amylopectin

Specificity of the purified SBE isoforms towards amylose and amylopectin were studied. The activity was followed by assay B (Amylopectin was assayed the same way but color change was followed at A_{540}).

Purification of starch debranching enzyme

DEAE-Sepharose column chromatography

DBE with pullulanase activity was precipitated at 20-50% ammonium sulfate. The pellet was collected by centrifugation at 10000 rpm for 1 hour at 4 °C and dissolved in Buffer B.

The supernatant was loaded onto a DEAE-Sepharose (2X30 cm, Amersham,Sweden) equilibrated with buffer B at 0.2 ml/min. The column was eluted with linear gradient of 0-0.6 M sodium in buffer B and fractions with DBE activity were collected

Sephadex G-150 column chromatography

DBE was applied to Sephadex G-150 column (1.5x90 cm) and eluted with buffer B containing 0.1 M NaCl at flow rate 10ml/hour. Fractions of 2 ml were collected and detected for DBE activity.

Starch debranching enzyme activity assay

DBE activity was determined by the reaction mixture containing 1% (w/v) pullulan, 100 mM acetate buffer pH 6.0 with appropriate amount of enzyme in total volume of 0.5 ml and incubated for 45 minutes at 37 °C. To determine amount of reducing sugar released, 0.5 ml of dinitrosalicylate reagent was added, boiled for 10 minutes and measured A_{540}. One unit of activity was defined as the release of 1 μmol of reducing sugar per minute.

Characterization fo starch debranching enzyme

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out as described for SBE.

Nondenaturing PAGE was performed in 4% stacking gel and 10% separating gel containing 3% soluble starch (Kanto-chemical, Japan). The electrophoresis was performed at a constant current

of 16 mA at 4°C. 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 followed by incubation in iodine solution (1% KI/0.1% I_2) for 15 minutes. The enzyme activity appeared as blue-band on the purple-blue background.

Results

Purification of SBE and DBE from cassava tuber.

Starch branching enzyme from cassava tuber was purified by polyethylene glycol precipitation, followed by chromatographies on two DEAE-Toyopearl and Q-sepharose columns. First chromatography on DEAE-Toyopearl was used for separation of the enzyme activity peak from other proteins by elution of SBE peak at 0.15 M NaCl. The pooled SBE fractions was loaded to a second column of DEAE-Toyopearl to separate isoforms by stepwise elution with 0.04 M, 0.07 M. and 0.1 M NaCl, respectively (Fig.1a).Each isoform was further cleaned up with Hitrap Q Sepharose column. The purification results were summarized in table 1a.

Starch debranching enzyme was precipitated at 20-50% ammonium sulfate and separated onDEAE-Sepharose column. The pullulanase activity was eluted at 0.15 M NaCl (Fig. 1b).The pooled DBE activity peak was further subjected to Sephadex G-150 column.

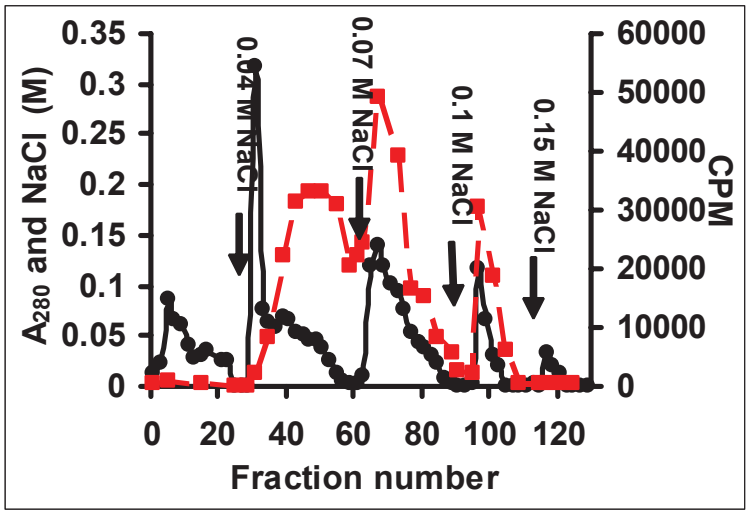

Fig 1a Second DEAE-Toyopearl chromatographic profile of SBE ● A_{280} ■ CPM

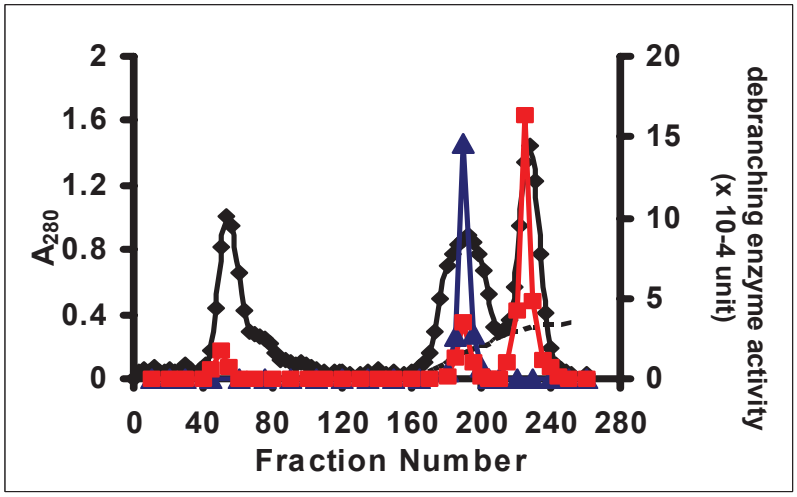

Fig 1b. DEAE-Sepharose chromatographic profile of Starch debranching enzyme

◆ A_{280} ■ Pullulanase ▲ Isoamylase

Table 1a Purification of cassava starch branching enzyme

Fraction	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg protein)	folds
Crude	1920	200	0.104	1
PEG supernatant	1113	184	0.165	1.6
1st DEAE	265	200	0.75	7
Isoform 1				
2nd DEAE	172	1012	6	58
Hitrap Q-Sepharose	39	503.5	13	125
Isoform 2				
2nd DEAE	133	321	2.41	23
Hitrap Q Sepharose	50	400	8	77
Isoform 3				
2nd DEAE	118	587	5	48
Hitrap Q Sepharose	32	822	26	250

Table 1b Purification of starch debranching enzyme

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

The purified SBE and DBE were subjected to nondenaturing PAGE and stained with iodine solution (Fig 2a and Fig 2b).

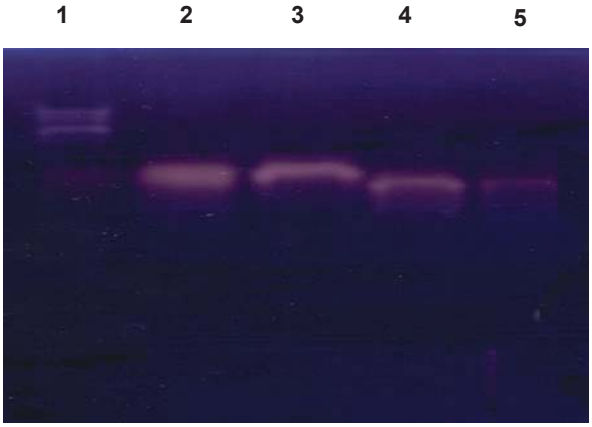

Fig 2 a Activity staining of cassava starch branching enzyme

Lane 1 Crude enzyme
Lane 2 First DEAE-Toyopearl
Lane 3 Isoform 1 from Sephadex G-150
Lane 4 Isoform 2 from Sephadex G-150
Lane 5 Isoform 3 from Sephadex G-150

Fig 2 b Activity staining of cassava starch debranching enzyme

Lane 1 Crude enzyme Lane 2 20-50% saturated ammonium sulfate precipitation

Lane 3 DEAE- Sepharose Lane 4 Sephadex G-150

Characterization of the purified enzymes

Molecular weight determination of SBE isoforms

The apparent molecular mass of the SBE isoforms on Sephadex G150 were 60, 65 and 65 kDa. Analysis by 10% SDS-PAGE of the purified SBE 2 and 3 revealed a major single protein band with molecular weight of 57 and 60 kDa, whereas SBE 1 showed 2 prominent bands with molecular weight of 57 and 108 kDa, respectively.

Molecular weight of DBE was 103 kD as determined by Sephadex G-150 and 35 kD as determined on SDS-PAGE.

Isoelectricfocusing gel showed that SBE 1, 2 and 3 had the pI values of 4.9, 4.9 and 5.0, respectively.

Determination of Optimum pH and temperature

The optimum pH and temperature of purified SBE 1, 2 and 3 were determined by using enzyme assay B. The enzyme displayed maximum SBE activities at pH 8.0, 6.5 and 7.5 for isoforms 1, 2 and 3, respectively. Optimum temperatures of SBE were determined to be 37 °C for isoforms 1 and 2 while isoform 3 showed higher activity at 30 °C . The optimum pH for DBE was pH 6.0 and the optimum temperature was 37 °C. Table 3 summarized the results on optimum pH and temperature for with SBE and DBE

Study of kinetic parameters and substrate specificity

The kinetic parameters of SBE isoforms were studied using amylase as substrate. The *Km* for amylose was 1.12, 1.37 and 2.17 mg/ml and *Vmax* were 1.03. 0.83 and 0.57 ΔA_{660}/30min for SBE 1,2 and 3 respectively. Isoforms 1 and 2 utilized amylose better than isoform 3 while isoform 3 was more active towards amylopectin as substrate.

The *Km* and *Vmax* of DBE for pullulanase was 0.88 mg/ml and 24.81 nmol maltose/min. respectively. It was also observed that in presence of DTT, the DBE activity was enhanced up to 300%.On the contrary, NEM and IAA showed inhibitory effect on the enzyme by 80 and 100% at the same concentration as DTT.

Table 3 Optimum pH and temperature of Branching enzyme and Debranching enzyme

	Optimum pH	Optimum temperature (°c)
Branching enzyme		
Isoform1	8.0	37
Isoform 2	6.5	30
Isoform 3	7.5	37
Debranching enzyme	6.0	37

Table 4 Kinetic parameters of Branching enzyme and Debranching enzyme

	Km (mg/ml)	*Vmax* (ΔA_{660}/30min)
Branching enzyme (amylose as substrate)		
Isoform 1	1.12	1.03
Isoform 2	1.37	0.83
Isoform 3	2.17	0.57
Debranching enzyme (pullulan as substrate)	*Km* (mg/ml) 0.88	*Vmax* (nmol maltose/min) 24.81

Discussion

We have described the purification and some characterizations of SBE isoforms from cassava tuber in cultivar KU50 which was developed in Thailand. This cultivar produces tuber with high starch content. Polyethylene glycol 6000 was employed to precipitate some contaminating proteins such as amylase and starch synthase (6). Although, this step did not provide significant purification but was essential because other starch metabolizing enzymes were removed. Ion exchange chromatography on DEAE-Toyopearl was performed in 2 steps.

The first run removed about 70%of contaminating proteins without loss of SBE activity. The second DEAE-Toyopearl column gave good separation of three SBE isoforms. Hitrap Q-Sepharose, a strong ion exchange column with gel filtration matrix further enhanced the purity of each isoforms. The strong anionic resin removed some of the protein co-eluted with SBE as unbound fractions. All of the three purified isoforms showed characteristics of SBE staining as reddish brown bands on nondenaturing starch PAGE stained with I_2 (14).

The molecular weight of all isoforms determined by gel filtration on Sephadex G-150 and SDS-PAGE were quite close in the range of 57-60 kDa.The appearance of SBE1 as 2 bands on SDS PAGE at 108 and 57 kDa either indicated that SBE1 existed as an 57 kDa protein and its dimer or the SBE1 fraction was contaminated with SBE2 . Several plants were also reported to have isoforms of almost similar molecular weight. Therefore, the molecular weight of the cassava SBE isoforms we have isolated complied with previous observations in other plants.

The three isoforms differed in their optimum pHs and temperatures and the and as observed in other plants (15,7,8).Their K_m and V_{max} values were also different. The K_m for amylose was 1.12, 1.37 and 2.17 mg/ml and V_{max} were 1.03, 0.83 and 0.57 Δ A_{660} / 30 min for isoforms 1, 2 and 3, respectively. This indicated that isoforms 1 and 2 were more active towards amylose than isoform 3.

It was reported that some SBE isoforms, although catalyzed branching of amylose, preferentially used amylopectin as substrate (2). Isoform 1 was most active in utilizing amylose, followed by isoforms 2 and 3. When amylopectin was used as substrate, isoform 1 and 2 were less active. Isoform 3 can utilize amylopectin 30% better than isoforms 1 and 2. This result agreed with

the *Km* for amylose of this isoform. SBE has been classified into two classes by amino acid sequences, class A preferentially branches amylopectin whereas class B preferentially branches amylose (3). It was reported that C-terminal domain of SBE is involved in substrate specificity whereas N-terminal domain is important for specificity of transferred chain length and require for maximum enzyme activity. From the above information, SBE 1 and 2 should belong to class B. whereas isoform 3 which was more specific for amylopectin should belong to class A.

DBE can exhibit isoamylase or pullulanase activity or both. In our study, activity peak with isoamylase activity alone was very minute. Only one peak with pullulanase activity was observed in the DEAE Sepharose column. This peak also contained some isoamylase activity. This result complied with the report that DBE with pullulanase activity was usually prominent in plant storage tissue (16).The observed molecular weight of DBE also was within the range of molecular weight of plant DBE reported (17).The enhancing effect of dithiothreitol, which stabilized SH group, on DBE activity suggested that -SH group played important role in the pullulanase activity. This was further confirmed by the inhibitory effect of Iodoacetic acid and N-ethylmaleimide, the -SH blocking reagents.

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