CHAPTER II

MATERIALS AND METHODS

2.1 Plant materials

Cassava tubers (*Manihot esculenta* Crantz) cultivar KU50 were harvested from Rayong field crop center, Rayong, Thailand

2.2 Chemicals

Acrylamide; Merck

Amylopectin, potato; Fluka

Amylose, potato; Fluka

Aquasorb; SM.lab

Coomassie blue R-250; Acros

Coomassie blue G-250; Fluka

DEAE – Sepharose; GE-Healthcare

3,5-Dinitrosalicylic acid; Merck

Dithiothreitol; Amersham Bioscience

Ethelenediaminetetraacetic acid; Carlo

Glutatione reduced form; Sigma

Glycine; Amersham Bioscience

Glycogen, Rabbit liver; Sigma

Hydrochloric acid; BDH

Iodoacetic acid; Sigma

Iodine; Fluka

Maltose; Sigma

Maltotriose; Sigma

β-Mercaptoethanol; Scharlau

Methanol; BDH

N,N,N',N'-Tetrametylene ethylene diamine; Fluka

N,N-methyl-bis-acrylamide; Amersham Bioscience

N-Ethylmaleimide; Sigma

Phenylmethyl sulfonyl fluoride; USB

Potassium iodide; Fluka

Potato starch soluble; Kanto chemical

Pullulan; US Biological

Sephacryl S200; Amersham Bioscience

Sodium chloride; BDH

Sodium citrate anhydrate; Carlo

Sodium laulyl sulphate; Sigma

Tris(hydroxymethyl) aminomethane; Amersham Bioscience

2.3 Equipments

Blender; Phillip

Bench top centrifuge; Labquip

Electrophoresis unit / Hoefer Mighty small; Amersham Bioscience

Electrophoresis power supply / EPS 300; Amersham Bioscience

Fraction collector; Pharmacia Biotech

Peristaltic pump; Pharmacia Biotech

pH meter; Metler

Refrigerator centrifuge /Avanti J-30I; Beckman

Spectrophotometer; Beckman

Water bath shaker; Sturt

2.4 Preparation of starch debranching enzyme (DBE) from cassava tuber

Cassava cultivar Kasetsart 50 (KU50) was grown at Rayong field crop Research Center, Rayong. Tuberous roots of 9-month-old cassava were collected and the skin was pealed off. The parenchyma was cut into small pieces and homogenized in blender. The homogenate was then centrifuged at 10,000 rpm for 1 hour at 4 °C. The crude extract from this step was adjusted to pH 7.5 by adding 50 mM Tris-HCl buffer pH 7.5 containing 10 mM β-mercaptoethanol, 3 mM $CaCl_2$ and 0.5 mM PMSF. The crude extract was kept at 4°C for the further purification.

2.5 Purification of DBE

2.5.1 Ammonium sulfate precipitation

Solid $(NH_4)_2SO_4$ was gradually added to the crude extract to attain 60% saturation and the precipitate formed was collected by centrifugation at 10,000 rpm for 1 hour at 4°C. The pellet was dissolved in 50 mM Tris-HCl buffer pH 7.5 containing 10 mM β-mercaptoethanol and 3 mM $CaCl_2$ (starting buffer) and dialyzed against the same buffer.

2.5.2 Ion exchange chromatography

DEAE Sepharose was washed with 1 M NaCl, followed by 0.1 M NaOH in 0.5 M NaCl and distilled water until the pH was natural, packed in a glass column (2.5 x 20 cm.) at the flow rate 12 ml/hr and equilibrated with starting buffer. The sample

from section 2.5.1 was loaded on the column and eluted with the starting buffer at the flow rate of 12 ml/hr. The column was then eluted with 0-0.3 M NaCl in starting buffer and finally eluted with 0.5 M NaCl in starting buffer until absorbance at 280 nm of the 3 ml fractions reached zero. The conductivity of the eluted fractions was measured and protein monitored by measuring absorbance at wavelength 280 nm. The DBE activity was detected by the method described in section 2.7. The fractions with DBE activity were pooled, concentrated and dialyzed in the starting buffer.

2.5.3 Gel filtration chromatography

Sephacryl S-200 was packed in a glass column (2.0 x 100 cm.) and equilibrated with starting buffer at the flow rate of 30 ml/hr. The enzyme from section 2.5.2 was loaded on the column and eluted with starting buffer at the flow rate of 20 ml/hr. Two milliliters were collected and monitored for protein at A_{280} nm and assayed for DBE activity.

2.6 Protein determination

Protein concentration was estimated by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. One hundred microliter of sample was mixed with 5 ml of coomassie blue reagent and left for 30 minutes before recording the absorbance at 595 nm.

2.7 DBE activity assay

2.7.1 Pullulanase activity

The enzyme (100 µl) was added into the reaction mixture containing 50 mM acetate buffer pH 6.0 and pullulan (20 mg/ml) as substrate in the final volume 0.5 ml. The reaction mixture was incubated at 37°C for 45 minutes. The reaction was stopped by adding 0.5 ml DNS reagent, then boiled for 10 minutes and 2 ml distilled water was added. The reducing sugar released into the reaction mixture was determined by measuring the absorbance at 570 nm. Maltose was used as standard, 1 unit of activity was defined as the release of 1 µmole of reducing sugar per minute.

2.7.2 Isoamylase activity

Isoamylase was monitored as described in section 2.7.1 but amylopectin (8 mg/ml) was used as substrate instead of pullulan.

2.8 Non-denaturing starch PAGE

Slab gel was prepared with the separating gel containing polyacrylamide 7.5% (w/v) with the soluble starch (0.4% w/v) or amylopectin (0.2% w/v) and stacking gel containing 4% (w/v) polyacrylamide. Cool Tris-Glycine buffer pH 8.3 containing 2 mM DTT was used as electrode buffer. Electrophoresis was run with the current of 16 mA at 4°C. Following electrophoresis, the gel was soaked in 50 mM acetate buffer pH 6.0 containing 1 mM DTT at 37°C for 2 hours. Then the gel was stained with iodine solution (0.1% I_2/KI) for 15 minutes. Pullulanase activity will appear as blue band and isoamylase activity will appear as clear band against a dark purple background.

2.9 SDS-PAGE

SDS-PAGE was performed in slab gel employing 10 % (w/v) separating gel and 4 % (w/v) stacking gel containing 0.1% (w/v) of SDS. Tris-glycine buffer pH 8.3 containing SDS was used as electrode buffer. Sample to be analyzed were treated with sample buffer and boiled for 5 minutes before apply to the gel. The electrophoresis was performed at constant current of 20 mA per slap, at room temperature.

2.10 Characterization of starch debranching enzyme

2.10.1 Determination of molecular weight.

2.10.1.1 Sephacryl S-200 chromatography

From gel filtration chromatography, the molecular weights of proteis were determined from calibration curve prepared from partition coefficient (K_{av}) and the molecular weights of standard marker proteins; chymotrypsinogen A (27,000 Da), ovalbumin (43,000 Da), albumin (66,000 Da), aldolase(158,000) and catalase(232,000 Da). Blue dextran and riboflavin were used to determine the void volume (V_o) and total bed volume (V_t), respectively. K_{av} of the standard marker proteins and DBE were calculated from the formula

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Where V_e = elution volume

2.10.1.2 SDS-PAGE

After electrophoresis, the protein bands in gel were visualized by coomassie blue stain. The molecular weight of DBE were determined from calibration curve obtain from R_f and molecular weight of standard proteins; phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa).

2.10.2 Effect of temperature on DBE

The partial purified pullulanase and isoamylase from ion exchange chromatography was used to study effect of temperature on its activity. The enzyme was incubated at 25, 37, 45, 50, 60, 70, 80 and 90°C for 45 minutes. The activity was measure as described in section 2.7. The result was expressed as percent relative activity.

2.10.3 Effect of pH on DBE

The enzyme (100 µl) was incubated in different pH of reaction mixture for 45 minutes at optimum temperature. The pH range of reaction mixture is 4.5-12. The buffers used were acetate buffer (pH 4.5-6), phosphate buffer (pH 6-7.5), Tris-HCl buffer (pH 7.5-9) and glycine buffer (pH 9-12). The reaction mixture was stopped the reaction by adding DNS reagent as described in section 2.7. The result was expressed as percent relative activity.

2.10.4 Temperature stability

The temperatures at 4, 25, 37, 50, 60 and 70°C were used to study temperature stability of DBE. The enzyme was incubated at the selected temperatures for 0, 2, 4, 6, 12, 24 and 48 hours and the activity was measured as described in section 2.7. The result was expressed as percent relative activity.

2.10.5 Effect of sulfhydryl reagents on DBE

Different final concentrations (1, 5 and 10 mM) of DTT, GSH, β-mercaptoethanol, IAA and NEM were added to the reaction mixtures. The reaction mixture was incubated at optimum temperature for 45 minutes. The DBE activity was determined as described in section 2.7 and the result was expressed as percent relative activity.

2.10.6 Effect of divalent metal ions

Various final concentrations (1, 5 and 10 mM) of $CuSO_4$, $CoCl_2$, $MnCl_2$, $MgSO_4$ and $NiCl_2$ were added to the reaction mixtures and DBE activity was detected as described in section 2.7.

2.10.7 Comparison of DBE activity with various substrates

Amylose, amylopectin, pullulan, glycogen and soluble starch at 1 % w/v were used as substrate for assay DBE activity. The enzyme was assayed and the activity measured as described in section 2.7

2.11 Determination of K_m and V_{max}

Purified pullulanase and isoamylase from Sephacryl S-200 column were used for kinetic study. Pullulan and amylopectin were used as substrate. The reaction mixture consisted with various concentrations of substrate (2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 30, 40 and 50 mg/ml). The reaction was incubated for 10 minutes at optimum temperature and pH. The reaction was stopped by adding DNS reagent and boiled for 10 minutes. The activity was measured as described in section 2.7.