CHAPTER II

MATERIALS AND METHODS

2.1 Plant material

Tubers of cassava, *Manihot ecculenta* Crantz from Pak-Khlong-Talad, Bangkok, Thailand

2.2 Equipment

Name of equipment	Companies
Autopipette: Pipetman	Gilson, France
Beta counter: Liquid scintillation counter LS 1801	Beckman, USA
Centrifuge, refrigerated centrifuge: Model J-21C	Beckman Instrument Inc, USA
Electrofocusing unit: model111 MINI IEF cell	Bio-Rad Applied Biosystem company, USA
Electrophoresis unit: Model Mini-protein II Cell	Bio-Rad, USA
Fraction collector: model 2211	Pharmacia LKB, Sweden
Incubater Shaker	Phyco-therm, New Brunswick Scientific Co., USA
Peristatic pump	Pharmacia LKB, Sweden
pH meter	Radiometer, Denmark
Spectrophotometer: UV-240	Shimadzu, Japan, and Du series 650, Beckman, USA
Vortex: Model k-550-GE	Scientific Industries, USA
Waterbath	Charles Hearson CO., Ltd., England
Waterbath, shaking	Heto lab Equipment, Denmark

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2.2 Chemicals

Name of chemicals	Companies
N-acetylimidazole	Sigma, USA
Acrylamide	Merck, USA
Aguasorb	Fluka, Switzerland
AMP	Sigma, USA
Amylose	Sigma, USA
Amylopectin	Sigma, USA
Ascorbic acid	Sigma, USA
Benzamidine	Sigma, USA
α-D-[^{14}C]-Glucose-1-phosphate	Amersham, England
Coomassie brillant blue G-250	Sigma, USA
Coomassie brillant blue R-250	Sigma, USA
α- and β-Cyclodextrin	Fluka, Switzerland
DEAE-cellulose, anion exchanger	Sigma, USA
DL-dithiothreitol	Sigma, USA
Dextrin	Sigma, USA
Glucose-1-phosphate	Sigma, USA
Glycine	Sigma, USA
Glycogen, oyster	Sigma, USA
Maltose	Sigma, USA
N, N'-methyl-bis-acrylamide	Sigma, USA
Pentose	Sigma, USA
Polyethyleneglycol$_{6000}$	Fluka, Switzerland
Phosphorylase a, rabbit-muscle	Sigma, USA
Standard molecular weight marker proteins	Pharmacia, USA
Standard pI marker proteins	Pharmacia, USA

The other common chemicals were of reagent grade.

2.4 Purification of cassava SBE

2.4.1 Preparation of crude enzyme

Freshly harvested cassava tubers were peeled and the cortex was removed. The parenchyma was chopped by knife into small pieces and homogenized in a blender. The cassava juice was left standing about 10 min to settlement of the starch fraction. The homogenate was centrifuged at 8,000 xg for 15 min. The pellet was discarded and the cassava juice or crude enzyme was kept at 4 ^{o} C.

2.4.2 Precipitation with polyethyleneglycol

The cassava crude enzyme was mixed with dry Tris-base, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), benzamidine and polyethyleneglycol 6000 (PEG_{6000}) in a glass beaker to give final concentrations of 50 mM Tris-HCl, 2 mM EDTA, 5 mM DTT, 1 mM benzamidine, 10 % PEG and a final pH of 7.2 to 7.8. The homogenate was left for 10 min on ice with continuous stirring and was subsequently centrifuged at 8,000 xg for 15 min. The pellet was discarded and the supernatant was collected for the DEAE-cellulose chromatography.

2.4.3 DEAE-cellulose chromatography

DEAE-cellulose was activated by washing sequentially with excess volume of 0.5 N HCl for 3 hr followed by distilled water until the pH was 4.0 and then washed with 0.5 N NaOH for 3 hr followed by distilled water until the pH was 8.0. The activated cellulose was equilibrated with 50 mM Tris-HCl pH 7.5 containing 2 mM EDTA and 5 mM DTT overnight. The prepared DEAE-cellulose was packed into the column (1.5 X 25 cm) at the height of 20 cm. The column was equilibrated with 50 mM Tris-HCl pH 7.5 containing 2 mM EDTA and 5 mM DTT and then the supernatant from PEG precipitation was loaded onto the column at flow rate of 45 ml/hr. The column was washed with equilibrating buffer until A_{280} was negligible, then the column

was subjected to 400 ml of 0-0.5 M NaCl linear gradient elution. Fractions of 4.5 ml were collected for measurement of A_{280} and SBE activity. The fraction containing SBE activity were pooled, dialyzed and kept for Q-Sepharose chromatography.

2.4.4 Q-Sepharose chromatography

Q-Sepharose was equilibrated with 50 mM Tris-HCl pH 7.5 containing 2 mM EDTA and 5 mM DTT overnight. The Q-Sepharose was packed into a 1.0 X 15 cm column with the height of 12 cm. The column was equilibrated with the same buffer and then the pooled active fractions from DEAE-cellulose column were applied to a column at flow rate of 20 ml/hr. The column was washed with equilibrating buffer until A_{280} was negligible, then the column was subjected to 150 ml of 0-0.5 M NaCl linear gradient elution. Fractions of 2.0 ml were collected for measurement of A_{280} and SBE activity. The fraction containing SBE activity were pooled and kept for Sephadex G-200 column chromatography.

2.4.5 Chromatography on Sephadex G-200 column

Sephadex G-200 column (2.4 x 55 cm) was equilibrated with 50 mM Tris-HCl pH 7.5 containing 2 mM EDTA and 5 mM DTT overnight. The pooled active fractions from Q-Sepharose column were applied to the column at flow rate of 20 ml/hr. The starch branch enzyme was then eluted with the same buffer and 2.0 ml fractions were collected for measurement of A_{280} and SBE activity.

The elution volume (Ve) of SBE was compared with standard molecular weight marker proteins; namely catalase (232 kD), bovine serum albumin (68 kD), chymotrypsinogen (27kD) and cytochrome c (12 kD). Blue dextran and potassiumdichromate ($K_2Cr_2O_7$) were used to determine the position of the void volume (Vo) and the total volume (Vt), respectively. The partition coefficient (Kav) of each standard marker protein calculated from (Ve–Vo) / (Vt–Vo) was plotted against log molecular weight of each protein on

semi-logarithmic graph paper to obtain a caiibration curve. The Kav of the SBE was calculated in the same way and was used to determine its native molecular weight from the calibration curve.

2.5 Protein determination

Protein concentration was determined by the Coomassie blue micro method according to Bradford (1976), using bovine serum albumin (BSA) as standard.

One hundred microlitres of sample was mixed with 5 ml of Coomassie blue reagent and left for 5 minutes before recording the absorbance at 595 nm

One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of ethanol, 100 ml of 85 % H_3PO_4 and distilled water.

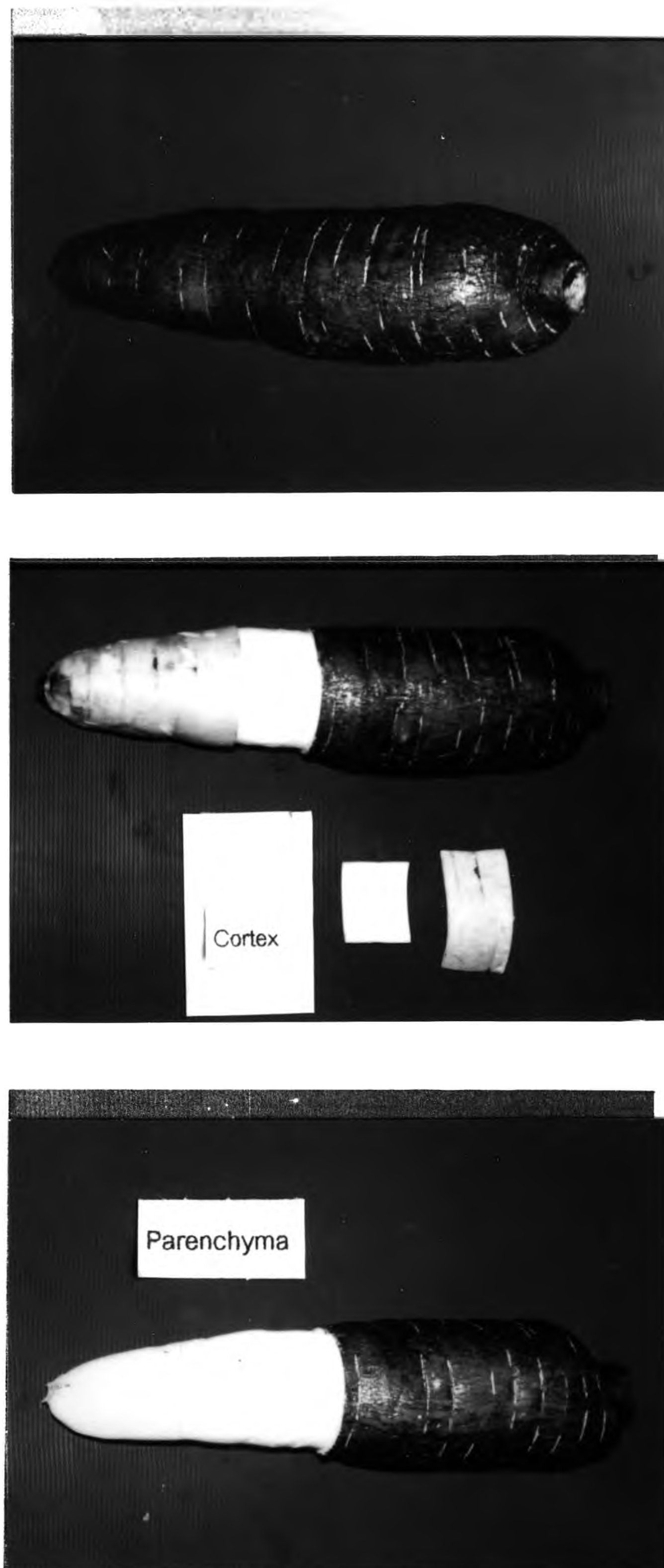

Figure 2.1 Cassava whole tuber and its tissue, cortex and parenchyma.

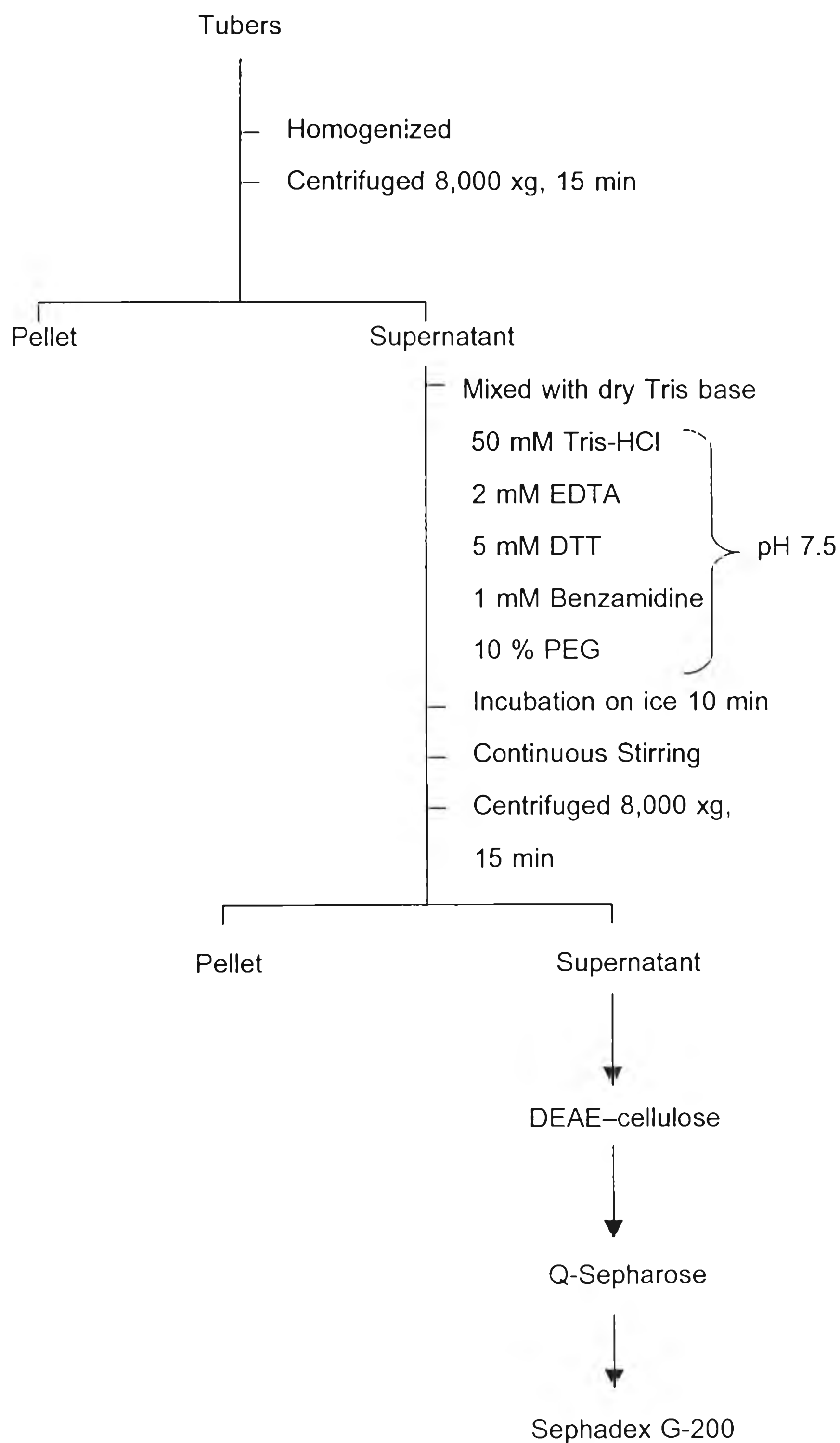

Figure 2.2 Flowchart of purification process of cassava SBE.

2.6 Assay of starch branching enzyme

Activity of starch branching enzyme was assayed by measuring the incorporation of ^{14}C-glucose into α-D-glucan synthesized by stimulation with rabbit-muscle phosphorylase a performed according to the method described by Mizuno *et al* (Mizuno, 1992).

The reaction was conducted in a 0.2 ml mixture 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 appropriate 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 forms glucans with 75 % 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 α-D-[^{14}C] glucose incorporation from α-D-[^{14}C] glucose-1-phosphate into the methanol-insoluble material per min under the conditions used (Appendix D).

2.7 Polyacrylamide gel electrophoresis (PAGE)

2.7.1 Non-denaturing Starch-PAGE

Non denaturing polyacrylamide gel was prepared as described in Appendix A, with addition of 1 % starch solution (w/v) in the gel solution on slap gel (10 x 8 x 0.75 cm) of 10 % (w/v) separating gel and 5.0 % (w/v) stacking gel. Tris-Glycine buffer pH 8.3 was used as electrode buffer. The electrophoresis was run from cathode towards anode, at constant current of 15 mA per slap at room temperature on a Mini-Gel Electrophoresis Unit. After electrophoretic separation the gel was soaked in 50 mM Tris-citrate buffer pH 7.0 containing 2 mM ascorbic acid for 4 hr at 22°C. Afterwards the gel stripes were rinsed with distilled water and incubated in an iodine solution for 15 min. The zones of cassava SBE activity appeared sharp red-brown bands on the blue stained background (Rammesmayer, 1992).

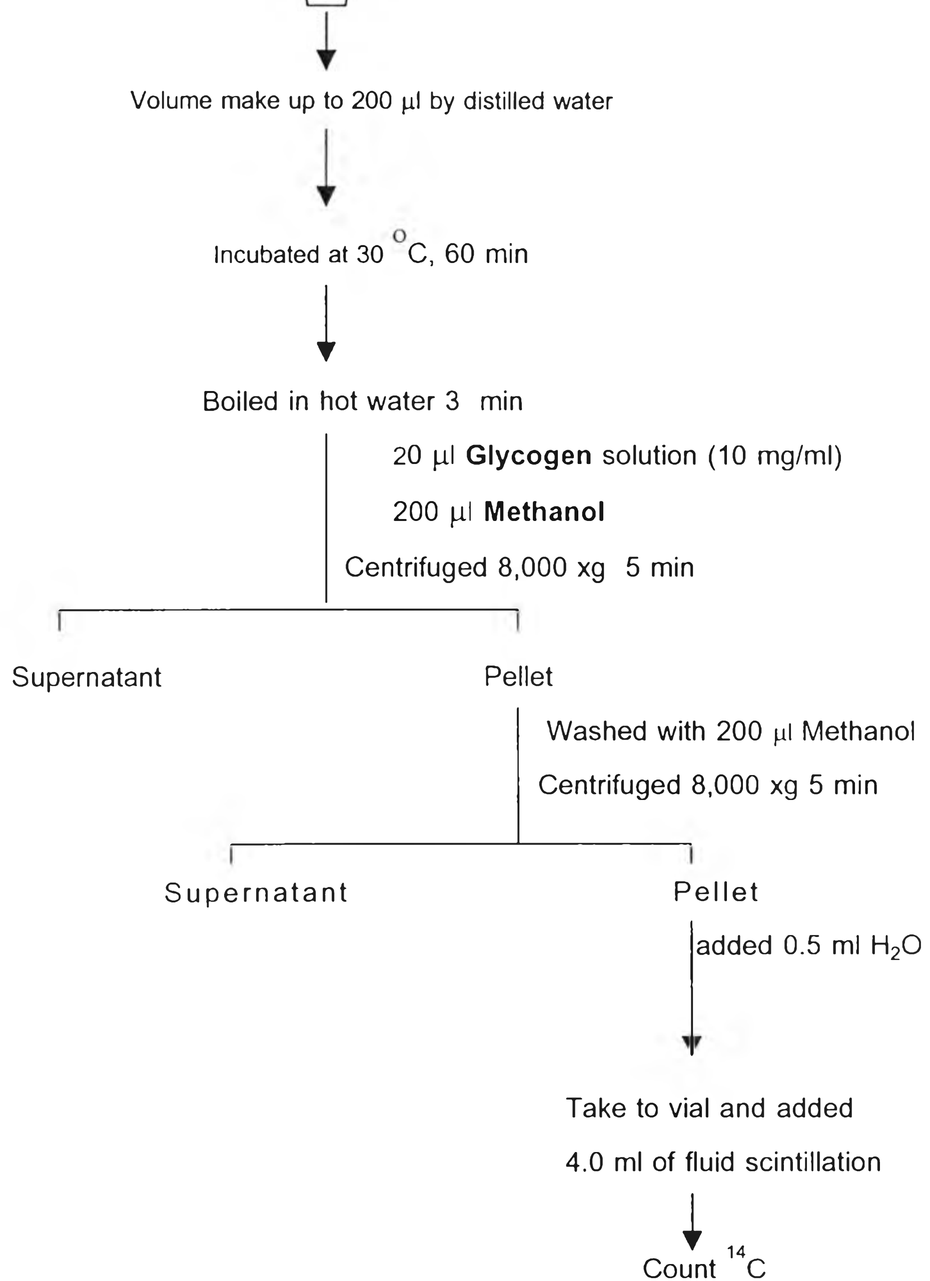

Figure 2.2 Flowchart of cassava SBE assay.

2.7.2 SDS-PAGE

The denaturing gel carried out with 0.1% (w/v) SDS in 7.5 % (w/v) separating gel and 5.0 % (w/v) stacking gel and Tris-Glycine buffer pH 8.3 containing 0.1% (w/v) SDS was used as electrode buffer (see Appendix A). Sample to be analyzed were treated with sample buffer and boiled for 5 min to application to the gel. The electrophoresis was performed at constant current of 15 mA per slap, at room temperature on a Mini-Gel Electrophoresis Unit from cathode towards anode.

After electrophoresis, proteins in the gel were visualized by Coomassie blue staining.

2.8 Effect of pH on SBE activity

The purified cassava SBE obtained from Sephadex G-200 column was used to study the effect of pH on the enzyme activity. The substrate solution containing universal buffer at various pH ranging 3–10 was mixed with the enzyme and measured the remained activity by the method as describe in section 2.6. The result was expressed as the percentage of that pH 7.0.

2.9 Effect of temperature on SBE activity

The purified cassava SBE obtained from Sephadex G-200 column was used to find out optimum temperature. The cassava SBE was determined by the method as describe in section 2.6 at various temperatures ranging from 25 $^{\circ}$C to 55 $^{\circ}$C (25 $^{\circ}$C , 30 $^{\circ}$C , 37 $^{\circ}$C , 45 $^{\circ}$C and 55 $^{\circ}$C). The enzyme blank at each temperature was also carried out under the same condition. The result was expressed as the percentage of the enzyme activity remained.

2.10 Temperature stability

Enzyme stability was investigated over the range of 25 °C to 65 °C (25 °C , 30 °C , 37 °C , 45 °C and 65 °C). The purified cassava SBE obtained from Sephadex G-200 column was incubated at various temperatures for 30 min and cooled immediately to 25 °C before measuring the remained activity by the method as describe in section 2.6. The result was expressed as the percentage of the enzyme activity remained.

The purified cassava SBE obtained from Sephadex G-200 column was kept at three storage temperatures, -20 °C, 4 °C and 30 °C for one month. Every week the enzyme solution was measured the remained activity by the method as describe in section 2.6. The result was expressed as the percentage of the enzyme activity remained.

2.11 Isoelectric focusing polyacrylamide gel electrophoresis (IEF)

2.11.1 Preparation of gel support film

A few drops of water was pipetted on to the plate. The hydrophobic side of the gel support film was then placed against the plate and flatly rolled the test tube to force excess water and bubbles. Subsequently, it was place down on the casting tray with the gel support film facing down so that they rest on the space bars.

2.11.2 Preparation of the gel

The gel solution of IEF was composed of the following solution (see Appendix B) :

1. 30 % acrylamide
2. 1% bis-acrylamide
3. 50 % sucrose
4. 10 % ammonium persulfate
5. TEMED

Mixed the solutions 1–3 and degased about 5 min, then added the solution 4 and 5 and carefully pipeited between the glass plate and casting tray with a smooth flow rate to prevent air bubbles. The gel was left about 45 min to allow polymerization, then lifted the gel plate from the casting tray by spatula. The gel was fixed on the gel support film and ready for used.

2.11.3 Sample application and running the gel

The sample was loaded on the small piece of filter paper to allow its diffusion into the gel foe 5 minute and the filter paper was carefully removed from the gel. The gel with the adsorbed samples was turned upside-down and directly placed on top of the graphite electrodes. Focusing is carried out constant voltage conditions in a stepwise fashion. The gel was firstly focused at 100 V for 15 minutes, followed by an increase in voltage up to 200 to 15 minutes and the finally run at 450 V for an additional 60 minutes. After complete electrofocusing, the gel was stained. Standard protein markers with known pI's in the range 3–10 were run in parallel. The pI's of sample proteins were determined by the standard curve constructed from the pI's of the standard proteins and their migration distance from cathode.

2.12 Effect of glycans on the cassava SBE activity

Different glycans were added to the reaction mixture for the assay of SBE activity at the concentration 1.0 mg/ml. The concentration of other components were the same as described in section 2.6 while the volume of the glycan solution added was adjusted with the original buffer solvent. The assay condition was the same as described in section 2.6. The result was expressed as the folds of the enzyme activity comparing with the control.

2.13 Effect of glycans on precipitation of SBE reaction products

Different glycans were test as carrier to enhance precipitation of SBE reaction product by methanol. SBE assay was performed according to section 2.6. After the reaction was stopped other glycans (glycogen, dextrin,

amylopectin and starch) was added in place of glycogen and the amount of the radioactive in the precipitate was measured using a liquid scintillation counter as described in section 2.6. The result was express as the folds of the ^{14}C counting that incorporated to methanol-insoluble glucan comparing with the control.