CHAPTER III
MATERIALS AND METHODS

3. 1 Materials

3.1.1 Laboratory instruments and others

Name	Company
Auto pipette	Bio-Rad, USA.
Balance	Mettler Toledo, PB3002
Balance	Satorius, AC2105
Slab gel dryer	SE1160
Centrifuge	Hitachi koki, Japan
Dialysis Tubing, Benzoylated	SIGMA-ALDRICH, Germany
ECONO Pump	Bio-Rad, USA
Ettan™ IPGphor	Amersham Biosciences, UK
Fraction collector (Model 2110, Bio-Rad)	Bio-Rad, USA
Freezer–70°C	Harris Manufacturing, USA
Gel dryer (SLAB, Model SE1160)	Hoefer Scientific Instruments, USA
Immobiline™ DryStrip pH 3-10, 7 cm	Amersham Biosciences, UK
Magnetic stirrer (Model MS115)	HL Instrument, Thailand
Micropipette	PYREX, Japan
Mixer (EYELA, Model MA-1)	Tokyo Rikakikai, Japan
Parafilm	Pechiney Plastic Packaging, USA
pH meter (Model pHI 34)	BECKMAN, USA
Quartz cell	Hellma, USA

3.1.1 Laboratory instruments and others (Cont.)

Name	Company
Scanning electron microscope (LEO 1450VP)	LEO Electron Microscopy, UK
Slab-gel Electrophoresis	Hoefer™ SE 260
Slab-gel Electrophoresis	Bio-Rad, USA
Spectrophotometer	Jas. Co, Japan
Spectrophotometer	Shimadzu, Japan
Speedvac concentrator (SpeedVac®Plus)	Thermosavant, USA
Strip holder	Amersham Biosciences, USA

3. 1.2 Chemicals and reagents

Name	Company
3,5-Dinitrosalicylic acid	Sigma, USA
Agar	Himedia, India
Agarose	Amersham Bioscience, USA
Acetic acid	Carlo Erba, France
Acrylamide	Bio-Rad, USA
Ammonium persulphate	Fluka, Switzerland
β-cyclodextrin	Sigma, USA
β-mercaptoethanol	Sigma, USA
Bromophenol blue-methanol solution	Sigma, USA
Bovine serum albumin (BSA)	GE Healthcare, Sweden
Calcium chloride	Rankem, India
Cobalt chloride	Rankem, India
Coomassie brilliant blue G250	Amersham Bioscience, Sweden
Coomassie Brilliant Blue R-250	Fluka, Switzerland
Cover fluid	Amersham Bioscience, Sweden
DEAE Toyopearl	Tosoh Corporation, Japan

3.1.2 Chemicals and reagents (Cont.)

Name	Company
Dithiothreitol (DTT)	USB, USA
EDTA	Rankem, India
Epoxy-activated sepharose 6B	Amersham Biosciences, USA
Ethyl alcohol	Carlo Erba, France
Glucose	Sigma, USA
Glycine, Laboratory	Scharlau, Spain
Glycerol, AR	Carlo Erba, France
Methanol, AR	Calo Erba, France
Manganese (II) chloride	Fluka, Switzerland
N, N-bis- methylene-acrylamide	Bio-Rad, USA
N, N, N', N'-tetramethylethylenediamine (TEMED)	Fluka chemika, Switzerland
Hydrochloric acid	Carlo Erba, France
Iodine	Sigma, USA
Iodoacetamide (IAA)	Amersham Bioscience, Sweden
IPG buffer pH 3-11	Amersham Bioscience, USA
Perchloric acid	Sigma, USA
Protein marker (Low molecular weight)	GE Healthcare, USA
Phophoric acid	Analar, England
Potassium chloride	Carlo Erba, France
Potassium Dihydrogen Phosphate	Rankem, India
Potassium iodide	Carlo Erba, France
Silver staining kit, Protein	Amersham Biosciences, Sweden
Sodium acetate	Rankem, India
Sodium chloride	Rankem, India
Sodium dodecyl sulphate (SDS)	Sigma, USA

3.1.2 Chemicals and reagents (Cont.)

Name	Company
Sodium hydroxide	Carlo Erba, France
Sodium thiosulphate	Merck, UK
Starch rice	Sigma, USA
Sulfuric acid	Carlo Erba, France
Triton X-100	Sigma, USA
Tris (Hydroxymethyl) methylamine	BDH, England
Zinc chloride	BDH, England

3. 2 Methods

3.2.1 Sample preparation

Ripening of Mon Thong durian (*D. zibethinus* Murr. cv. Mon Thong) was collected from Tomandee durian orchard, Rayong province, Thailand. The criteria for fruit selection are as follow: [1] There is hollow sound when either the fingertips, [2] The tips of the spines turned brown upon maturation, [3] The spines also become more flexible and can be pushed more easily than those of immature fruit., [4] The fruit emits its characteristic unique odour when it has begun to ripen. (Subhadrabandhu and Ketsa, 2001)

The fruit was photographed and peeled. Fruit pulp was weighted and sliced into small pieces. Fruit pieces were ground in a mortar and starch content was determined. Large particles were removed from the aqueous extract by filtering through many layers of gauze. Filtered samples were centrifuged at 10,000*g* for 15 minutes. Then, the supernatant was kept at -70 °C.

3.2.2 Starch and reducing sugar content determination

Starch and reducing sugar content were estimated by Iodine test and dinitrosalicylate (DNS) method to determine the ripe stage in Mon Thong durian, respectively.

Iodine test (Carter and Neubert, 1954) was used to determine starch content in Mon Thong durian. Five grams of durian pulp was ground and homogenized with 20 ml of 7.8 N perchloric acid for 10 minutes. After that, it was filtered through borosilicate glass wool and collected supernatant. The supernatant was diluted with double distilled water (1:1, 1:10, 1:50 and 1:100, respectively). The 0.8 ml of iodine solution was added in 2 ml of each dilution. For Blank, 0.8 ml of iodine solution and 0.8 ml of 0.16 N Sodiumthiosulfate was added in 2 ml of each dilution. The absorbance was measured at 620 nm.

DNS method was used to determine reducing sugar content in Mon Thong durian. In 5 ml of 100 times diluted of fruit extract, 2 ml of DNS (3, 5-dinitro-salicylic acid) reagent was added. The mixture was heated in a boiling water bath for 5 minutes. After that, the tubes were cooled to stop reaction of DNS reagent. Spectrophotometer was used to observe optical density at 540 nm. Standard curve of glucose was established and used to calculate the amount of reducing sugar.

3.2.3 Protein determination and protein clean up for analysis

Protein concentration was measured by Bradford method (Bradford, 1976). One µl of fruit extract was added with 1 ml Bradford solution and incubated for 10 minutes. The absorbance was read at 595 nm. Bovine serum albumin (BSA) was used as protein standard. BSA 2, 4, 6, 8 and 10 µg was made up to 1 ml with Bradford solution. Calibration curve of bovine serum albumin (Bio-Rad, USA) standard protein was plotted and the protein sample concentration was calculated showing as termed mg protein/ g fresh weight.

2D-Clean up kit protocol (Amersham Bioscience, Sweden) was used to prepare protein and remove contaminated substances. The 300 µl of precipitant reagent was added with 0.5-1 ml sample, stirred by vortex and incubated for 15 minutes on ice. Then, 200 µl of co-precipitant reagent was added with the reaction mixture and centrifuged at 8000g for 10 min. The supernatant was removed and discarded by using a pipette tip. The distilled water was added on the top of the layer pellet and shaken by vortex for several seconds. After that, the aliquot of 1 ml of chilled washing buffer and 5 µl of wash additive buffer was added and incubated at least 1 hr at -20 °C. The supernatant was discarded. The pellet was dried under vacuum and solubilized with rehydration buffer.

3.2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Laemmli, 1970)

Protein pattern and molecular weight of protein in fruit extract was analyzed by SDS-PAGE. The glass plate and spacers was cleaned, dried and assembled. The 13% separating gel was prepared and poured into the gel chamber bewaring of air bubbles. Double distilled water was carefully overlaid above the gel. The mixture was left for three hours for complete polymerization. After it was polymerized, 4% stacking gel was filled to the gap of the chamber. The comb was inserted and the gel was allowed to stand for one hour. After the stacking gel was set, the comb was removed. The gel was fixed in the electrophoresis equipment and filled with electrode buffer. Fruit extract (20 µg protein) was mixed with 2X solubilizing buffer with and without β-mercaptoethanol ratio 1:1 (v/v). and then, boiled at 100 °C for 5 min. After that, the mixtures were loaded and separated at 150 volt until bromophenol blue reached the bottom of the gel by using Hoefer™ SE 260

apparatus. After that, the gel was stained with Coomassie R 250 for 30 min and detained with destaining solution until the background cleared and protein appeared. Molecular weight was determined with *Rf* value by comparing with standard proteins marker (Amersham Bioscience, Sweden).

3.2.5 Zymographic method

Activity of amylase was detected by Zymographic method (Masakasu, et al., 2008) with modification. Zymographic method was performed using similar procedures as with SDS-PAGE. The 13% separating gel containing 0.1% w/v of soluble starch as final concentration was prepared and poured into the gel chamber then gently overlaid with double distilled water. The mixture was stood for three hours. After it was complete polymerization, 4% stacking gel was filled to the chamber. The comb was inserted and the gel was allowed to complete polymerize for one hour. After the stacking gel was set, the comb was removed. The gel was set in the electrophoresis equipment. Protein samples (20 µg) were mixed with solubilizing buffer with and without β-mercaptoethanol ratio 1:1 (v/v) and then, the sample was mixed with solubilizing buffer with β-mercaptoethanol was heated at 100 °C for 5 min. Samples were loaded and separated at 150 V until the tracking dye reached the bottom of the gel. The gel was incubated in 1% triton X-100 solution for 1 hr to remove SDS and incubated in activity solution (mixture of 0.2% starch with 10 mM Sodium acetate buffer pH 4.8, 2 mM $CaCl_2$) for 2 hr at 37 °C and incubated gel was stained with activity staining solution (1% acetic acid containing 10 mM I_2 and 14 mM KI). After incubation, amylase activity was located as a transparent band.

3.2.6 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Liquid chromatography tandem mass spectrometry (LC-MS/MS)

2D-PAGE and LC-MS/MS strategies were used to analyze the partial amino acid sequence amylases. The clean protein (20 µg) were separated in the first dimension with IPG dry strip (pH 3-11 NL, 7 cm). The sample was mixed with rehydration buffer containing DTT 0.001 grams and 1 µl of IEF buffer. The strips were rehydrated for 12 hr. After loading, the strip was covered with the immobiline strip cover fluid to certify that strip did not dry out during running and then, focusing started at 500 V for 30 minutes, continually focusing on 1,000 V for 30 minutes the focusing finished at 5,000 V for 100 minutes. The isoelectric focusing, which total at

8 kVh was performed by using the Ettan™IPGphor™Isoelectric Focusing System (Amersham Biosciences, USA). After completion of the IEF and reduction and alkylation steps, the protein separated on IPG strips were separated on second dimension electrophoresis. The focused strip was equilibrated in equilibration buffer containing DTT and followed by equilibration buffer with IAA, which were incubated in room temperature for 15 minutes, respectively. The second dimension was separated on 13% SDS-PAGE (Hoefer™ SE 260, USA) and performed for 15 min at 10 mA/gel, approximately 2 hr at 20 mA/gel until the bromophenol blue front reached the bottom of the gel. Separated gel was fixed with fixing solution for 60 minutes. Then, the gel was stained with coomassie brilliant blue G 250 staining solution for 1 hr and destained with double distilled water for 1 hr. The protein spots were analyzed by Image master 2D platinum program. The interesting spots were analyzed by LC-MS/MS at Genome Institute, National Center for Genetic Engineering and Biotechnology Thailand. The ion spectra of peptides were searched against NCBI database using MS/MS Ion Search mode of MASCOT (http://www.matrixscience.com /search_form _select. html).

3.2.7 Purification of amylase from Mon Thong durian

3.2.7.1 Ammonium sulphate fractionation

All purification steps were done at 4 °C. The crude extract was precipitated with 70% ammonium sulphate saturation to remove contaminated substances. The ammonium was placed over night in 120 °C drying oven in a large beaker or drying dish (ammonium sulfate decomposes at 220 °C) and ground dry ammonium sulphate to a fine powder in a mortar. The ground powder (21.8 gram) was added with fruit extract (50 ml) slowly but steadily with thorough mixing and do not allow clumps to form. After the ammonium sulphate was completely dissolved to 70% saturation, the saturation was allowed to stir for another 1 hours to complete equilibration between the dissolved and aggregated proteins. Protein precipitation was collected by centrifugation at 10,000 *g* for 30 minutes to pellet the protein. Then, it was dissolved in a small volume of buffer and dialyzed with 50 mM acetate buffer, pH 6.0 containing 5 mM $CaCl_2$ for 4 hr and overnight with three changes of buffer to remove ammonium sulphate.

3.2.7.2 Purification of amylase from Mon Thong durian using Epoxy-activated Sepharose 6B affinity chromatography (modified from Kumari et al., 2010, Elarbi et al., 2009, Tripathi et al., 2007)

Affinity chromatography was used to purify amylase from Mon Thong durian using Epoxy-activated Sepharose 6B was ligated with β-cyclodextrin (Dominic et al., 2000, GE Healthcare). Epoxy-activated Sepharose 6B was weighed to 1 g (1 g freeze-dried powder gives about 3.5 ml final volume of medium) and suspended in distilled water. The medium will be swelled immediately and should be washed for 1 hour on a sintered glass filter. Approximately 200 ml distilled water per gram freeze-dried powder was added in several aliquots. The 0.794 gram (700 μmoles) of β-cyclodextrin was mixed with 3.5 ml of 0.1 M NaOH. After that, the medium was mixed with β-cyclodextrin solution (ligand) in a stoppered vessel using a shaker in a water bath for 24 hours at 37 °C. The mixtures were washed away excess ligand using 0.1 M NaOH and blocked any remaining active groups, which the medium was transferred with 2.5 % glucose solution. After standing for overnight at 37 °C, the mixture (resin) was washed thoroughly with at least three cycles of alternating pH. Each cycle should consist of a wash with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer pH 8 containing 0.5 M NaCl.

The resins were resuspended and carefully poured down using the glass rod to the column (1.5×15 cm) that sealed the bottom with cotton wool. After washing and equilibrating with 20 mM sodium acetate buffer, pH 6.0 for 3 column volumes, the partial purified proteins from ammonium sulphate fractionation (2 mgProtein) was applied to a column, pre-equilibrated with 50 mM acetate buffer, pH 6.0 containing 5 mM $CaCl_2$ at a flow rate of 0.5 ml/min. Then, the column was washed with 20 mM acetate buffer, pH 6.0 containing 25 mM $CaCl_2$ and 0.5 M NaCl. The fractions were collected 1.5 ml per fraction and measured the absorbance at 280 nm until the absorbance of column returned to base line. After that, column was eluted with 10 mg/ml β-cyclodextrin in washing buffer. The fractions were measured at 280 nm to verify elution profile and assayed the activity of amylase as described by step 3.2.8. The purified fractions were checked for purity by SDS-PAGE analysis. Then, the active fractions were pooled and dialyzed with 50 mM acetate buffer, pH

6.0 containing 5 mM $CaCl_2$ and concentrated using speedvac concentrator for further purification.

3.2.7.3 Purification of amylase from Mon Thong durian using DEAE Toyopearl Anionic Exchange Chromatography

DEAE Toyopearl anionic exchange beads were stirred in 0.5 M NaOH for 30 min at room temperature. The beads were filtered by suction and washed with double distilled water until the pH of filtrate was neutral. The beads were stirred in 0.5 M HCl for 30 min at room temperature. After filtering and washing, the beads were repeatedly stirred with 0.5 M NaOH once, then filtering and washing again. The column bottom (1×5 cm) was sealed with glass cotton wool and carefully packed with the beads. The bead was resuspended and filled in the column using glass rod. The beads were set down and the excess supernatant was discarded. Before filling the column again, the top of the gel was stirred for completely setting. The column was equilibrated with 50 mM phosphate buffer, pH 7.5 for 2 column volumes at a flow rate of 0.5 ml/min. The partial purified proteins from Epoxy-activated Sepharose 6B affinity chromatography were loaded and washed with 50 mM phosphate buffer, pH 7.5 for 3 column volumes. The fractions were collected at 0.25 ml per fraction and measured the absorbance at 280 nm. When the absorbance drop to baseline, the protein in column was eluted with 0 to 0.5 M NaCl. The fractions were measured at 280 nm to monitor the elution profile and assayed activity of amylase. Purity of fractions at the peak was checked by SDS-PAGE analysis.

3.2.7.4 Activity analysis of amylase (modified from Noman et al., 2006)

Activity of amylase was assayed by the dinitrosalicylate (DNS) method. Reaction mixtures of three sets (blank, control and sample) contained 1 ml of 1% soluble starch solution in 0.1 M phosphate buffer pH 7.0 containing 20 mM NaCl and 2 mM $CaCl_2$. The reaction mixtures were mixed and incubated at 37 °C for 10 minutes. After, 0.25 ml of enzyme was added to sample tube. For control tube, 0.25 ml of 2 M NaOH and 0.25 ml of enzyme were added. In blank tube, 0.25 ml of distilled water was added in place of enzyme. Then, three set of tubes were incubated at 37 °C for 15 min with 1,000 rpm shaking speed and 0.25 ml of 2 M NaOH was added to sample and blank tubes to stop the reaction. After that, 0.25 ml of DNS

reagent was added and it was vortexed. Then, the tubes were heated at 100 °C for 5 minutes. The absorbance was measured at 540 nm. Standard curve of glucose was established and used to calculate the amount of reducing sugar. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of reduced sugar per minute under standard assay conditions.

3.2.8 Enzyme characterization using DNS method

3.2.8.1 Optimum pH determination

The activity of amylase was analyzed as described on Amylase activity assay (Step 3.2.7.4) by varying pH values from pH 4-9. The pH conditions were adjusted using the following buffers: 0.1 M sodium acetate buffer for pH 4-6, 0.1 M potassium phosphate buffer pH 6-8 and 0.1 M Tris-HCl buffer pH 8-10. After that, % Relative of activity (percentage of the maximum activity) was calculated.

3.2.8.2 Optimum temperature determination

The activity of amylase was analyzed as described on Amylase activity assay (Step 3.2.7.4) by increasing temperature from 30 to 100 °C. The temperatures were controlled by water bath. After that, % Relative activity (percentage of the maximum activity) was calculated.

3.2.9 Enzyme characterization using Zymographic method

3.2.9.1 Optimum pH determination

The optimum pH of amylase was determined by the activity assay in different pH values. The partial purified amylase (0.01 μg Protein) was mixed with solubilizing buffer without β-mercaptoethanol ratio 1:1 (v/v). Then, the sample was analyzed by Zymographic method (Step 3.2.5) by varying pH values of activity solution from pH 4 to 10 and incubated 6 hr at 37 °C. The pH conditions were adjusted using the following buffers: 10 mM sodium acetate buffer for pH 4-6, 10 mM potassium phosphate buffer pH 6-8, 10 mM Tris-HCl buffer pH 8-10.

3.2.9.2 Optimum temperature determination

The optimum temperature was determined by amylase activity assay at different temperature. The partial purified amylase (0.01 μgProtein) was mixed with solubilizing buffer without β-mercaptoethanol ratio 1:1 (v/v). Then, the sample was analyzed following Zymographic method (Step 3.2.5) by increasing

temperature of incubation from 30 to 100 °C in activity solution (pH 7.0) for 6 hr. The temperatures were controlled by water bath.

3.2.9.3 pH stability determination

Stability of the enzyme at different pH values was determined by measuring the residual activity after incubating the partial purified amylase (0.005 μg Protein) in buffers with various pH. The partial purified amylase was incubated in different pH values at pH 4-10 for 30 min at 37 °C. After incubation, the enzyme solution was cooled and mixed with solubilizing buffer without β-mercaptoethanol ratio 1:1 (v/v). The remaining activity was measured by Zymographic method (Step 3.2.5) at incubation 6 hr, 37 °C. The pH condition were controlled by 50 mM sodium acetate buffer for pH 4-6, 50 mM potassium phosphate buffer pH 6-8, 50 mM Tris-HCl buffer pH 8-10.

3.2.9.4 Heat stability determination

Stability of the enzyme at different temperature values was determined by measuring the residual activity after incubating the partial purified amylase (0.05 μgProtein). The partial purified amylase was incubated at 30 to 100 °C for 30 min in 50 mM potassium phosphate buffer pH 7.0. After incubation, the enzyme solution was cooled and mixed with solubilizing buffer without β-mercaptoethanol ratio 1:1 (v/v). The remaining activity was assayed by Zymographic method (Step 3.2.5) at incubation 6 hr, 37 °C.

3.2.9.5 Effect of metal ion and EDTA

The effect of metal ion and EDTA on enzyme activity were determined by pre-incubating the partial purified amylase (0.01 μgProtein) with different metal ions and EDTA (5 mM of final concentration) for 30 min. After incubation, the enzyme solution was mixed with solubilizing buffer without β-mercaptoethanol ratio 1:1 (v/v). The remaining activity was measured by Zymographic method (Step 3.2.5) at incubation time for 6 hr, 37 °C.