CHAPTER 3

METERIALS AND METHODS

1. Materials

1.1 Instruments

Instrument	Model	Company
Autoclave	HA-300MTI	Hirayama, Japan
Automatic fraction collector	2110	Bio-rad, USA.
Analytical balance 2 dig	SE 2020	OHAUS, USA
Analytical balance 4 digits	BP110S	Sartorius, Germany
Bench- top refrigerated centrifuge	5804R	Eppendorf, Germany
Freeze-dryer	DW6-85	Heto Drywinner, Denmark
Fourier transform infrared spectrophotometer (FTIR)	FTIR-8900	Shimadzu, Japan
Hot air oven	LR-270	The grieve Co., USA
Micro-pipettes	P200, P1000	Gilson, France
Micro-pipettes	P10, P50, P250	Merck, France
Micro-pipettes	P2.5	Eppendorf, Germany
Magnetic stirrer	HB 502	Bibby Sterilin, UK
Micro tube pump	MP-3	Eyela, Japan
Minicentrifuge	C-1200	National Labnet, USA
pH meter	Ecoscan	Utechinstrument, Singapore
Rotary evaporator	R-314	Büchi, Germany
Superspeed refrigerated centrifuge	JA-21	Beckman, USA

Instrument	Model	Company
Ultracentrifuge	L8-70M	Beckman, USA
UV-Vis recording spectrophotometer	UV 160 A	Shimadzu, Japan
Vortex-2 Genie	G-560E	Scientific Industries, USA
Water bath	WB22	Scientific Industries, USA

1.2 Chemicals (Analytical grade)

Chemical	Company
Ammonium sulfate	BDH Chemical Ltd.
Acetic acid	J.T. Baker
Bovine serum albumin	Sigma Chemical Co.
Calcium chloride	Merck
3,5-Dinitrosalicylic acid	Fluka
Ethyl acetate	Lab-scan
Ethanol	Merck
Filter paper	Whatman
Alpha-glucosidase (*Saccharomyces cerevisiae*)	Sigma Chemical Co.
Hexanes	Lab-scan
Hydrochloric	Merck
Invertase (Yeast sucrase)	Sigma Chemical Co.
β-mercaptoethanol	BDH
Methanol	Lab-scan
Magnesium sulfate	J.T. Baker
Liquid nitrogen	TIG Co.

Chemical	Company
Potassium chloride	Ferak
Polyvinyl polypyrrolidone(PVP)	Sigma Chemical Co.
Phenylmethylsulfonyl fluoride(PMSF)	Sigma Chemical Co.
Salivary α-Amylase	Sigma Chemical Co.
Sephadex G-75	Phamacia
Sodium chloride	Lab-scan
Sodium acetate	Carlo erba
di-Sodium hydrogen orthophosphate	APS Finechem
Sodium dihydrogen phosphate dihydrate	Sigma Chemical Co.
Tris (hydroxymethyl) amino methane	Sigma Chemical Co.
Glucose oxidase Kit	Wako Pure Chemical Industries, Ltd

1.3 Plant and insect materials

Fresh green pericarps of Sataw (*Parkia speciosa*) were purchased from local market in Songkhla province, Thailand. Mung bean seeds (*Vigna radiata* Wilczek.) were purchased from local market. *Callosobruchus chinensis* and *Callosobruchus maculates* were obtained from the Department of Biochemistry, Kasetsart University Bangkok, Thailand. *Sitophilus oryzae* Linnaeus were collected from rice (*Oryza sativa*).

2. Methods

2.1 Preparation of *Parkia speciosa* Hassk. pericarp powder

Freshly purchased *Parkia speciosa* Hassk. was cleaned and seeds were separated from their pods. The pericarps were ground into powder with a mortar under liquid nitrogen. The powder was weighed and subjected for studies.

2.2 Assay of α-amylase activity

In this study, amylase activity from various source including saliva, pancreas and insects was assayed according to Bernfeld method (Bernfeld, 1955). Amylase solution in 0.02 M sodium phosphate buffer pH6.9 containing 0.01 M NaCl (Buffer A) was measured for its activity by hydrolyzing 0.2 %(g/v) starch solution in Buffer A at 37°C for 3 min and the reaction was assayed for mg maltose produced with DNS reagent (3,5-dihydrosalicylicacid in 0.4 M NaOH) spectrometrically at 540 nm.

One unit of amylase activity was defined as the amount of enzyme that liberated 1 mg maltose from starch under pH 6.9 at 37°C in 3 min.

2.3 Inhibition assay of amylase inhibitor on α-amylases

Alpha-amylase inhibitory activity was measured by adding equal amount of test sample to α-amylase solution (constant activity 0.26 Unit activity) in 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl (Buffer A), preincubated for 30 min at 37 ^{o}C and assayed for nonbinding amylase activity by adding 2%(g/v) starch solution as substrate. After the incubating of the mixture for 3 min at 37^{o}C, the reaction was stopped by the addition of DNS (3, 5-dinitrosalicylic acid in 0.4 M NaOH), boiled, diluted with water and measured for its absorbance at 540 nm using Shimadzu spectro photometer model UV 160 A.

One unit of amylase inhibitory activity was defined as the amount of inhibitor that inhibits one unit of amylase activity under the assay conditions. And the concentration of inhibitor required to inhibit 50% of α-amylase activity under the assay conditions was defined as IC_{50} value. To obtain IC_{50} value, the sample was diluted to various concentrations and subjected to the above assay procedure. Percent inhibition, calculated on the basis of the left over α-amylase activity divided by the initial activity, was plotted against diluted sample concentrations and the IC_{50} value was obtained from the plot.

2.4 Comparative study of five methods for α-amylases inhibitor extraction efficiency

The powder of fresh green pericarp of *Parkia speciosa* Hassk. was weighed and extracted for α-amylases inhibitor according to the following five methods.

1. Method of Grant *et al.*, (1995)

Powder of the pericarp was extracted (1: 5 w/v) with 0.02 M sodium phosphate buffer pH 6.9 containing 0.15 M NaCl for 16 hours at 4 °C, followed by centrifugation at 50,000 x g for 20 min at 4 °C using Beckman L8-70M. The precipitate was discarded and the supernatant was heated at 70°C for 10 min, cooled on ice and centrifuged as before. The clear supernatant was used for the estimation of AI content according to 2.3

2. Method of Grant *et al.*, (1995) without heat treatment

Powder of the pericarp was extracted (1: 5 w/v) with 0.02 M sodium phosphate buffer pH 6.9 (1 : 5 w/v) containing 0.15 M NaCl for 16 hours at 4 °C, followed by centrifugation at 50,000 x g for 20 min at 4 °C using Beckman L8-70M. The precipitate was discarded and supernatant was used for the estimation of AI content as described in 2.3.

3. Method of Pueyo and Delgado- Salinas (1997)

Powder of pericarp was extracted (1: 10 w/v) with 10 mM Tris-HCl pH 7.5 containing 10 mM β-mercaptoethanol, 0.1 M NaCl, 0.2 mM phenylmethyl-sulfonyl fluoride (PMSF) by stirring for 10 min at 4 °C, followed by centrifugation at 15,000 x g for 10 min at 4°C using Beckman JA-20. The precipitate was discarded and the supernatant was used for the analysis of AI content as described in 2.3

4. Method of Giri and Kachole, (1998)

Powder of pericarp was defatted with hexane for 5 min and filtered through Whatman paper #1. The defatted pericarp powder was added 0.1 M HCl containing 0.1 M NaCl and 1% PVP (1: 6 w/v) and was stirred for about 2 hours at 4 °C. The extract was centrifuged at 12,000 x g for 10 min at 4 °C using Beckman JA-20. Supernatant was adjusted to pH 7.0 with 1 M NaOH. The precipitate obtained was removed by centrifugation at 12,000 x g for 10 min at 4 °C using Beckman JA-20. The supernatant was used for the analysis of AI content (see 2.3).

5. Method of Marshall and Lauda, (1975)

Powder of pericarp was extracted (1: 3 w/v) with 1% sodium chloride solution (NaCl) by stirring for 1 hour at room temperature. After extraction, the extract was centrifuged at 27,000 x g for 30 min at room temperature using Beckman L8-70 M, the supernatant solution was heated at 70 °C for 15 min. Coagulated protein was removed by centrifugation (27,000 x g, 60 min at 25°C) and the supernatant was used for analysis of α-AI content (see 2.3).

2.5 Efficacy of ammonium sulfate ($AmSO_4$), ethanol and methanol on α-amylase inhibitor precipitation

Precipitation is one of the simplest methods for partial purification of interested compound from other contaminants. This study aimed to find a most suitable chemical in precipitating α- amylase inhibitor with high activity and yield.

Powder of fresh pericarp of *P. speciosa* Hassk. was weighed and extracted (1: 5 w/v) in buffer according to the method of Grant *et al*., (1995) without heat treatment as previously described. The supernatant (crude extract) was added the chemicals as follows.

Ammonium sulfate precipitation : Crude extract was added $AmSO_4$ to obtain 30, 40, 45, 50, 60, and 80 % saturations and gently stirred with glass rod and kept at 4 °C for 2 hours. The precipitate was recovered by centrifuging at 10,000 x g for 20 min at 4°C and redissolved in Buffer A to the initial volume. The redissolved solution of each % $AmSO_4$ was assayed for α- amylase inhibitory activity, mg protein (Lowry, 1951) and specific inhibitory activity.

Ethanol precipitation : Crude extract was added ethanol at 20, 30, 40, 50, 60, 80 and 95 % (crude extract : ethanol 1: 4 v/v) gently stirred with glass rod and kept at 4 °C for 2 hours and centrifuged at 10,000 x g for 30 min at 4 °C. The precipitate was collected and redissolved in Buffer A to the initial volume used for precipitation. The clear supernatant of ethanol was evaporated at 175 mb, 40°C and the remaining liquid was lyophilized to dry powder and redissolved with distilled water to its initial volume. The redissolved solution of precipitated and supernatant from each %ethanol was assayed for α- amylase inhibitory activity, mg protein and specific inhibitory activity.

Methanol precipitation : Crude extract was added methanol at 20, 30, 40, 50, 60, 80 and 95 % (crude extract : methanol 1: 4 v/v) gently stirred with glass rod and kept at 4 °C for 2 hour and centrifuged at 10,000 x g for 30 min at 4 °C. The precipitate was collected and redissolved in Buffer A to the initial volume used for precipitation. The clear supernatant of methanol was evaporated at 337 mb, 40°C and the remaining liquid was lyophilized to dry powder and redissolved with distilled water to its initial volume. The redissolved solution of precipitated and supernatant from each %methanol was assayed for α- amylase inhibitory activity, mg protein and specific inhibitory activity.

2.6 Partial purification of α-amylase inhibitor by gel filtration

2.6.1 Extraction of α-amylase inhibitor

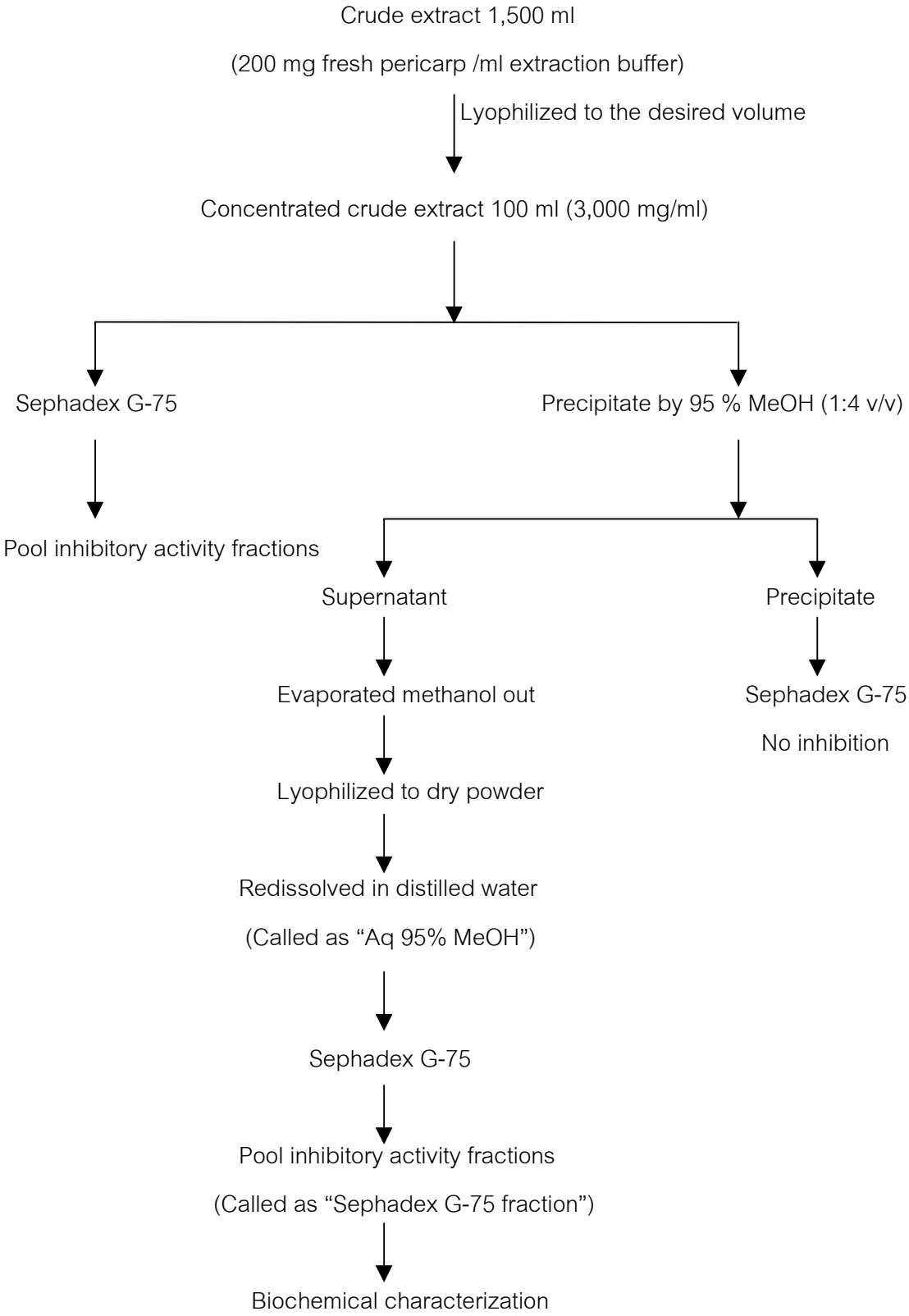
The powder of fresh green pericarp (300g) was extracted (1: 5 w/v) in 1,500 ml of 0.02 M sodium phosphate buffer pH 6.9 containing 0.15 M NaCl for 16 hours at 4 °C according to method of Grant *et al*., (1995) without heat treatment, followed by centrifugation at 50,000 x g for 20 min at 4 °C and precipitate was discarded. Supernatant (crude extract, 0.2 g pericarp powder/ml) was lyophilized and concentrated by freeze dryer to obtain 15-fold of the original crude extract concentration (3 g pericarp powder/ml). The concentrated crude extract was kept at 4 °C for further study, including inhibitory activity assay, protein determination, and purification and other studies.

2.6.2 Precipitation of α-amylase inhibitor with methanol

An aliquot of 4 ml of the concentrated crude extract was added 95% methanol (1: 4 v/v) gently stirred with glass rod and kept at 4 °C for 2 hour and centrifuged at 15,000 x g for 30 min at 4 °C. The precipitate was collected and redissolved in Buffer A to the initial volume (4 ml). One half of its volume was loaded onto Sephadex G-75 for partial purification while the other half was used for protein determination and inhibitory activity assay.

The supernatant was collected and methanol evaporated off at 337mb, 40°C. The aqueous was lyophilized to dry powder. The powder was redissolved in distilled water to its initial volume (4 ml) (Aq 95% MeOH) and subjected to partial purification, the inhibitory activity assay and protein determination as the redissolved solution of the precipitate.

Diagram of the Purification Process



2.6.3 Partial purification of α-amylase inhibitor by gel filtration.

Crude extract, redissolved precipitate and redissolved solution of Aq 95% MeOH were separately loaded onto Sephadex G-75 column (117 x 0.6 cm) equilibrated with buffer A. The column was eluted with buffer A at 30 ml/hour flow rate until the absorbance unit at 280 nm fell to 0-0.1. Fractions (3 ml) were collected and assayed for their ability to inhibit amylase activity. Inhibitory active fractions were combined and lyophilized to powder. The powder was redissolved in water and named as "Sephadex G-75 fraction" and this solution was used for further studies.

2.6.4 Verification of amylase inhibitor purity

Purity of amylase inhibitor in the pooled fractions obtained from Sephadex G-75 column was checked by thin layer chromatography (TLC). An appropriate amount (2 µl) of sample was spotted onto a 10 x 1.5 cm glass plate coated with Merck silica gel powder. The plate was developed with H_2O: ethyl acetate : glacial acid (0.5 : 5 : 1 v/v/v). After the plate was dried, the separation was detected under UV lamp at wavelength 366 nm.

2.7 Property studies of the inhibitor against salivary α-amylase

2.7.1 Effect of temperature on inhibitory activity of the inhibitor

To study the effect of temperature on the inhibitory activity of α-amylase inhibitor, test samples were preincubated with the salivary α-amylase in Buffer A for 30 min at various temperature from 4 °C, 25 °C, 37°C, 40 °C, 50 °C, 60 °C, 80 °C and 90°C respectively. After adjusting temperature to 37 °C, 2%(g/v) starch was added to the reaction mixture and further incubated at 37 °C for 3 min. The reaction was stopped by adding DNS and the remaining amylase activity was spectrophotometrically determined at 540 nm as described previously.

2.7.2 Stability of the inhibitor in various temperatures

To study thermal stability of inhibitor, test samples in Buffer A were maintained at 4 °C, 25 °C, 37°C, 40 °C, 50 °C, 60 °C, 80 °C and 90°C respectively for 30 min. The samples were adjusted to 37 °C and salivary α-amylase was added and determined for its activity as previously described.

2.7.3 Effect of pH on inhibitory activity of the inhibitor

To determine the effect of pH on the inhibition of the inhibitor against α-amylase, the following 0.02 M buffers containing 0.015 M NaCl were prepared: sodium acetate pH 5.0; phosphate buffer pH 6.0, 6.9, 7.0 and 8.0; and Tris-HCl pH 9.0. The test samples were preincubated with the salivary α-amylase at each pH for 30 min at 37 °C. After that, 2% (g/v) starch was added to start the reaction, incubated for 3 min at 37 °C and the reaction was stopped by adding DNS. The remaining amylase activity was spectrophotometrically determined at 540 nm.

2.7.4 Stability of the inhibitor in various pH

The effect of pH on stability of amylase inhibitor was measured at pHs 5.0, 6.0, 6.9, 7.0, 8.0 and 9.0 using the same buffer systems as a described in effect of pH on inhibitory activity of the inhibitor. Test samples were maintained at each various pHs buffer system for 30 min at 4 °C, adjusted to pH 6.9 and incubated with salivary α-amylase for 30 min at 37 °C. The remaining amylase activity was determined using 2%starch as substrate as previously described.

2.7.5 Effect of salt on inhibitory activity of the inhibitor

To study the effect of salt on α-amylase inhibitory activity tested samples were preincubated with α-amylase in 0.02 M phosphate buffer pH 6.9

containing 0.015 M of various salts as follow :- sodium chloride, calcium chloride, potassium chloride and magnesium sulfate for 30 min at 37 °C, then the remaining amylase activity was determined as previously described.

2.8 Potential application of α-amylase inhibitor on blood glucose reduction *via* luminal enzyme *i.e.* pancreatic α-amylase, α-glucosidase and sucrase

2.8.1 Effect of inhibitor on pancreatic α-amylase

Hog pancreas obtained from slaughterhouse (Department of animal science, Faculty of Natural Resource, Prince Songkla University) was sliced and homogenized (1 : 5 w/v) with 0.02 M sodium phosphate buffer pH 6.9 containing 0.15 M NaCl and then followed with centrifugation at 50,000 x g for 20 min at 4 °C using Beckman L8-70M. The supernatant was collected and designated as crude porcine pancreatic α-amylase solution. Its amylase activity was measured as a previously describe.

The test inhibitor sample was preincubated with known activity unit of the crude porcine pancreatic α-amylase for 30 min at 37°C and nonbinding α-amylase was determined using 2% (g/v) starch solution as a substrate as previously described.

2.8.2 Effect of the inhibitor on α-glucosidase

In this study α-glucosidase from yeast (Sigma Co.) and porcine small intestine were used. Maltase (α-glucosidase) was dissolved in buffer A to obtain its activity equivalent to salivary α-amylase (0.560 mg glucose production at 37°C 3 min). Porcine small intestine from the slaughterhouse was chopped into small pieces and homogenized (1 : 5 w/v) in 0.02 M sodium phosphate buffer pH 6.9 containing 0.15 M NaCl. After centrifugation at 50,000 x g for 20 min at 4 °C the supernatant was

collected, divided into small aliquots and kept at -20°C for further studies. This supernatant was named as crude porcine α-glucosidase solution.

Alpha-glucosidase activity of these solutions were determined by adding 2% maltose solutions in buffer A as a substrate and incubated at 37°C for 30 min. The reaction was stopped in boiling water for 5 min, cooled to room temperature, added glucosidase kit and incubated at 37°C for 10 min. The solution was diluted with distilled water and measured for the absorbance at 505 nm along with standard glucose. One unit of α-glucosidase activity activity was defined as the amount of enzyme that liberates 1 mg glucose under the assay conditions, pH 6.9, 37°C for 3 min.

For the inhibitory activity assay, equal amount of inhibitor and enzyme solutions were preincubated at 37°C for 30 min and the nonbinding α-glucosidase was determine as the described above. One unit of inhibitory activity is the amount of inhibitor that inhibits one unit of α-glucosidase activity under the assay conditions. The concentration of inhibitor required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC_{50} value. IC_{50} value of the sample was obtained from the plot of %inhibition of α-glucosidase against diluted sample concentrations.

2.8.3 Effect of inhibitor on yeast sucrase

In this study sucrase from yeast (Sigma Co.) and porcine small intestine (from the slaughterhouse) were used.

Sucrase activity of these solutions were determined by adding of 2% sucrose solutions in buffer A as a substrate and incubated at 37°C for 30 min. The reaction was stopped in boiling water for 5 min, cooled to room temperature, added glucosidase kit and incubated at 37°C for 10 min. An aliquot of the mixture was removed to a new tube and diluted 10 times before measurement of the absorbance at 505 nm. One unit of sucrase activity was defined as the amount of enzyme that liberates 1 mg glucose under the assay conditions, pH 6.9, 37°C for 3 min.

For the inhibitory activity assay equal amount of inhibitor and enzyme solutions were preincubated at 37°C for 30 min and the nonbinding sucrase was determine as described above. One unit of inhibitory activity is the amount of inhibitor that inhibits one unit of sucrase activity under the assayed conditions. And, the concentration of inhibitor required to inhibit 50% of sucrase activity under the assay conditions was defined as the IC_{50} value. IC_{50} value of the sample was obtained from the plot of %inhibition of sucrase against diluted sample concentrations.

2.8.4 Potency of α-amylase inhibitor against maltase and sucrase comparing to α-amylase

To compare the potency of the inhibitor in inhibiting maltase and sucrase in comparison to salivary α-amylase, kinetic study of the there enzymes were determined as a follows.

Inhibition modes of test samples against salivary α-amylase activities were measured with various concentrations of starch, the substrate in the absence or presence of test sample at concentrations that provided 25% inhibition (IC_{25}) and 50% inhibition (IC_{50}) of the enzyme activity. Starch solution at 0.25, 0.5, 1.0 and 2.0% (g/v) were added to the mixture of salivary amylase and test samples to start the reaction and incubated at 37 °C. After 3 min and DNS solution was added to stop the reaction. The remaining amylase activity was determined as previously described. Inhibition type was determined by Lineweaver- Burk plot, (v) is initial velocity and [s] is the substrate concentration used.

Kinetic of the inhibitor against α-glucosidase was determined similarly to those α-amylase with the exception that the substrate was maltose.

Kinetic of the inhibitor against sucrase was determined similarly to those α-amylase with the exception that the substrate was sucrose.

2.9 Potential application of the inhibitor on pest control

2.9.1 Extraction of insect α-amylase

Alpha-amylase was extracted from the 3 day old adults of two selected insects, *Callosobruchus chinensis* and *Callosobruchus maculatus.* After freezing to death at -20°C, the insect were homogenized with a mortar in buffer A (2 g insect : 8 ml buffer for *C. chinensis* and 2 g insect : 4 ml buffer for *C. maculatus)* under ice-cool condition. The homogenate was centrifuged at 10,000 x g for 20 min at 4°C and the supernatant was collected and designated as crude amylase solution. The crude amylase solution of each insect was subjected for determination of amylase activity and amount of protein. The rest of solution was divided into small aliquots and kept at -20 °C for further studies.

2.9.2 Inhibition of the inhibitor on insect amylase activity

Inhibitory activity of the test samples against insect amylase activity was performed by adding 0.1 ml of crude amylase solution to 0.1 ml of tested samples in buffer A. The mixture was preincubated for 30 min at 37 °C and assay for nonbinding amylase activity as described previously.

2.9.3 Effect of inhibitor on the growth of insect pest in artificial beans

Dry mung bean (*Vigna radiata* Wilczek.) seeds were ground with a mortar into powder. One gram of the powder was mixed with 4 ml of crude extract, Sephadex G-75 fraction, buffer A or distill water (as a control) respectively. The mixture was formed into a columnar shape, 5mm in dia. (about 7 artificial beans/1 g) with a hand compressor, the artificial bean (1g) were placed in a plastic dish where 14 adults of the adzuki bean weevil (7 males and 7 females) were introduced. The dishes were kept at

room temperature for 3 days, after which the adults were removed. After 35 days, the numbers of adult insects emerging from the artificial beans was counted.

2.9.4 Effect of inhibitor on the growth of insect pest in mungbean seeds

Dry mungbean (*Vigna radiata* Wilczek.) seeds (1g) were coated with 4 ml of crude extract, sephadex G-75 fraction, buffer A and distill water (as a control) respectively. The coated mung bean seeds were left for dryness at room temperature for overnight. The seeds were placed in a plastic dish where adults of the adzuki bean weevil were introduced. The dishes were kept at room temperature for 3 day, after which the adults were removed. After 35 days, the number of adult insects emerging from the artificial beans was counted.

2.10 Identification of Sephadex G-75 fraction

According to literature review many of nonproteinaceous amylase inhibitors from plants belong to a group of phenolic compound (Kandra *et al*., 2004; McCue *et al*., 2004). To preliminary identify if the active amylase inhibitor in Sephadex G-75 fraction is in a group of phenolic compound three following methods were used.

2.10.1 Reacting with Folin Reagent

An aliquot 0.1 ml of test sample plus 0.9 ml distilled water, were added with 0.5 ml 50% Folin-Ciocalteu's phenol reagent (stock 2 N; Sigma Chemical Co.,), vortexed, and allowed to incubate for 5 min. Next, 1 ml of 5% (w/v) sodium bicarbonate solution was added to each sample, vortexed, and incubated in a dark cupboard for 1 hour. After 1 hour, the samples were vortexed again and observed for the change of colour to a positive blue colour of phenolic compound.

2.10.2 **Fourier transform infrared** spectrophotometer (FTIR)

A powder of SephadexG-75 fraction and that was hydrolyzed in 2 M HCl (see 2.10.3) were each ground thoroughly with KBr (potasium bromide) at approximate ratio of test sample per KBr at 1:3 or 1:4 by weight and pressed it into a thin disk with a thickness of about 0.5 mm. The KBr pellet was analyzed by Fourier transform infrared spectrophotometer (FTIR-8900 Shimadzu, Japan), The spectra were recorded from 400 cm^{-1} to 4000 cm^{-1} using a Shimadzu FTIR 8900 spectrometer, Pure KBr containing no sample was used as reference background. Gallic acid and tannic acid were also run under the same condition as a known standard compounds.

2.10.3 Thin layer chromatography (TLC)

A powder of Sephadex G-75 fraction at 0.105 g (equivalent to 1 ml of concentrate crude extract) was hydrolyzed in 2 M HCl (1 : 1 v/v) at room temperature for 30 min. Then, the ether solution was added to hydrolysate at 1:1 volume ratio, shake gently, the phenols were taken into ether and the ether extract was washed, dried and evaporated to dryness. The residue was redissolved in 0.1 ml ether, and its 2 μl (0.026 g of fresh sataw pericarp) were spotted on silica gel TLC plate and performed two-dimension chromatography using acetic acid-chloroform (1 : 9 v/v) in the 1^{st} dimension and ethyl acetate-benzene (9 : 11 v/v) in the 2^{nd} dimension. After the development each separated spots were detected by spraying with Folin reagent, followed by fuming the plate with ammonia (NH_4OH) vapour. Folin reagent gave blue color with gallic acid, 3, 4-dihydroxy benzoic acid, and hydroquinone while the following compounds gave a blue colour with Folin reagent after fuming with ammonia; 2, 5-dihydroxybenzoic acid, rhododendrol, orcinol, *p*-hydroxybenzoic acid, syringic acid, vanillic acid and salicylic acid. The retention factor (R_f) values were calculated from the distance traveled by the compound divided per the distance traveled by the solvent

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

The R_f values of sample were compared with the R_f values of each following standards : gallic acid, *p*-hydroxybenzoic acid, 2, 5-dihydroxybenzoic acid, salicylic acid and hydroquinone. The standards were each prepared by dissolving 0.01 g powder in 1 ml 95% ethanol and 1 μl of each standard solution was spotted on the TLC plates, the first plate was for solvent system 1 (acetic acid – chloroform, 1:9 v/v) and the second plate was for solvent system 2 (ethyl acetate – benzene, 9:11 v/v). Running pattern of the standards were detected as described for the sample.