CHAPTER 2

METERIALS AND METHODS

1. Materials

1.1 Instruments

Instruments	Model	Company
Autoclave	HA-300M	Hirayama**,** Japan
Analytical balance 2 digits	SE 2020	OHAUS, USA
Analytical balance 4 digits	BP110S	Sartorius, Germany
Bench- top refrigerated centrifuge	5804R	Eppendorf, Germany
Blood glucose meter and test strips	Accu-CHEK Advantage	Roche Germany
Electrophoresis unit	ATTO /AE-6450	ATTO**,** Japan
Fraction collector	2110	Bio-Rad, USA.
Freeze drier	DW6-85	Heto Drywinner, Denmark
Hot air oven	LR-270	The grieve Co., USA
Micro-pipettes	P1000	Gilson, France
Micro-pipettes	P10, P50, P100, P250	Merck**,** France
Magnetic stirrer	HB 502	Bibby Sterilin, UK
Micro tube pump	MP-3	Eyela**,** Japan
Mini centrifuge	C-1200	National Labnet, USA
Power supply	Hoefer	Hoefer science instrument, USA
pH meter	EcoScan	Utech instrument, Singapore
Superspeed refrigerated centrifuge	JA-21	Beckman, USA

Instruments	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	Memmert	Scientific Industries, USA

1.2 All Chemicals are analytical grade.

Chemical	Company
Acrylamide	Merck, France
Ammonium persulfate	Merck, France
Ammonium sulfate	BDH Chemical Ltd.
Alpha-glucosidase from *Saccharomyces cerevisiae*	Sigma Chemical Co.
Bromophenol blue	
Bovine serum albumin	Merck, France
Calcium chloride	Sigma Chemical Co.
Coomassie brilliant blue R-250	Merck, France
Carboxylmethyl cellulose (CMC)	Fluka, Switzerland
3,5-Dinitrosalicylic acid	Sigma Chemical Co.
Dialysis bag cut off 12,000	Fluka, Switzerland
Ethyl acetate	Sigma Chemical Co.
Ethanol	Lab-Scan, Ireland
Express-Ion exchanger D free base (DEAE-cellulose)	Merck, France
Folin reagent	Sigma Chemical Co.
Glucobay acarbose 50 mg/tablet	
Glucose	BDH Chemical Ltd. PT Bayer, Germany Riedel de Haen AG, Seelze-Hannover, Germany

Chemical	Company
Glucose Oxidase Kit	Wako pure chemical industries, Ltd, Japan
Hydroxyapatite	Sigma Chemical Co.
Invertase (Yeast sucrase)	Sigma Chemical Co.
Isopropyl alcohol	Lab-Scan, Ireland
Magnesium sulfate	J.T. Baker, USA
Maltose	Sigma Chemical Co.
β-mercaptoethanol	BDH Chemical Ltd.
Methanol	Lab-Scan, Ireland
N-(1-naphthy) ethylenediamine	Panreac, E.U.
Potassium chloride	Ferak, Geramany
Salivary α-Amylase	Sigma Chemical Co.
Sephadex G-100	Phamacia
Sodium chloride	Lab-Scan, Ireland
Sodium acetate	Carlo erba
Sodium carbonate anhydrous	APS Finechem, Australia
di-Sodium hydrogen orthophosphate	APS Finechem, Australia
Sodium dihydrogen phosphate dihydrate	Sigma Chemical Co.
stardard sugars (maltotriose, maltotetraose, maltopentaose, maltphexaose and maltoheptaose)	Sigma Chemical Co.
Tris (hydroxymethyl) amino methane (Tris-HCl)	Sigma Chemical Co.
Thin layer chromatography (silica 60 F_{254})	Merck, Geramany
Standard molecular weight marker for electrophoresis	Sigma Chemical Co.
Standard molecular weight marker for gel filtration	Amersham
Starch, soluble	AJAX Finechem, Australia
Sucrose	Sigma Chemical Co.
Sulfuric acid	Lab-Scan, Ireland

2. Methods

2.1 Preparation of red kidney bean extract

Red kidney beans were purchased from supermarket. The seeds of red kidney beans were ground into powder with a mortar. The amount of 500 g powder was weighed, added 0.02 M sodium phosphate buffer pH 6.9 containing 0.15 M NaCl in the ratio of 1:5, w/v according to Grant *et al*.(1995). The mixture was stirred continuously for 16 h at 4 °C and centrifuged at 50,000 x g for 20 min at 4 °C. The supernatant was collected and assayed for α-amylase inhibitory activity.

2.2 Assay of α-amylase activity and its inhibitory activity

Activity of saliva or prancreatic amylase were measured by method of Bernfeld (1995). A solution of 2% starch in 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl (Buffer A) was incubated with α-amylase at 37 °C for 3 min, stopped the reaction with 3, 5-dinitrosalicylic acid solution (DNS) and measured the reaction colour at 540 nm using various concentration of maltose as a standard curve. One unit of amylase activity is defined as the amount of enzyme that liberates 1 mole of maltose from starch solution under assay condition at 37 °C in 3 min.

Alpha-amylase inhibitory activity was measured by preincubate the test sample with equal volume of known activity of α-amylase solution at 37 °C for 30 min and further assayed for the nonbinding amylase activity at 37 °C for 3 min as the above described paragraph. One unit of amylase inhibitor is defined as one unit amylase that is inhibited under the given assay condition. For the assay of 50% inhibition of α-amylase activity (IC_{50}), the sample was diluted to various concentrations and subjected to the above assay procedure. Then IC_{50} value was obtained from the plot of %inhibition of the enzyme against diluted sample concentrations.

2.3 Protein analysis

Samples were determined for their protein concentration by the Lowry's method (Lowry *et al*., 1951) using bovine serum albumin as the standard.

2.4 Preparation of proteinaceous amylase inhibitor

According to Sitthipong (2005), 50% ammonium sulfate ($AmSO_4$) was used for partial purified AI protein from crude extract of Royal red kidney bean. Its redissolved dialyzed pellet was further purified through series of chromatography column as follows: anion exchanger by DEAE, size of column 12 × 2.5 cm, flow rate 30 ml/h; gel filtration by Sephadex G-100, size of column 60×2.0 cm, flow rate 20 ml/h; and adsorption by hydroxyapatite, size of column 6×0.5 cm, flow rate 10 ml/h. In this study, the amount of crude extract preparation was increased, the size of each column types were increased together with the amount of loaded protein as described below.

2.4.1 Ammonium sulfate precipitation

A 300 ml crude extract of red kidney bean (see 2.1) was added ammonium sulfate to obtain 50% saturation, stirred gently at 4 °C, kept for 5 h and centrifuged at 10,000 x g for 15 min. The pellet was dissolved in a minimum amount of 0.01 M sodium phosphate buffer, pH 6.9 (buffer B) and dialyzed against in buffer B for 24 h. The pellet was dissolved and adjusted to a desired volume with buffer B was and called as dialysate. This dialysate was subjected for amylase inhibitory activity assay and protein determination.

2.4.2 Ion-exchange chromatography

The dialysate containing 250 mg protein was applied to a DEAE-cellulose column (17 × 3.5 cm) that already equilibrated with buffer B. The bound proteins were gradually eluted with linear gradient solution of 0-1.0 M NaCl in buffer

B at a flow rate of 30 ml/h. Three milliliter fractions were collected and measured for their protein content at 280 nm until reach zero. The obtained fractions was assay for their inhibitory activity against salivary α-amylase every 10 tube as previously described. Fractions showing inhibitory activity were pooled, concentrated with carboxymethyl cellulose (CMC) to a desired volume, and subjected to the determination of its protein concentration and inhibitory activity.

2.4.3 Gel filtration chromatography

Known amount of mg protein (100 mg) was loaded on a Sephadex G-100 column (109 × 1.6 cm) equilibrated with buffer B. The proteins were eluted with a solution of buffer B at a flow rate of 20 ml/h. One milliliter fraction was collected, measured for their protein content at 280 nm until reach zero. The obtained fractions was assay for their inhibitory activity against salivary α-amylase every 10 tube as previously described. Fractions showing activity were pooled, concentrated with CMC to a desired volume, and estimated for its protein concentration and inhibitory activity.

2.4.4 Adsorption chromatography

The pooled Sephadex G-100 fraction of 50 mg protein was loaded on a hydroxyapatite column (5.2 × 3 cm) equilibrated with the 0.001 M phosphate buffer, pH 6.8. The bound proteins were eluted with a linear gradient of increasing ionic strength 0.001-0.07 M sodium phosphate buffer, pH 6.8 at a flow rate of 10 ml/h. One milliliter fractions were collected, monitored for their protein content at 280 nm, and tested for their inhibitory activity against salivary α-amylase. Fractions with inhibitory activity were pooled, concentrated with CMC, adjusted to 3 ml and measured for its protein concentration and inhibitory activity**.**

2.4.5 Verification of proteinaceous amylase inhibitor purity and its molecular weight

(1). Native polyacrylamide gel electrophoresis, native-PAGE

Hydroxyapatite pooled fractions with AI activity was checked for its purity and native molecular weight by native-PAGE as follows.

A slab gel composed of stacking gel (3 cm) and separating gel (6 cm) was used. Native 14-17 % gradient PAGE was performed according to the method of Davis (1964). The samples and standard markers were mixed with a sample buffer (0.2 M tris-HCl, pH 6.8, 40% glycerol, 8 mM EDTA and 0.4% bromophenol blue) in a ratio of 3:1 (v/v). They were then electrophoresed in the electrode buffer (0.25 M Tris-HCl, 0.192 M glycine buffer, pH 8.3) at a constant current of 15 mA for 2.5 h. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R-250 (0.08% Coomassie Brilliant Blue R-250-40% methanol - 10% acetic acid) for 2 h and then the gel background was removed using destained solution (40% methanol-10% acetic acid) and the specific protein band was detected and compared with standard molecular weight marker: phosphorylase b (94 kDa), albumin (67 kDa), oval bumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14 kDa).

(2). Sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE

Hydroxyapatite pooled fraction with AI activity was checked for its subunits molecular weight by SDS-PAGE as follows.

The samples were electrophoresed in a slap gel, composed of stacking gel (3cm) and separating gel (6 cm). SDS 14-17 % gradient PAGE was performed according to the method of Laemmli (1970). The sample and standard protein markers were mixed with a sample buffer (0.2 M Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 8 mM EDTA, 0.4% β-mercaptoethanol and 0.4% bromophenol blue as the tracking dye) in a ratio of 3:1,v/v. They were then boiled for 5 min and loaded on the gel. Electrophoresis was carried out in the electrode buffer (25 mM tris-HCl 0.192 M glycine, 0.1% SDS, pH 8.3) at a constant current of 15 mA for 3 h. After

electrophoresis protein band was stained with Coomassie Blue. Mobilites of the sample, standard marker and bromophenol blue were measured and calculated for theirs relative mobility (R_f) using the following relationship:

$$\text{Relative mobility } (R_f) = \frac{\text{Mobility of proteins (cm)}}{\text{Mobility of bromophenol blue (cm)}}$$

A curve of standard protein molecular weight was plotted between R_f and the log of the molecular weight of the standard markers. The molecular weight of purified proteins was estimated by compairing their R_f values to those of standard markers which covered a broad range of molecular weights; phosphorylase b (94 kDa), albumin (67 kDa), oval bumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14 kDa).

(3). Gel filtration chromatography

In order to estimate the apparent molecular weight (M_r), amylase inhibitors was passed through a Sephadex G-100 column (109 × 1.6 cm) already equilibrated with buffer B and its was eluted with buffer B at flow rate 20 ml/h. Standard protein (bovine serum albumin, 68 kDa.; ovalbumin, 43kDa.; chymotrypsinogen A, 25 kDa.; ribonuclease A, 14 kDa) was applied to the same column, eluted and measured A_{280} nm in the same manner. The K_{av} values of the band with AI activity and the band of standard proteins were calculated using the equation given below.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e : eluting volume for protein

V_0 : voil volume for blue dextran

V_t : total volume for $K_2Cr_2O_4$

The M_r of the eluting AI was calculated by comparing its distribution coefficient (K_{av}) to those of standard proteins curve which plotted between K_{av} and M_r of each protein.

2.5 Preparation of nonproteinaceous amylase inhibitor

Nonproteinaceous amylase inhibitor was prepared as described by Kim *et al*. (2002) and Sitthipong (2005) with a modification in the ratio of sample: ethanol from a ratio of 1:8, v/v to ratio of 1:4, v/v. The details of preparation were as follows.

2.5.1 Preparation by solvent fractionation

The crude extract of red kidney bean was added methanol in the ratio of 1 : 4, v/v, stirred gently at 4 °C for 5 min and centrifuged at 4,000 x g, 4 °C for 5 min. The colleted supernatant was added ethanol in the ratio of 1:4, v/v and stirred gently at 4 °C for 5 min to obtain the precipitate. The precipitate was collected after centrifugation at 4,000 x g, 4 °C for 5 min and dissolved in buffer A to a desired volume (called as a nonproteinaceous amylase inhibitor, NAI). The NAI was assayed for its inhibitory activity, sugar concentration, protein concentration, identified by thin-layer chromatography (TLC), and kept for further studies including property and animal.

2.5.2 Verification of nonproteinaceous amylase inhibitor

The NAI was checked for the purity on the Whatman K6F silica gel TLC plates developing with a solvent system of ethyl acetate/ isopropyl alcohol/ water (1:3:1 by volume). After the development the TLC plate was air dried at room temperature, sprayed with a solution containing 0.3% (w/v) *N*-(1-naphthy)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and heated at 110 °C for 10 min. The visualized spots was compared with the mobility of standard sugars (maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) concurrently run on the same plate.

To confirm the separate spots with AI activity each spots on the non-spray lane of the sample was scraped, extracted with buffer A and centrifuged at 4,000 x g, room temperature 10 min. The collected supernatants were each added known activity salivary amylase, incubated for 30 min at 37 °C and 2% starch incubated 37 °C for 3 min, observed for the blue colour after the adding of iodine solution. The solution with the blue colour indicates the inhibition of α-amylase activity in hydrolyzing starch.

2.6 Property studies of the nonproteinnaceous amylase inhibitor against salivary α-amylase

In these studies a known unit amylase (0.3 U) and 2% starch solution were used.

2.6.1 Effect of temperature on the inhibitory activity

To study the effect of temperature on the inhibitory activity of the inhibitor, test samples were preincubated with the salivary α-amylase in Buffer A for 30 min at various temperatures from 4 ^{o}C, 25 ^{o}C, 37 ^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C and 80 ^{o}C respectively. After the samples were brought back to 37 ^{o}C, 2% starch was added to the reaction mixture and further incubated at 37 ^{o}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.

2.6.2 Effect of temperature on the stability of the inhibitor

To study thermal stability of the inhibitor, test samples in buffer A were maintained at 4 ^{o}C, 25 ^{o}C, 37 ^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C and 80 ^{o}C respectively for 30 min. The samples were immediately maintained warm temperature at 37 ^{o}C and amylase was added and determined for the inhibitory activity as previously described.

2.6.3 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 system containing 0.015 M NaCl were prepared: sodium acetate buffer pH 5.0; phosphate buffer pH 6.0, 6.9, 7.0 and 8.0; and Tris-HCl buffer pH 9.0. The test samples were preincubated with the salivary α-amylase at each pH for 30 min at 37 ^{o}C. After that, 2% starch was added to the reaction mixture, incubated for 3 min at 37 ^{o}C and the reaction was stopped by adding DNS. The remaining amylase activity was spectrophotometrically determined at 540 nm.

2.6.4 Effect of pH on the stability of the inhibitor

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 α-amylase inhibitor. Tested AI samples were incubated in buffer systems at various pH for 30 min at 4 ^{o}C, the samples were adjusted to pH 6.9 and incubated with salivary α-amylase for 30 min at 37 ^{o}C. The remaining amylase activity was determined using 2% starch as substrate as previously described.

2.6.5 Effect of salts on inhibitory activity of the inhibitor

To study the effect of salts on α-amylase inhibitory activity the tested samples were preincubated with α-amylase in 0.02 M phosphate buffer pH 6.9 containing 0.015 M of various salts as following : sodium chloride, calcium chloride, potassium chloride and magnesium sulfate for 30 min at 37 ^{o}C, then the remaining amylase activity was determined as previously described.

2.6.6 Mechanism of the inhibition

Inhibition modes of test samples against the activity of salivary α-amylase, starch solution at 0.25, 0.5, 1.0 and 2.0% were added to the mixture of salivary α-amylase in the absence or presence of the test samples at 25% inhibition or 50% inhibition concentration to start the reaction and incubated at 37 ^{o}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.

2.7 Potential application of the inhibitor on blood glucose reduction *via* luminal enzymes *in vitro*

2.7.1 Preparation of luminal enzymes

(1). Porcine pancreatic extract

Porcine pancreatic and small intestine were obtained from the slaughterhouse in the Department of Animal Science, Faculty of Natural Resource, Prince of Songkla University. Hog pancrease obtained from slaughterhouse was sliced and homogenized with buffer A in blender, and then followed with centrifugation at 50,000 x g for 20 min at 4 °C. The supernatant was collected and designated as crude porcine α-amylase solution.

(2). Porcine jejunum extract

Porcine jejunum from the slaughterhouse was scraped for the epithelial cells lining and homogenized in buffer A. After centrifugation at 50,000 x g for 20 min at 4 °C, the supernatant was collected and named as crude extract of porcine jejunum.

(3). Yeast maltase solution

Maltase from yeast (Sigma Co.) was dissolved in buffer A to obtain its activity equivalent to salivary α-amylase (0.6 mg glucose production at 37 °C 3 min).

(4). Yeast sucrase solution

Sucrase from yeast (Sigma Co.) was dissolved in buffer A to obtain its activity equivalent to salivary α-amylase (0.6 mg glucose production at 37 °C 3 min).

2.7.2 Assay of enzyme activity and inhibitory activity

(1). Alpha-amylase

Amylase activity in porcine pancreatic extract was measured as a previously.

(2).Maltase

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

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

(3). Sucrase

Sucrase activity was determined by adding of 2% sucrose solutions in buffer A as a substrate and incubated at 37 °C for 3 min. The reaction was stopped in boiling water for 5 min, cooled to room temperature. A 0.2 ml aliquot was transferred

to a new tube, added 1 ml of glucosidase kit and incubated at 37 °C for 10 min. The mixture was diluted with 2 ml of distilled water and measured for the absorbance at 505 nm along with standard glucose. One unit of sucrase activity was defined as the amount of enzyme that hydrolyzed of sucrose to invert sugar per min under the assay conditions, pH 6.9 at 37 °C for 3 min.

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

2.7.3 Effect of inhibitor on pancreatic α-amylase

The test inhibitor samples 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% starch solution as a substrate as previously described.

2.7.4 Effect of the inhibitor on maltase

In this study maltase from yeast (Sigma Co.) and the crude extract of porcine jejunum were used and each source was diluted with buffer A to obtain its maltase activity equivalent to salivary α-amylase. Then the test samples was preincubated with each of the diluted source of maltase for 30 min at 37 °C and nonbinding maltase was measured as a previously.

2.7.5 Effect of inhibitor on yeast sucrase

In this study sucrase from yeast (Sigma Co.) and crude extract of porcine jejunum were used and each source was dilute with buffer A to obtain its sucrase activity equivalent to salivary α-amylase. Then the test samples was preincubated with each of the diluted source of sucrase for 30 min at 37 °C and nonbinding sucrase were measured as a previously.

2.8 Potential application of the inhibitor on blood glucose reduction *via* luminal enzymes *in vivo*

2.8.1 Comparative assay of rat blood glucose level by glucose oxidase kit and glucometer

To reduce the suffering of animals from taking a large portion of blood the analysis of blood glucose level was aimed to replace the glucose oxidase kit with the glucometer. Determination of the rat blood glucose levels by the glucosidase kit and glucometer was thus compared as detailed below.

Blood was collected from the tail vein of 6 rats by making a snip in terminal of the tail with sharp scissors, struck the tail gently with thumb and finger to enhance the flow of blood onto two glucometer strips for the value reading *via* glucometer and then into the eppendorf tube approximately 200 µl and cap locked. The collected blood was subjected to centrifugation at 1,000 x g for 15 min at 4 °C to obtain the serum. The glucose levels in serum were determined triplicately using a glucose oxidase kit (WAKO, Japan) by adding 3 ml of the glucose oxidase kit solution, mixed, incubated at 37 °C for 5 min, boiled, added 2 ml of distilled water and measured for the absorbance at 505 nm along with the standard glucose.

2.8.2 Effect of the inhibitors on fasting blood glucose level in rats

Four-week-old male Wistar rats weighting between 200 ± 10 g, were obtained from the Animal House in Faculty of Science, Prince of Songkla University. They were randomly divided into nine groups of 6 rats, kept in stainless steel cages in a room controlling temperature at 22 ± 2 °C and light/dark at 12/12 h. Each of the animal group was then designated as group 1 to 9. The day before the treatment animals were measured for their body weights and blood glucose levels (BG) with the glucometer. Then the animals were fed with the test substance for 14 days at 9.00 am in the test group as follow.

Group 1 or control group: The animal were fed with 1 ml of buffer A, then allowed for normal diet and water.

Group 2: The animal were fed with 1 ml of crude extract, then allowed for normal diet and water.

Group 3: The animal were fed with 1 ml of diluted concentration of crude extract at IC_{50} inhibition against salivary α-amylase, then allowed for normal diet and water.

Group 4: The animals were fed with 1 ml of PAI solution which obtained from the concentrate eluate from the hydroxyapatite column, then allowed for normal diet and water.

Group 5: The animals were fed with 1 ml of diluted concentration of PAI at IC_{50} inhibition against salivary α-amylase, then allowed for normal diet and water.

Group 6 : The animal were fed with 1 ml of NAI solution which obtained from the solvent fractionation of crude extract, dissolved in buffer A 10 ml, then allowed for normal diet and water.

Group 7 : The animal were fed with 1 ml of diluted concentration of NAI at IC_{50} inhibition against salivary α-amylase, then allowed for normal diet and water

Group 8: The animals were fed with 1 ml of 2.5 mg acarbose which had 100% inhibition against salivary α-amylase, then allowed for normal diet and water.

Group 9: The animal were fed with 1 ml of acarbose at IC_{50} inhibition against salivary α-amylase, then allowed for normal diet and water.

On the 14th days the animals were all fast overnight and theirs blood glucose concentration were determined using the glucometer on the 15th day at 9.00 am.

2.8.3 Oral tolerance test

(1). Oral glucose tolerance test

On the 15th day after the blood collection at 9.00 am the animals in each group were again fed with the test samples as described in the previous section. After 5 min rest an aliquots of 0.2 g/ml glucose was given orally to obtain 0.2 g glucose per 100 g body weight. Blood glucose concentrations were measured 30, 60 and 120 min after glucose administration. Blood glucose concentration time curves were plot, the Zenith blood glucose concentration (ZBG) and the area under the curve (AUC) were determined. The formula for AUC calculation was as follows:

AUC (mg/dl/hr)

$$= [(BG_0 + BG_{30}) \times 0.5 \div 2] + [(BG_{30} + BG_{60}) \times 0.5 \div 2] + [(BG_{60} + BG_{120}) \times 1 \div 2]$$

Where BG_0, BG_{30}, BG_{60} and BG_{120} represented blood glucose concentrations at 0 (before loading of glucose) 30, 60 and 120 minutes after loading.

(2). Oral maltose tolerance test

On the 17th day the rats were subjected to blood collection at 9.00 am and fed with the test samples of each group. After 5 min rest an aliquots of 0.4 g/ml maltose solution was given orally to obtain 0.4 g maltose per 100 g body weight. Blood glucose levels at 30, 60 and 120 min, its time curves and calculation for AUC were determined as in the test of oral glucose tolerance.

(3). Oral sucrose tolerance test

On the 19th day the rats were subjected to blood collection at 9.00 am and fed with the test samples of each group. After 5 min rest an aliquots of 0.4 g/ml sucrose solution was given orally to obtain 0.4 g sucrose per 100 g body weight. Blood glucose levels at 30, 60 and 120 min, its time curves and calculation for AUC were determined as in the test of oral glucose tolerance.

(4). Oral starch tolerance test

On the 21st day the rats were subjected to blood collection at 9.00 am and fed with the test samples of each group. After 5 min an aliquots of 0.3 g/ml starch solution was given orally to obtain 0.3 g starch per 100 g body weight. Blood glucose levels at 30, 60 and 120 min, its time curves and calculation for AUC were determined as in the test of oral glucose tolerance.

2.8.4 Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Statistical significance was evaluated by one-way analysis of variance using SPSS 11.5 (SPSS Institute, Cary. NC, USA) and individual comparisons were obtained by Duncan's multiple-range test and $p<0.05$ was considered as significant.