CHAPTER 3

RESULTS

1. Preparation of proteinaceous amylase inhibitor

Alpha-amylase inhibitor was extracted from royal red kidney beans (*Phaseolus vulgaris*) using method by Grant *et al*., 1995. The crude extract was precipitated with 50% ammonium sulfate. The dialysate was applied to a DEAE-cellulose column, Sephadex G-100 column and hydroxyapatite column and each fraction was tested for its inhibitory activity.

Figure 4 showed the elution chromatographic profile from DEAE cellulose column. Fractions showing inhibitory activity against salivary α-amylase were pooled, concentrated and applied on to a Sephadex G-100 column. The eluted chromatogram of proteins was showed in Figure 5. The fraction with inhibitory activity were pooled, concentrated and applied onto a hydroxyapatite column. The bound proteins were eluted and each fraction was tested for its inhibitory activity, as shown in Figure 6. Fractions with inhibitory activity were pooled, concentrated and used in further studies.

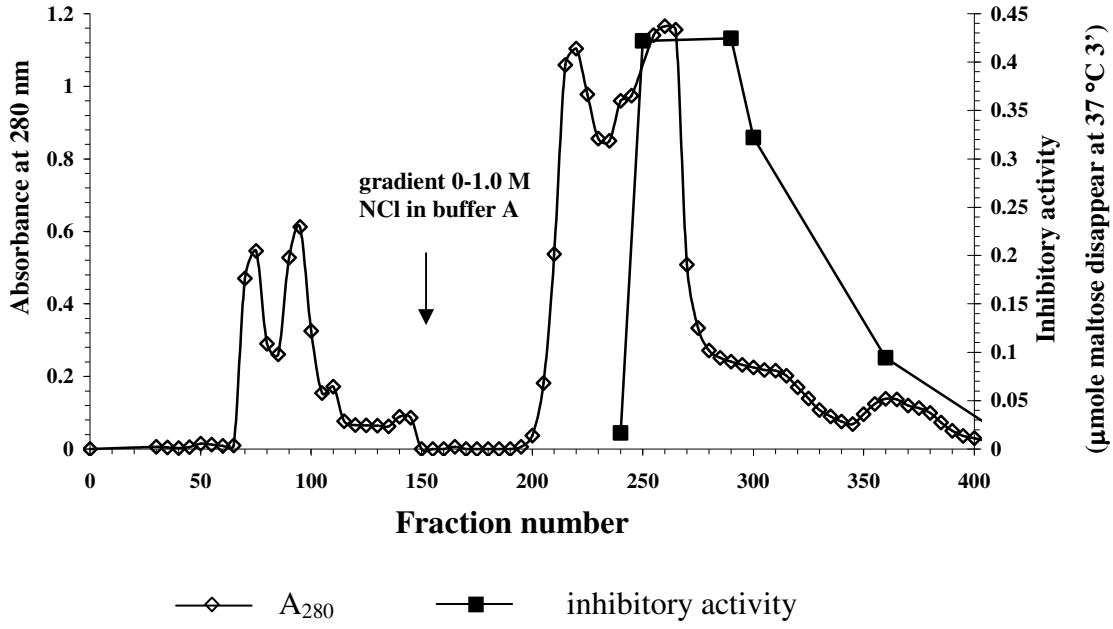


Figure 4 DEAE-cellulose column chromatogram (17 × 3.5 cm) of the dialysate. The bound proteins were eluted with linear gradient solution of 0-1.0 M NaCl in 0.01 M phosphate buffer, pH 6.9 at a flow rate of 30 ml/h.

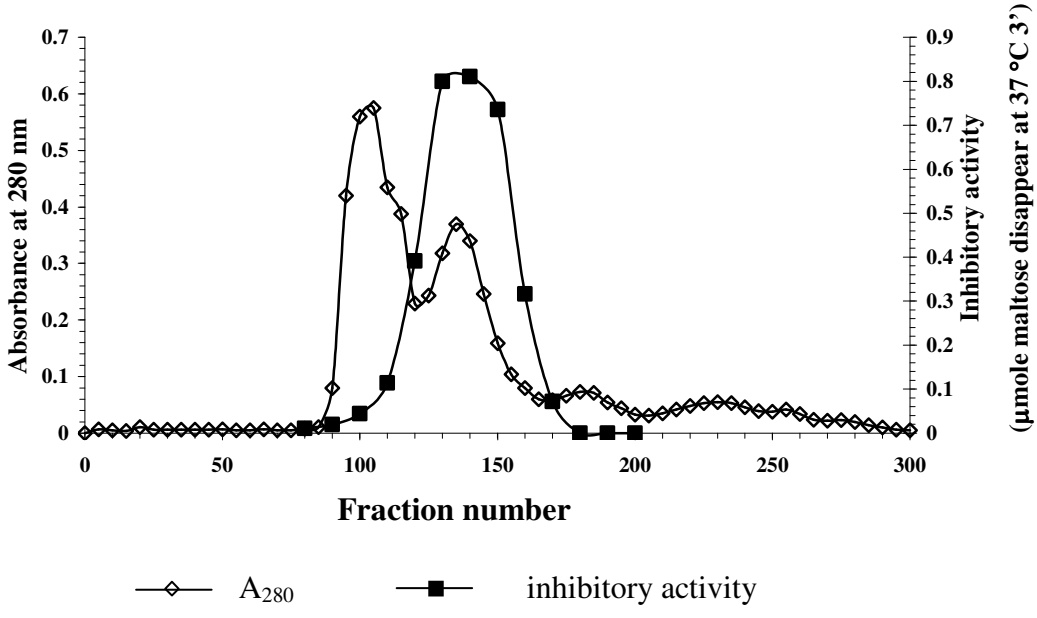


Figure 5 Sephadex G-100 column chromatogram (109 × 1.6 cm) of the concentrated DEAE pooled fraction with amylase inhibitory activity. Fractions were eluted with 0.01 M phosphate buffer, pH 6.9 at a flow rate of 20 ml/h.

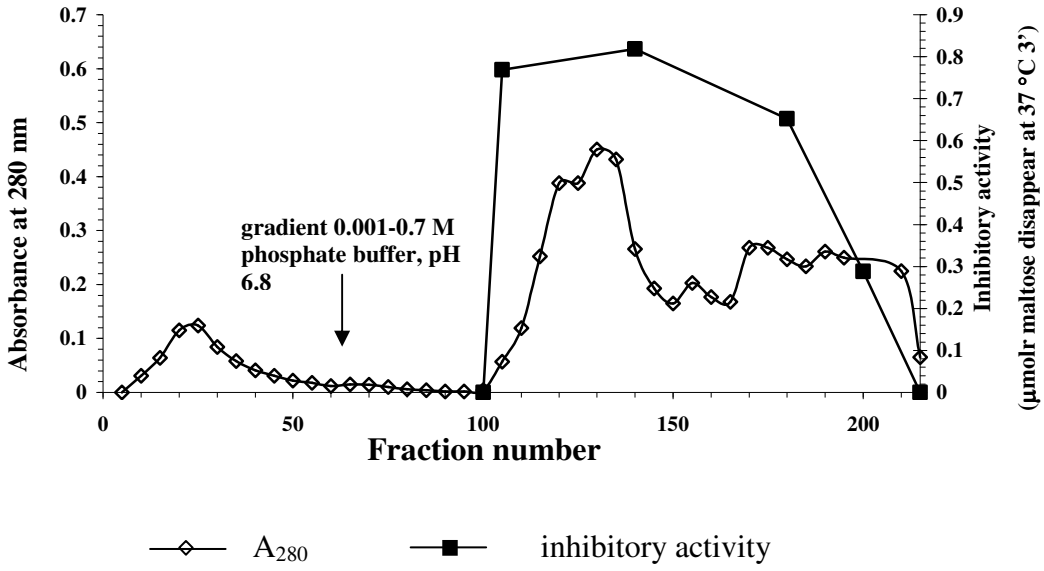


Figure 6 Hydroxyapatite column chromatogram (5.2 × 3 cm) of the concentrated Sephadex G-100 pooled fraction with amylase inhibitory activity. The bound proteins were eluted with a linear gradient of 0.001-0.07 M phosphate buffer, pH 6.8 at a flow rate of 10 ml/h.

2 Verification of proteinaceous amylase inhibitor purity and its molecular weight

The proteinaceous amylase inhibitor from hydroxyapatite column was verified for its purity by 14-17% native-PAGE as shown in Figure 7A the purified inhibitor contained a single protein band of a native molecular weight 36.3 kDa.

When the purified proteinaceous amylase inhibitor was subjected to SDS-PAGE, it resolved into three protein bands as shown in Figure 7B under the addition of β-mercaptoethanol. According to the standard curve (Figure 9), this proteinaceous amylase inhibitor contained three subunits of 15.5, 17 and 18.2 kDa.

This proteinaceous amylase inhibitor was analyzed for its native molecular weight by gel filtration using a Sephadex-100 column as shown in Figure 10 and 11. A molecular weight of purified proteinaceous amylase inhibitor was calculated to be 47.7 kDa from the plot between respective values on the K_{av} of standard proteins and logarithm of their molecular weight and as shown in Figure 11.

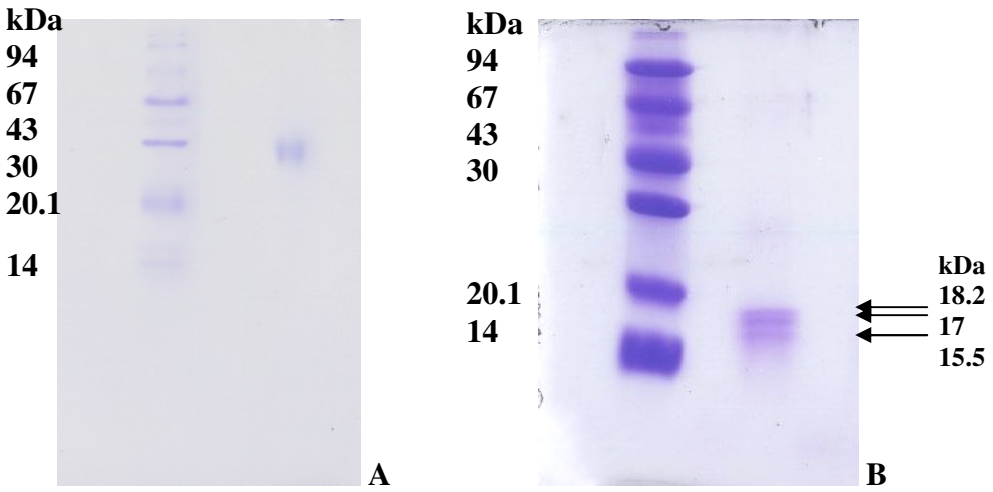


Figure 7 Native-PAGE of the purified proteinaceous amylase inhibitor on 14-17% gradient polyacrylamide slab gel (A). SDS-PAGE of purified proteinaceous α-amylase inhibitor on 14-17% gradient polyacrylamide slab gel (B). The protein bands were stained with Coomassie Blue. Left lane; molecular weight marker: phosphorylase b (94 kDa), albumin (67 kDa), oval bumin (43 kDa), carbonic Anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14 kDa), Right lane; purified proteinaceous amylase inhibitor.

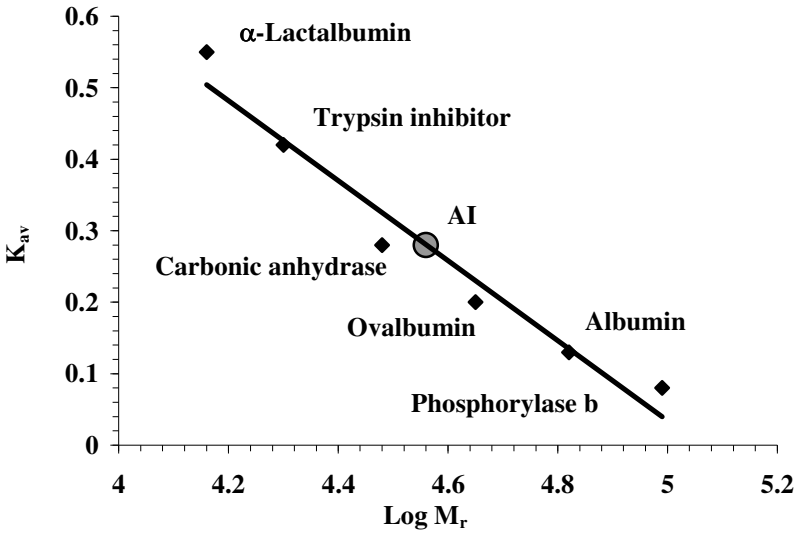


Figure 8 Calibration curve for molecular weight determination of purified proteinaceous amylase inhibitor by 14-17% Native-PAGE.

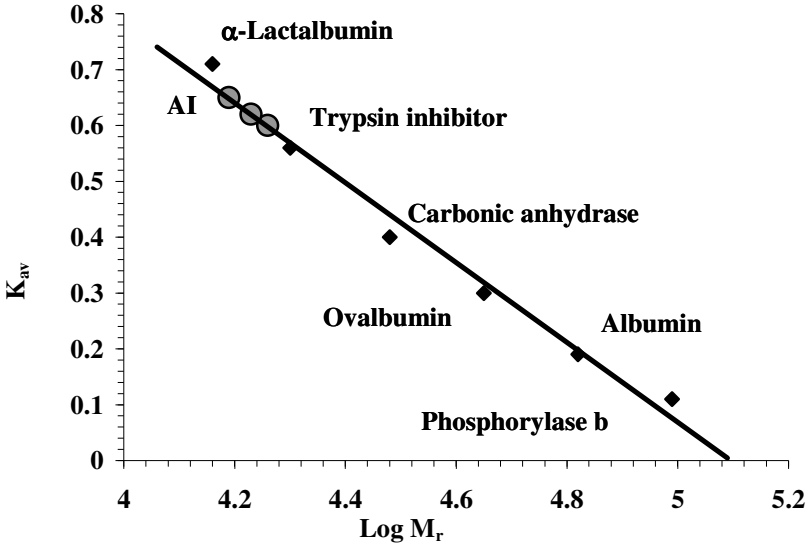


Figure 9 Calibration curve for molecular weight determination of purified proteinaceous amylase inhibitor by 14-17% SDS-PAGE.

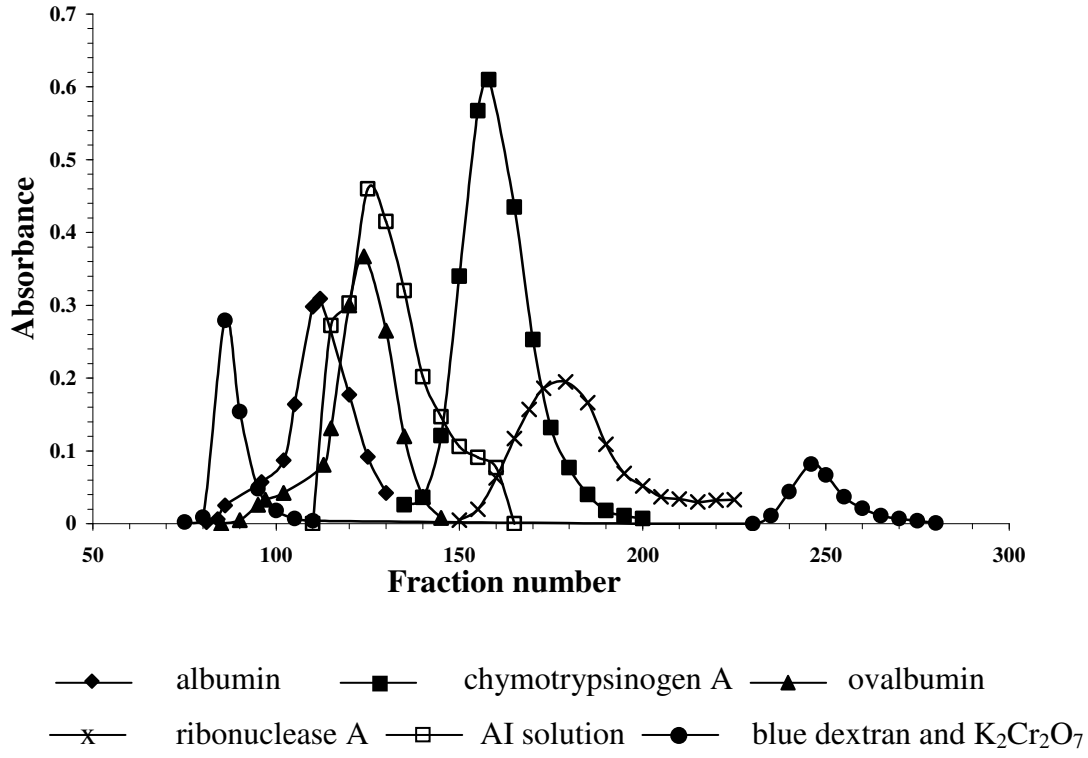


Figure 10 Chromatogram of the molecular weight markers and amylase inhibitor from Sephadex G-100 column (109 × 1.6 cm) eluted with 0.01 M phosphate buffer pH 6.9 at a flow rate of 20 ml/h. Proteins were measured at A_{280} while blue dextran and $K_2Cr_2O_4$ were measured at A_{620} and A_{480}, respectively.

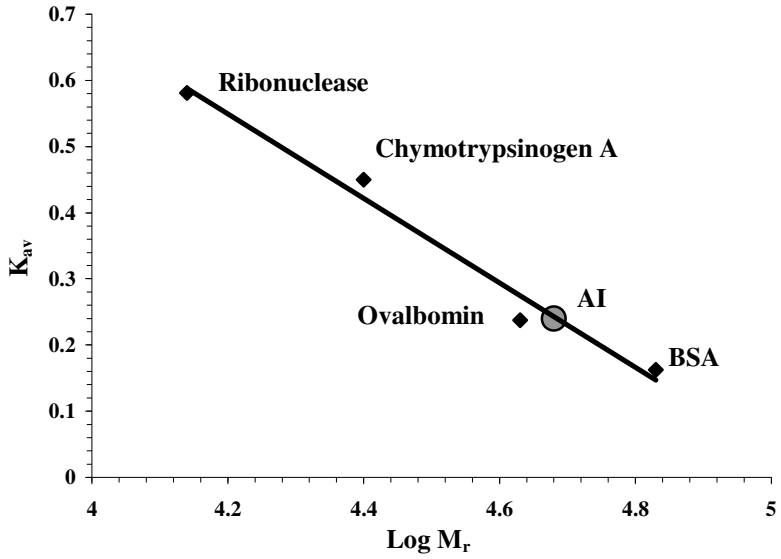


Figure 11 Calibration curve for molecular weight determination of the purified proteinaceous amylase inhibitor by a Sephadex G-100 column chromatography.

3. Verification of nonproteinaceous amylase inhibitor purity

The nonproteinaceous α-amylase inhibitor (NAI) identified by thin layer chromatography, according to method of Kim *et al.* (2002). The resulted showed in Figure 12A. The nonproteinaceous α-amylase inhibitor was separated into 3 spots at $R_f = 0.624$ for spot 1, $R_f = 0.581$ for spots 2 and $R_f = 0.109$ for spot 3. Spot 1 had its R_f value equivalent to glucose (G1) standard ($R_f = 0.624$) and spot 2 had its R_f value closed to maltose (G2) standard ($R_f = 0.538$).

To confirm the separated spots for the ability in inhibiting salivary α-amylase each spot on the non-spray lane of the sample was scraped, extracted with buffer A and qualitative assay for the inhibitory activity. The blue colour of spot 2 (tube5 in Figure 12B) indicated that it had inhibitory activity in inhibiting α-amylase to hydrolyze starch when compared with positive control (tube 1). Spot 1 (tube 4) and spot 2 (tube 6) showed no inhibition of amylase activity when compared with a negative control (tube 2).

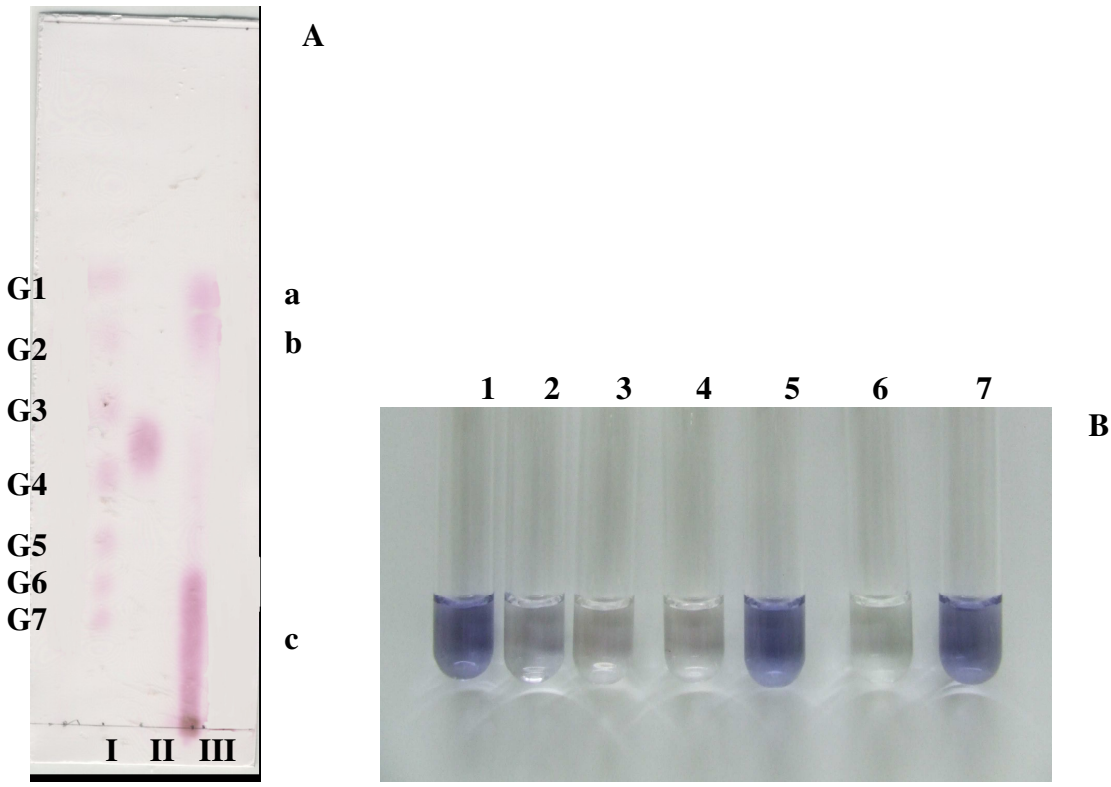


Figure 12 A: Thin layer chromatrogram of nonproteinaceous inhibitor (NAI) on Whatman K6F_{254} plate (5 × 10 cm) developing with ethyl acetate/ isopropyl alcohol/ water (1:3:1 by volume) sprayed with *N*-(1-naphthy)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and heated at 110 °C for 10 min. Lane I: standard sugar; lane II: acarbose; lane III: NAI. B: Confirmation of the separated spots for amylase inhibitory activity through blue colour of nonhydrolyzing starch with I_2 solution. Tube 4, 5, 6 were the non sprayed spots of NAI which corresponded to the sprayed lane IIIA spots a, b, c. Tube 7 was the solution of NAI. For positive and negative control solution of 2% starch, salivary amylase in the 0.02 M phostphate buffer pH 6.9 with 0.015 M NaCl were use in tube 1, 2.

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

Several factors are able to modify the inhibitory activity of α-amylase inhibitor such as temperature, pH and salt (Giri and Kachole, 1998; Kluh, *et al*., 2005; Gibbs and Alli, 1998).

4.1 Effect of temperature on the inhibitory activity

Figure 13A shows the effect of temperature on amylase activity with or without inhibitor. Alpha-amylase activity without the inhibitor was constant between 0.27-0.28 activity (0.3 unit) from 4-37 °C and gradually decreased at 40-50 °C, drop down to 0.04 and ceased at 80 °C. The addition of inhibitor reduced α-amylase activity from its initial value 0.3 unit to 0.18 at 4 °C, 0.13 at 25 °C, 0.12 at 37 °C, 0.06 at 40 °C and 0.05 at 50 °C. Above 50 °C-80 °C the inhibitor decreased α-amylase activity in similar pattern to that of enzyme activity without the addition of inhibitor. Figure 13B showed inhibitory activity of inhibitor in various temperatures which was calculated by the subtraction of initial activity of α-amylase by the left over activity of α-amylase after binding with the inhibitor under the study conditions. The inhibitor had the highest inhibitory activity at 40-50 ^{o}C.

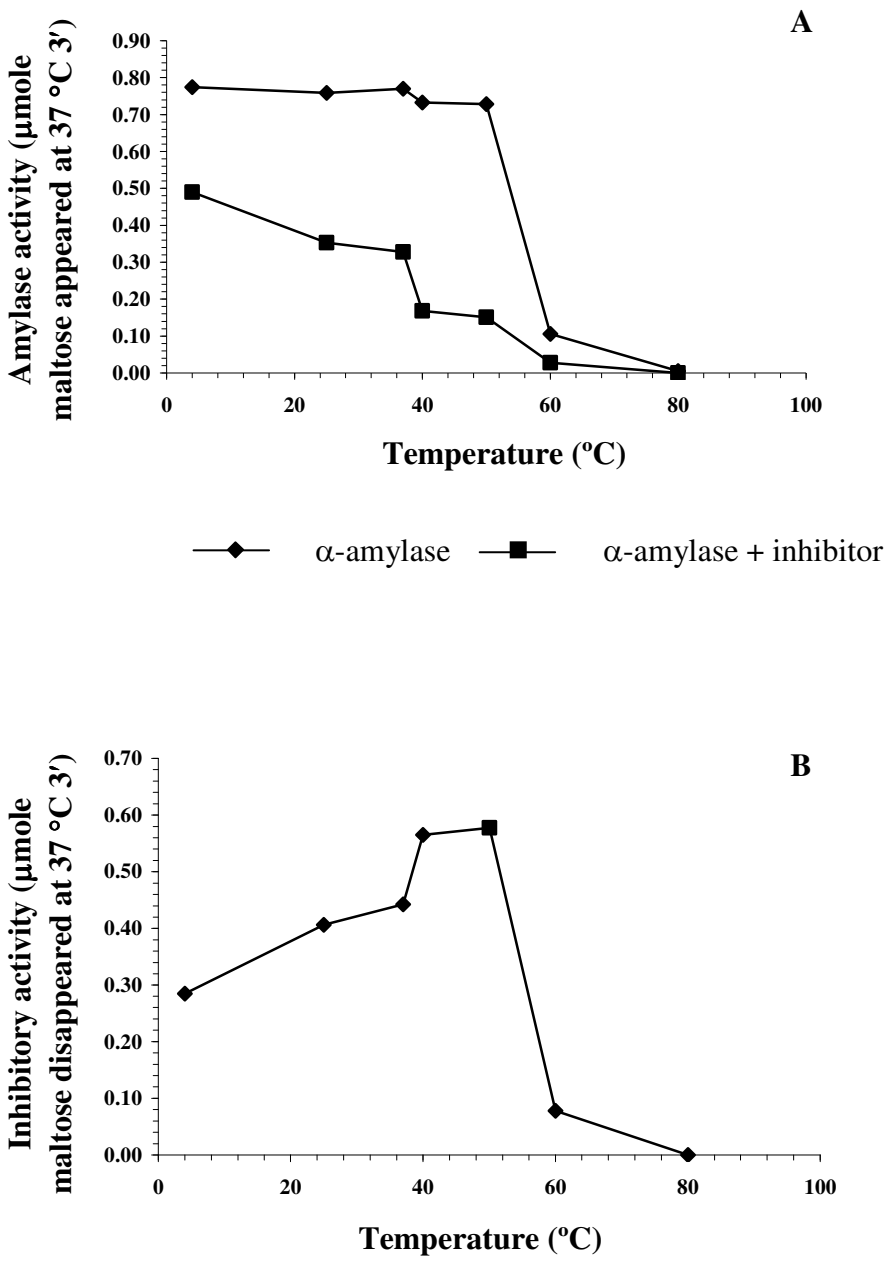


Figure 13 Effect of temperature on the inhibitory activity. The salivary amylase was incubated with or without the inhibitor in 0.02 M sodium phosphate buffer pH 6.9 containing 0.015 M NaCl for 30 min at various temperatures from 4 ^{o}C, 25 ^{o}C, 37 ^{o}C, 40 ^{o}C, 50 ^{o}C, 60 ^{o}C and 80 ^{o}C respectively. The remaining amylase activity was determined at 540 nm. The values were from duplicate data sets. A: the effect of temperature on amylase activity with or without inhibitor, B: the effect of temperature on the inhibitory activity.

4.2 Effect of temperature on the stability of the inhibitor

Preincubation of the inhibitor for 30 min at 4 , 25, 37, 40 and 50 ^{o}C did not affect on ability of AI in inhibiting α-amylase activity under normal assay condition as shown in Figure 14. Preincubation of the inhibitor for 30 min at 60 ^{o}C and 80 ^{o}C reduced ability of AI drastically.

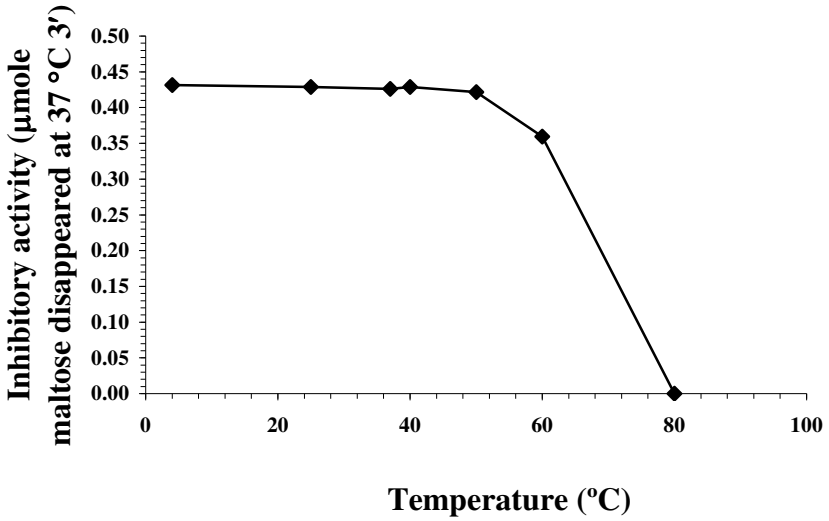


Figure 14 Effect of temperature on the stability of the inhibitor. The test samples in 0.02 M sodium phosphate buffer pH 6.9 containing 0.015 M NaCl were maintained at 4, 25, 37, 40, 50, 60 and 80 ^{o}C respectively for 30 min and adjusted back to 37 ^{o}C. Amylase was added, incubated at 37 ^{o}C, 30 min and the remaining amylase activity was determined at 540 nm using 2% starch as a substrate.

4.3 Effect of pH on inhibitory activity of the inhibitor

Inhibitor and enzyme were incubated together at 37 ^{o}C for 30 min at various pH from pH 5.0, 6.0, 6.9, 7.0, 8.0 and 9.0. After incubation the solution was adjusted the pH back to pH 6.9, substrate 2% starch solution were added for the assay of amylase activity. Figure 15A showed effect of pH on α-amylase activity with or without inhibitor. Alpha-amylase activity without the inhibitor sharply increased from pH 5 to 6.9 and reached its maximal activity at pH 7.0 then gradually decreased

from pH 8.0 to 9.0. Alpha-amylase activity with the inhibitor gave similar pattern to that of amylase activity without the inhibitor, but with lower value of amylase activity and the shift of the maximal activity at pH 7.0 to pH 8.0. These were due to the inhibitor binding to the enzyme.

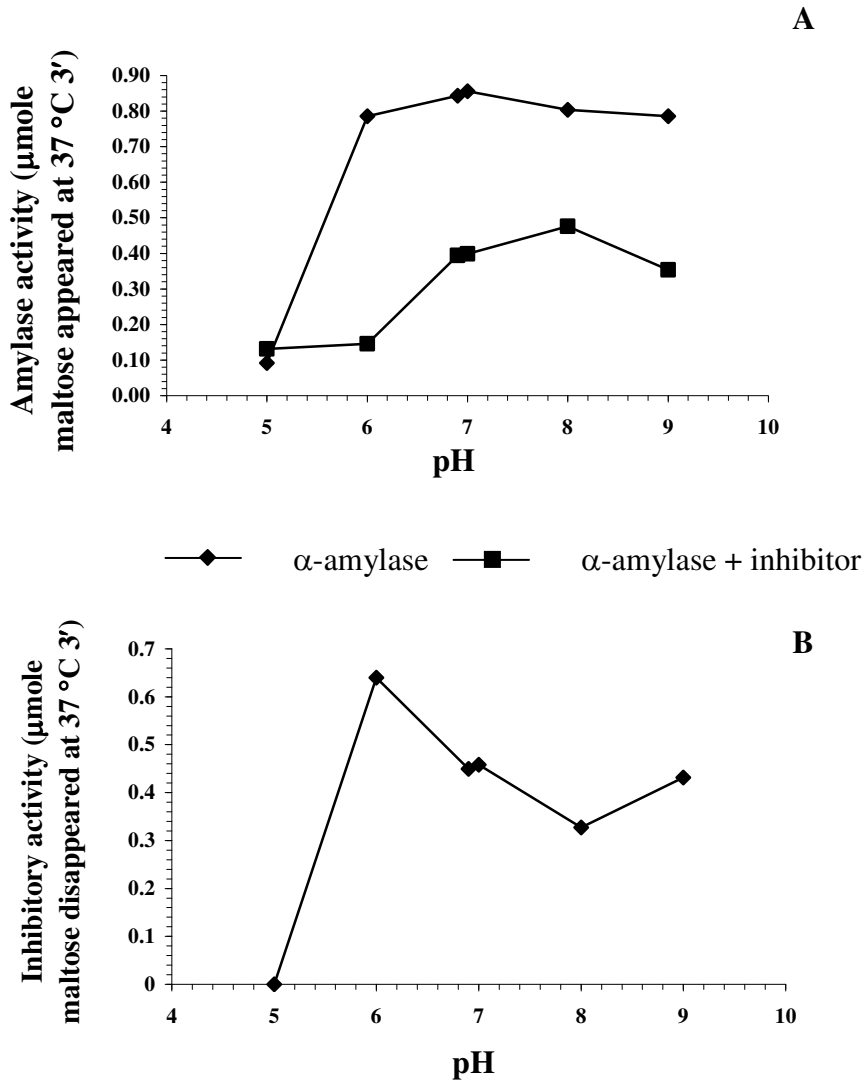


Figure 15 Effect of pH on activity of amylase with or without inhibitor. The test samples were preincubated with the salivary α-amylase at pH 5.0, 6.0, 6.9, 7.0, 8.0 and 9.0 for 30 min at 37 ^{o}C. The remaining amylase activity was determined at 540 nm. A: the effect of pH on α-amylase activity with or without inhibitor, B: the effect of pH on the inhibitory activity of the inhibitor.

4.4 Effect of pH on the stability of the inhibitor

Preincubation of the inhibitor for 30 min at various pH 5.0, 6.0, 6.9, 7.0, 8.0 and 9.0. Figure 16 showed alpha-amylase inhibitory activity of AI had increased from pH 5.0 to 6.0, maximal inhibitory activity at pH 6.0 and reduced ability of amylase inhibitory at 6.0 to 9.0.

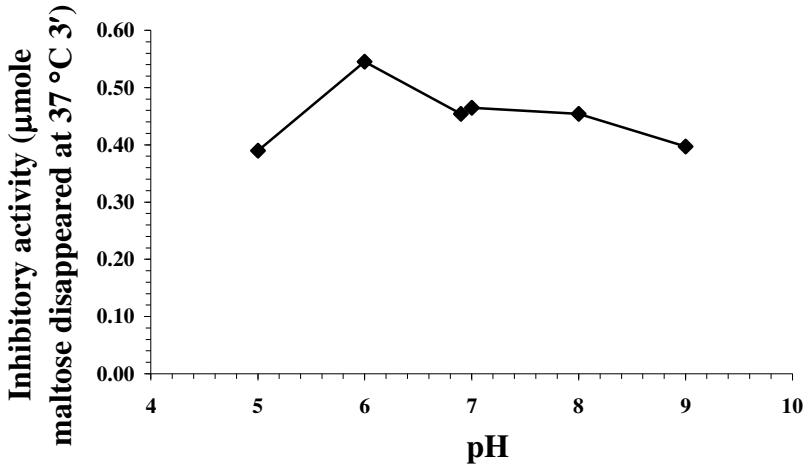


Figure 16 Effect of pH on the stability of the inhibitor. The tested samples were incubated in buffer systems at various pH for 30 min at 4 ^{o}C and incubated with salivary α-amylase for 30 min at 37 ^{o}C. The remaining amylase activity was determined at 540 nm.

4.5 Effect of salts on inhibitory activity of the inhibitor

Preincubation of the inhibitor for 30 min in 0.015 M of various salts either sodium chloride, calcium chloride, potassium chloride or magnesium sulfate. Magnesium sulfate and chloride ions enhanced the binding between amylase and inhibitor compared to the control (no salts added). Potassium chloride and calcium chloride showed similar effect with sodium chloride as shown in Table 4.

Table 4 Effect of salts on inhibitory activity of the inhibitor

Condition	Amylase activity	Inhibitory activity
Control*	0.90 ± 0.004	0.32 ± 0.010
0.015 M NaCl	0.80 ± 0.002	0.40 ± 0.012
0.015 M KCl	0.88 ± 0.002	0.40 ± 0.000
0.015 M $CaCl_2$	0.90 ± 0.004	0.41 ± 0.012
0.015 M $MgSO_4$	0.86 ± 0.004	0.47 ± 0.004

Amylase activity was 0.3 unit

Inhibitor was 0.66 mg maltose/ml

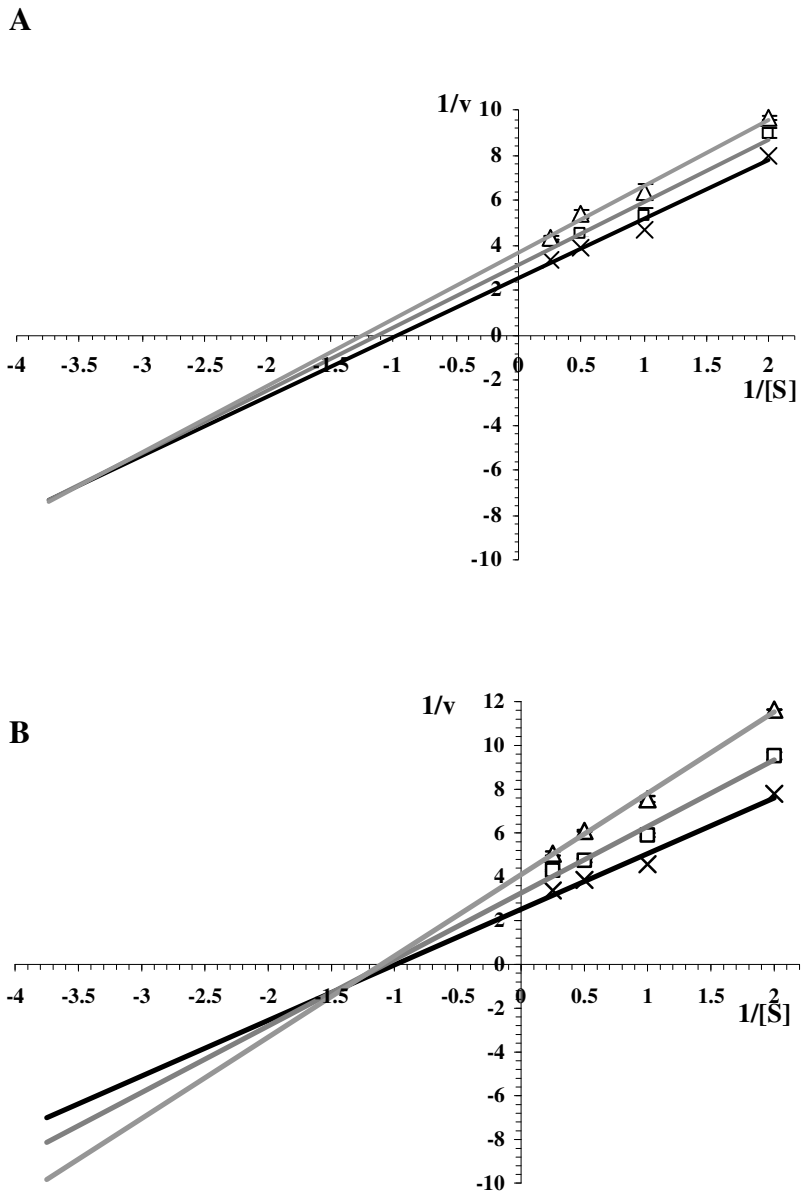
*Control contained no salt in 0.02 M sodium phosphate buffer pH 6.9

4.6 Mechanism of the inhibition

Type of mechanism which the inhibitor acts on salivary α-amylase was shown by the double reciprocal plot or Lineweaver-Burk plots

Kinetics of the inhibitor was studied using Lineweaver-Burk plot. Initial velocity (ν) was determined at different substrate concentrations [S] in the presence and absence of fixed inhibitor concentration. Figure 17A shows the Lineweaver-Burk plot for human salivary α-amylase (0.3 U) inhibited by nonproteinaceous. The plot gave straight lines with the intercept in the third quadrant at a single point. The inhibition is therefore of the mixed noncompetitive type for nonproteinaceous with *Km* 1.056 mg/ml for salivary α-amylase; K_i 1.571 mg/ml and K'_i 36.968 mg/ml for nonproteinaceous.

Figure 17B shows the Lineweaver-Burk plot for human salivary α-amylase (0.3 U) inhibited by acarbose. The plot gave straight lines with the intercept in the third quadrant at single point. The inhibition is therefore of the mixed noncompetitive type for acarbose with *Km* 1.007 mg/ml for amylase; K_i 0.578 mg/ml and K'_i 3.133 mg/ml for acarbose.



—×— Enzyme —□— concentration 0.25 mg maltose/ml or 0.88 mg acarbose/ml

—△— concentration 0.66 mg maltose/ml or 2.08 mg acarbose/ml

Figure 17 Lineweaver-Burk plot (1/[S] vs 1/v) for human salivary α-amylase inhibited by nonproteinaceous (**A**) and acarbose (**B**), [S] mg starch/ml, [v] mg maltose /ml appeared under assay condition.

5. Potential applications of the inhibitor on blood glucose reduction *via* luminal enzymes *in vitro*

Table 5 showed potential applications of α-amylase inhibitor on blood glucose reduction *via* luminal enzymes *in vitro*. Crude extract showed its inhibitory activity on the tested enzymes from high to low as following: human salivary α-amylase, porcine pancreatic α-amylase, maltase from yeast and not inhibited sucrase from yeast. Proteinaceous amylase inhibitor (PAI) showed its inhibitory activity on: human salivary α-amylase and porcine pancreatic α-amylase equally but not inhibited maltase and sucrase from yeast. Nonproteinaceous amylase inhibitor (NAI) showed its inhibitory activity on: porcine pancreatic α-amylase more than human salivary α-amylase without any inhibition with and not inhibited sucrase and maltase from yeast.

Table 5 Potential application of α-amylase inhibitor on various enzymes on IC_{50} consideration

	g bean/ml at dilution for IC_{50}			
Type of enzyme	α-amylase		maltase	sucrase
Source	Saliva[1]	Porcine[2] pancreas	Yeast[1]	Yeast[1]
Crude	0.0037	0.0043	0.0680	no inhibition
PAI	0.0002	0.0002	no inhibition	no inhibition
NAI	0.0873	0.0694	no inhibition	no inhibition

[1] Product by Sigma Co.

[2] Crude extract enzyme from porcine pancreas.

Amylase activity from saliva and porcine pancreatic were 0.3 ± 0.05 mg maltose at 37 °C 3 min.

Maltase and sucrase activity from yeast were 0.6 ± 0.05 mg glucose at 37 °C 3 min

Table 6 showed potential applications of α-amylase inhibitor on the luminal luminal enzymes which theirs activity was adjusted equally at product liberation 0.2 mmole under the assay conditions. Crude extract showed its inhibitory activity on the tested enzyme from high to low as following: human salivary α-amylase, yeast maltase, porcine pancreatic α-amylase, maltase, sucrase from jejunum but not inhibited sucrase from yeast. Crude extract at IC_{50} showed its inhibitory activity on: human salivary α-amylase, porcine pancreatic α-amylase and sucrose from yeast at 54.77, 44.19 and 12.96% without any inhibition on yeast maltase, jejunum maltase and jejunum sucrase. Both proteinaceous amylase inhibitor and its IC_{50} inhibited human salivary α-amylase porcine pancreatic α-amylase and maltase from jejunum, but not inhibited maltase from jejunum, maltase and sucrase from yeast. Nonproteinaceous amylase inhibitor showed its inhibitory activity on the test enzyme from high to low as following: porcine pancreatic α-amylase, human salivary α-amylase, sucrase and maltase from jejunum at 97.67, 92.73, 33.33 and 3.57% but not inhibited maltase and sucrase from yeast. Nonproteinaceous amylase inhibitor at IC_{50} inhibited porcine pancreatic α-amylase more than human salivary α-amylase and maltase from jejunum at 69.30, 55.68, and 19.23% inhibition without any effect on function of maltase from jejunum, maltase and sucrase from yeast (0% inhibition).

The test samples in Table 5 and 6 preparing from the extract of Royal red kidney bean had 100% inhibition against salivary α-amylase and 50% at its 54.17 dilution. The solution of NAI contained 15 mg maltose equivalence with 100% inhibition against the amylase and 50% inhibition at its 2.29 dilution. The solution of PAI contained 315 mg protein with 100% inhibition against α-amylase and 50% inhibition at its 85.88 dilution. For porcine pancreatic α-amylase, yeast maltase and yeast sucrase the extract gave 100% inhibition against their activities and 50% inhibition at its 46.75 and 2.94 dilution, respectively. For yeast sucrase 50% inhibition by the extract could not be determined. NAI solution gave 100% inhibition against porcine pancreatic α-amylase and 50% at its 2.88 dilution. PAI solution gave 100% inhibition against porcine pancreatic α-amylase and 50% at its 84.84 dilution. For yeast sucrase and maltase 50% inhibition by PAI solution could not be determined.

Table 6 Effect of amylase inhibitors on the luminal enzymes *in vitro* on %inhibition consideration.

	% Inhibition					
Type of enzyme	α-amylase		maltase		sucrase	
Source	Saliva[1]	Porcine[2] pancreas	Yeast[1]	Jejunum[3]	Yeast[1]	Jejunum3
Activity (mole products under assay condition)	0.00022	0.00022	0.00022	0.00028	0.00030	0.00027
Crude	100	90.70	100	44.64	0	59.26
Crude at IC_{50} of α-amylase	54.77	44.19	0	0	0	12.96
PAI	98.64	90.70	0	0	0	18.52
PAI at IC_{50} of α-amylase	79.32	69.30	0	0	0	1.85
NAI	92.73	97.67	0	3.57	0	33.33
NAI at IC_{50} of α-amylase	55.68	69.30	0	0	0	19.23

[1] Product by Sigma Co.

[2] Crude extract enzyme from porcine pancreas.

[3] Crude extract enzyme from porcine small intestinal.

The values were from the average of duplicate experiment *in vitro*.

Each enzyme was adjusted for equal activity in average at 0.2 mmole product liberation under the assay condition.

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

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

Fasting blood glucose levels (FBG) were compared between two assay methods *i.e*. glucose oxidase kit and glucometer and the results were shown in Table 7. BGL obtained by glucose oxidase kit and glucometer were statistically nonsignificance ($p < 0.05$).

Table 7 Comparative blood glucose level assayed by glucose oxidase kit and glucometer

Rat No.	Glucometer (mg/dl)*	Glucose oxidase kit (mg/dl)*
1	145.5 ± 0.41	152.6 ± 5.97
2	136.5 ± 4.50	134.6 ± 4.49
3	127.0 ± 13.00	107.5 ± 4.97
4	145.0 ± 1.00	131.8 ± 4.13
5	105.0 ± 14.00	110.9 ± 3.65
6	82.5 ± 9.50	84.4 ± 6.71

*The presented values were mean ± S.E. obtained from duplicate blood test of each rat by glucometer and from triplicate analysis of serum from each rat by glucose kit. Each obtained values from the two assay methods was used in SPSS-test.

6.2 Effect of the inhibitor on fasting blood glucose levels in rat

Fasting blood glucose levels (FBG) measuring on day 1 were not statistically significant difference between each group($p<0.05$), after treatment with the tested inhibitor as described in the method for 15 days, blood glucose level of the rats in each group were measured by glucometer. Their mean values ± S.E. were shown in Table 8 with the baseline level on day 1 and their weight. Statistical analysis by

Duncan's multiple test indicated nonsignificant difference (p-value = 0.05) of blood glucose level on day 1 amongst each group with the range of mean ± S.E. varied from 66.50 ± 2.05 mg/dl to 73.67 ± 2.54 mg/dl. On the 15th day the fasting blood glucose levels (FBG) showed the effectiveness of the fed substances in reducing blood glucose level significantly ($p < 0.05$) when compared with the control group. The mean ± S.E. values of FBG in mg/dl were 72.33 ± 2.81 for control, 66.67 ± 2.46 for PAI, 65.33 ± 1.87 for PAI at IC_{50}, 63.67 ± 2.46 for NAI at IC_{50}, 58.33 ± 2.43 for crude extract at IC_{50}, 57.83 ± 2.77 for crude extract and 56.83 ± 3.58 for NAI. Fasting blood glucose levels of the positive amylase inhibitor control group fed with 2.5 mg/ml acarbose and its solution at IC_{50} dilution were 63.83±2.04 and 69.50±1.41 in mg/dl, respectivery.

In consideration on the potency amongst the tested substances, the statistic test confirmed that they all had comparable potency in decreasing blood glucose level though the average blood glucose levels were difference. Statistical test indicated that NAI was powerful in reducing blood glucose of rats more than the extract, the extract at IC_{50} and NAI at IC_{50}, accordingly.

Proteinaceous solution and its solution at IC_{50} showed nonsignificant difference ($p < 0.05$) in reducing blood glucose level after the treatment for 15 days when compared with the control group. But they were significantly difference with the other treatments similar to the results of the control group. This indicated that both proteinaceous solution and its solution at IC_{50} had no affect in reducing blood glucose level within 15 days of treatment.

The treatment of acarbose 2.5 mg/ml significantly reduced blood glucose level in comparison to the control group, but the values were not as effective as the test inhibitors. The dose at its IC_{50} showed no significant difference in the blood glucose level comparing to the control group. Future study a suitable dose of acarbose in significantly decreasing rat blood glucose level comparing to the control group should be found and used in the case of a using it as a positive amylase inhibitor control group.

Weight of rats show in Table 8 on day 1 were not significantly difference amongst the groups, similary to the test for statistical different of day 15. The change of weight between day 15 and day 1 did not significantly difference when compared in each group. This result indicated that the test substances did not affect the growth of the rats in consideration to the normal increasing of their weight along the 15 days of treatment.

Table 8 Effect of amylase inhibitor on fasting blood glucose levels (FBG) and body weight in rats

Group	FBG (mg/dl)		% FBG decrese compare with control	Weight (g)	
	D1	D15		D1	D15
1. Control	66.50 ± 2.05	72.33 ± 2.81^{d}	-	207.33 ± 9.32	254.17 ± 9.79
2. Crude extract	72.83 ± 2.95	57.83 ± 2.77^{ab}	20.05	210.50 ± 5.33	244.33 ± 6.51
3. Crude extract solution at IC_{50} of α-amylase	72.83 ± 2.39	58.33 ± 2.43^{ab}	19.37	200.33 ± 5.57	239.50 ± 8.54
4. PAI	73.50 ± 2.59	66.67 ± 2.51^{cd}	7.83	198.50 ± 1.23	252.00 ± 4.12
5. PAI solution at IC_{50} of α-amylase	72.33 ± 1.87	65.33 ± 1.87^{bcd}	9.68	196.83 ± 0.91	239.50 ± 3.21
6. NAI	66.83 ± 1.47	56.83 ± 3.58^{a}	21.43	198.83 ± 2.07	243.83 ± 6.50
7. NAI solution at IC_{50} of α-amylase	71.67 ± 3.44	63.67 ± 2.46^{abc}	11.97	198.67± 0.95	236.50 ± 1.76
8. Acarbose solution 2.5mg/ml	73.67 ± 2.54	63.83 ± 2.04^{abc}	11.75	210.50 ± 4.83	255.50 ± 7.09
9. Acarbose solution at IC_{50} of α-amylase	73.17 ± 2.80	69.50 ± 1.41^{cd}	3.91	199.83 ± 5.10	247.67 ± 5.11

Presented value were mean ± S.E. from 6 rats

The different letters in the same column indicate the significant differences ($p < 0.05$)

a and d were significantly difference at *p*-value 0.004 for NAI, Crude extract, Crude extract solution at IC_{50}; 0.018 for NAI solution at IC_{50}, 0.020 for Acarbose solution 2.5mg/ml.

b and d were significantly difference at *p*-value 0.054 for PAI solution at IC_{50}

c and d were significantly difference at *p*-value 0.116 for PAI; 0.428 for Acarbose solution at IC_{50}

6.3 Oral tolerance test

The animals in each group were given either 0.2 g glucose, maltose, sucrose or starch per 100 g body weight after the feeding 1 ml of buffer A in control group and 1 ml of test samples in the other groups as described in the method section.

Figure 18 shows the plot of blood glucose level base line (at 0 min), 30′, 60′ and 120 min intervals of each treatment. Their rate of glucose adsorption (RGA), the zenith blood glucose concentration (ZBG) values and the area under the curve (AUC) were calculated for the mean ± S.E. values and all data were tested for the significant different at *p*-value 0.05 with the SPSS as shown in Table 9

In glucose tolerance the plot, the values of either zenith blood glucose concentration (ZBG), area under the curve (AUC) or the rate of glucose adsorption (RGA) at 30 min of all treatments were not significantly difference with the control group ($p < 0.05$).

Maltose, sucrose and starch tolerance tests also showed the similar pattern of the plots (Fig 18 B, C, D) and their values of ZBG, AUC and RGA at 30 min (Table 9) amongst the treatment to the result of glucose tolerance test, that is no significantly difference between the substance fed comparing to their control groups.

Its is noted that glucose blood level of amongst the group of tolerance test at 30 min and 60 min were vary from high to low depending on the type of fed carbohydrate as follows: glucose > maltose > sucrose > starch. This phenomina were due to chemical character of the carbohydrates not the test substances. Starch requires time for digestion before liberating glucose for absorption more than sucrose and maltose accordingly while glucose is ready for immediate absorption.

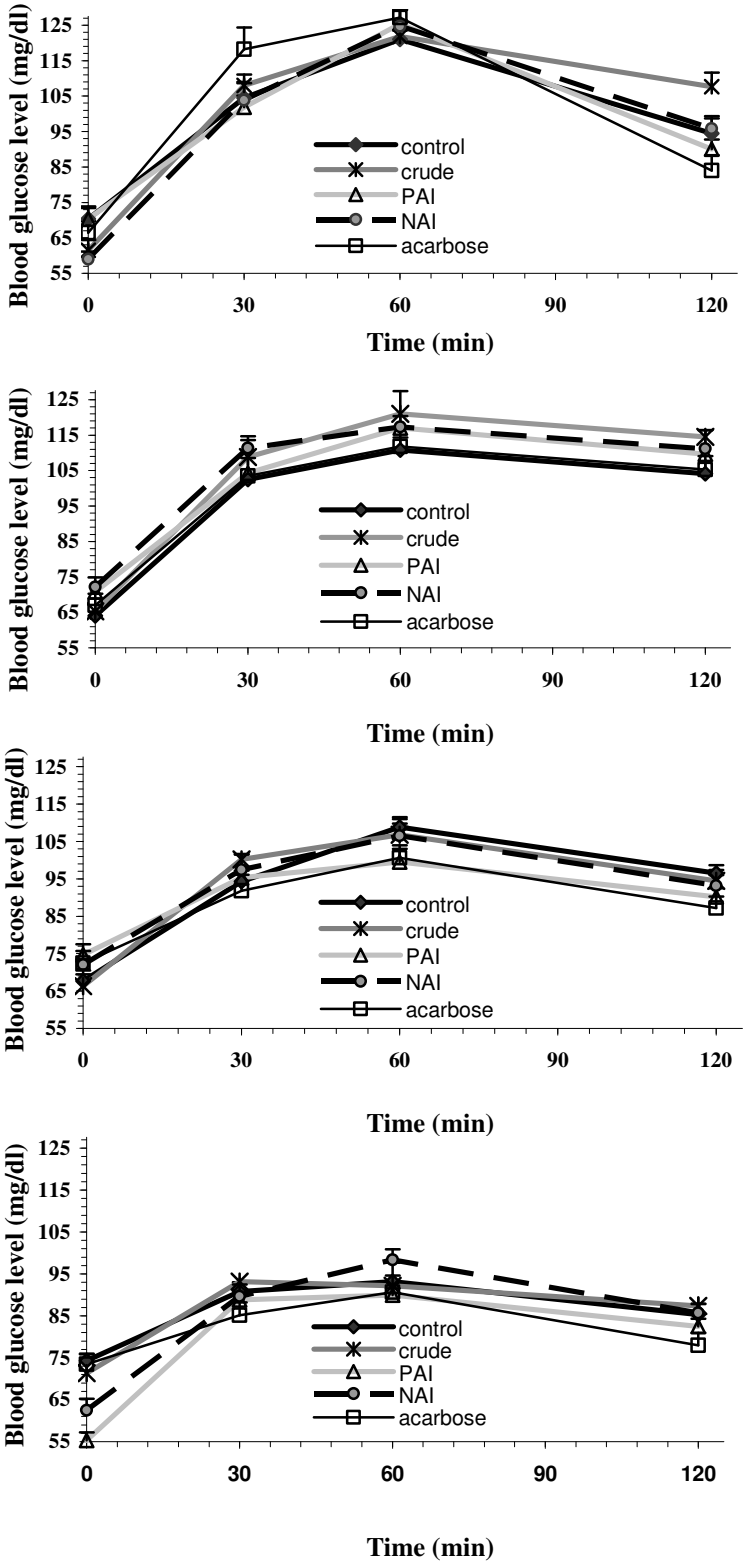


Figure 18 Effect of amylase inhibitors on tolerance test in rat

A: glucose tolerance test **B**: maltose tolerance test

C: sucrose tolerance test **D**: starch tolerance test

Table 9 Effect of amylase inhibitor on blood glucose concentration after substrate loading in normal rats

Group No.	Glucose loaded		
	RGA (mg/dl.min)	ZBG (mg/dl)	AUC (mg/dl)
Control	1.18 ± 0.28	121.00 ± 4.75	207.83 ± 4.44
Crude extract	1.56 ± 0.89	121.83 ± 4.50	214.58 ± 5.41
PAI	1.16 ± 0.21	125.33 ± 4.70	207.50 ± 4.31
NAI	1.71 ± 0.33	124.67 ± 4.69	208.08 ± 6.01
Acarbose (2.5 mg/ml)	1.85 ± 0.23	127.17 ± 2.10	213.08 ± 4.11

Group No.	Maltose loaded		
	RGA (mg/dl.min)	ZBG (mg/dl)	AUC (mg/dl)
Control	1.31 ± 0.11	110.83 ± 4.88	202.46 ± 5.39
Crude extract	1.78 ± 0.58	121.00 ± 6.42	218.75 ± 6.93
PAI	1.24 ± 0.22	117.00 ± 4.73	212.46 ± 6.95
NAI	1.61 ± 0.25	117.00 ± 3.04	217.29 ± 5.18
Acarbose (2.5 mg/ml)	1.24 ± 0.17	111.83 ± 2.52	205.04 ± 5.05

Group No.	Sucrose loaded		
	RGA (mg/dl.min)	ZBG (mg/dl)	AUC (mg/dl)
Control	1.06 ± 0.18^{ab}	108.83 ± 2.65	193.96 ± 4.63
Crude extract	1.21 ± 0.15^{b}	106.83 ± 2.96	194.04 ± 2.93
PAI	0.69 ± 0.10^{a}	99.50 ± 4.46	186.04 ± 5.40
NAI	1.03 ± 0.20^{ab}	106.50 ± 4.51	193.21 ± 5.50
Acarbose (2.5 mg/ml)	0.65 ± 0.17^{a}	100.67 ± 2.35	183.13 ± 4.17

Group No.	Starch loaded		
	RGA (mg/dl.min)	ZBG (mg/dl)	AUC (mg/dl)
Control	0.74 ± 0.18^{ab}	93.17 ± 4.50	176.54 ± 5.34
Crude extract	0.80 ± 0.16^{ab}	92.17 ± 2.62	177.21 ± 2.28
PAI	1.14 ± 0.18^{b}	90.00 ± 1.55	167.00 ± 1.39
NAI	1.05 ± 0.50^{ab}	98.33 ± 2.55	177.04 ± 3.33
Acarbose (2.5 mg/ml)	0.58 ± 0.19^{a}	90.67 ± 3.96	167.96 ± 4.79

The different letters in the same column indicate the significant differences ($p < 0.05$)