CHAPTER 4

DISCUSSION

1. Purified proteinaceous amylase inhibitor

In this study, the purified amylase inhibitor from crude extract was precipitated with 50% ammonium sulfate and dialyzed in 0.01 M phosphate buffer pH 6.9. The dialysate was further purified by anion exchanger, gel filtration and adsorption technique accordingly. Percent yield calculated from the total inhibitory activity of the concentrated fraction from each step in comparison to the crude extract were 43.0% for DEAE-cellulose column, 34.2% for Sephadex G-100 and 20% for hydroxylapatite column. Anion/Cation exchangers are often used in the purification of various proteins including amylase inhibitor of proteinaceous type. Dayler *et al.* (2005) purified the inhibitor from common bean through the DEAE-cellulose column. The fractions from the column had two peaks which peak I shown no inhibitory activity against porcine pancreatic α-amylase (PPA), while peak II could reduce *Z. subfasciatus* α-amylase (ZSA) activity. Farias *et al.* (2007) purified the dialysated of papaya seeds by CM-cellulose column and the retained peak submitted to an analytic reversed-phase column HPLC (Vydac C-18TP) yielding several peaks. Only one fraction, with molecular mass of 4562 Da, showed significant inhibitory activity against *C. maculates* α-amylase. Iulek *et al.* (2000) purified a new α-amylase inhibitor from Rye (*Secale cereale*) endosperm by using two ionic exchange chromatography columns; DEAE-Sepharose and CM-Sepharose column. Which inhibitory activity against human salivary α-amylase was in Peak BIII. Reversed phase chromatographic technique is also used in protein purification such as C-18TP (Farias *et al*., 2007). Gibbs and Alli (1998) fractionated four fractions from extract of white kidney beans with reverse phase chromatography. The isolated fraction with the highest α-amylase inhibitory activity and characterized to be a glycoprotein. Le Berre-Anton *et al*. (1997) also used ammonium sulfate and gel filtration techniques in purifying α-amylase inhibitor from kidey bean (*Phaseolus vulgaris*) seed. To obtain the inhibitor precipitate they use 40% saturation of ammonium sulfate. In the final step of their

process Sephadex G-200 column (100 × 1.5 cm, 177 ml bed volumn) was used. According to their purification procedure 100 g seed flour yielded about 50 mg α-AI isoform 1 and 20 mg α-AI isoform 2. Guzman-Partida *et al*. (2007) purified the α-amylase inhibitor from Palo Fierro seeds (αAI-PF) was purified using affinity chromatography on a fetuin-fractogel column followed by anionic exchange chromatography.

2. Verification of proteinaceous amylase inhibitor purity and its molecular weight

Concentrate fraction from hydroxylapatite column of the purified amylase inhibitor showed a single protein band on 14-17% Native-PAGE with 65 mg loading protein after coomassie blue staining. This support that the obtained protein was pure with less contamination of other proteins. In comparison to molecular weight markers and their plot with the migration distance on the gel this single band has the size of 36.3 kDa.

Results of molecular weight determination by Sephadex G-100 shows its molecular weight 47.7 kDa which is in the same range of that found in the Native-PAGE, result of Sitthipong (2005) and Marshall *et al.* (1975). Sitthipong (2005) reported the Native-PAGE size of her purified proteinaceous α-amylase inhibitor from red kidney beans 56.73 by Sephadex G-100 and 72.6 kDa by 10% Native-PAGE. Purification procedures of Sitthipong (2005) were similar with this study but by the smaller scale. Mashall *et al.* (1975) reported that the molecular weight of kidney bean (*P.vulgalis*) in size range 45-50 kDa by Sephadex G-100.

In the denature from and under the breaking of disulfide bonds this protein has 3 subunits of 15.5, 17.0 and 18.2 kDa on the 14-17% SDS-PAGE. Sitthipong (2005) reported that the purified inhibitor contained 2 subunits with 17.38 and 16.60 kDa by 10% SDS-PAGE. Yamada *et al.* (2005) reported that the purified αAI-PA2 from tepary bean have three subunits of the size range from 14-20 kDa 0n 13.5% SDS-PAGE.

3. Purified nonproteinaceous amylase inhibitor

Nonproteinaceous amylase inhibitor was prepared as described by Kim *et al*. (2002) from 300 ml of crude extract of Royal red kidney bean dissolved in 0.02 M phosphate buffer, pH 6.9 with 0.015 M NaCl with final volume 10 ml. The inhibitors have 1.5 mg maltose/ml and 4.4 mg protein/ml with IC_{50} = 0.66 mg maltose/ml.

Hansawasdi *et al*. (2000) isolated hibiscus acid from a roselle tea extract with 50% methanol and analyzed structure with mass spectrophotometer. McCue *et al.* (2004) studied potential inhibition of nonproteinaceous α-amylase inhibitor of selected American and Asian foods against α-amylase and α-glucosidase by homogenizing each of them in distilled water for 1 min using a Waring laboratory blender set on "HIGH". The homogenate was centrifuged at 10,000 rpm at 4 °C for 20 min. The supernatant was filtered through Whatman filter paper # 1 and subsequently optimized for phenolic content. Jamaluddin *et al*. (1995) extracted the nonproteinaceous α-amylase inhibitor, stigmast-4-en-3-one from empty pods of *P. speciosa* with petroleum ether, chloroform, dichloromethane, ethyl acetate, 25% ammoniacal chloroform and methanol. A general extraction procedure was filtered for each solvent by soaking the powdered pods overnight, the solution filtered and the solvent then evaporated. The extraction was repeated three times, each using a fresh solvent. Kim *et al.* (1999) prepared isoacarbose by the transglycosylation reaction of *Bacillus stearothermophilus* maltogenic amylase (BSMA) and purified by gel filtration on Bio-Gel P2. Yoon and Robyt (2003) purified acarbose analogues; maltohexose-Aca, maltododecaose-Aca and maltooctadecaose-Aca, were purified by Bio-gel P2 column. All of them can inhibited *A. oryzae* α-amylase, *B. amyloliquefaciens* α-amylase HSA and PPA.

4. Verification of nonproteinaceous amylase inhibitor

The TLC analysis of nonproteinaceous amylase inhibitors revealed two spots, with R_f = 0.624 and 0.581 with the big band of non identified pattern on the plate after sprayed land heated as described in the method. To indicate the spot with amylase inhibitory activity each separated spot on the non sprayed lane corresponded to the spots on the sprayed lane was scrapped, extracted with buffer A and checked for its inhibitory

against salivary α-amylase using 2% starch solution as a substrate. After the incubation at 37 °C for 3 min the remaining non hydrolyzed starch was detected by the addition of I_2 solution. The spot with R_f value = 0.581, which located between G1 and G2 of standard sugars R_f 0.624 and 0.538 show positive blue colour of I_2-starch complex. This spot thus contained an inhibitory activity against salivary α-amylase.

Sitthipong (2005) reported that the nonproteinaceous α-amylase inhibitor from Royal red kidney bean has its R_f value to acarbose. Kim *et al*. (2002) purified α-D-Glucopyranosyl-α-acarviosinyl-D-glucopyranose (GlcAcvGlc) from high-molecular-weight acarbose (HMWA) by solvent fractionation and chromatography. This purifed GlcAcvGlc can inhibited PPA more effectively than acarbose and has the same molecular weight as acarbose

5. Property studies of the nonproteinaceous 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). Knowledge of specific nature and unique behavior or properties of inhibitor on enzyme functions can be assigned for various applications. Since this work is study on the nonproteinaceous amylase inhibitor from Royal red kidney beans, the data obtained would be valuable for control postprandial plasma glucose levels.

Thermal effect on inhibitory activity and stability of nonproteinaceous amylase inhibitor from red kidney beans (*P.vulgaris*) at various temperatures was investigated. The optimum temperature for the inhibition of α-amylase by nonproteinaceous amylase inhibitor was at a constant level from 40-50 °C. This finding was in the difference range to that found by Le Berre-Anton *et al*. (1997) that is optimum temperature at 37 °C for inhibition of human and porcine pancreatic amylase by purified *P.vulgaris* L., cv.

Keeping inhibitor at various temperatures 30 min prior it inhibitory activity revealed it stability at temperature range from 4-50 °C but its activity was dropped seriously above 50 °C. This result was similar to the study of Grant *et al*. (1995) and Gili and Kachole (1998) which reported that proteinaceous amylase inhibitor from

P.vulgaris was not stable at 40-80 °C but opposite to with the study of Kim *et al*. (2005) which found that amylase inhibitor in pine bark extract was stable at 90-100 °C.

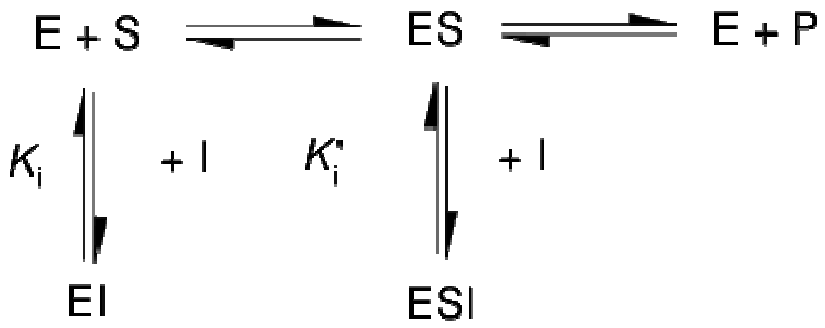
pH effect on nonproteinaceous amylase inhibitor has inhibitory against α-amylase optimum pH for the inhibition at pH 7.0. These data were similar to the pH optimum of the proteinaceous amylase inhibitor from various sources (*P.vulgaris* and *Zea mays*) between 6.0 and 7.0 reported by Gibbs and Alli (1998) and Figueira *et al*. (2003), but were different from a nonproteinnaceous amylase inhibitor from acarbose with its optimum pH between 4 and 8 (Talamon *et al*., 2002). Le Berre-Anton *et al*. (1997) reported that purified α-amylase inhibitor from white bean (*P.vulgaris*) had its optimum pH at 4.5. Marshall *et al*. (1995) reported that α-amylase inhibitor from white bean (*P.vulgaris*) has its optimum pH at 5.5.

Keeping inhibitor at various pH temperatures 30 min prior it inhibitory activity revealed it optimum stability at pH 6.0. This result was similar to α-amylase inhibitor from pine bark extract which was stable in acidic condition (Kim *et al*., 2005).

The effect of ions on inhibitory activity from higher to lower was $MgSO_4$>$CaCl_2$>NaCl = KCl> no salts added. Gibb and Alli (1998) reported that chloride ions were important for full inhibitory activity of α-amylase while calcium ions increased the initial rate of binding but magnesium or sulfate ions did not have an effect on inhibition. Sitipong 2005 reported that effect of metal ions on the inhibitory activity of proteinaceous amylase inhibitor from *P.vulgaris* as following $CaCl_2$ > NaCl > $MgSO_4$ > KCl > no salts added.

Kinetic inhibition, of α-amylase inhibitors of nonproteinaceous amylase inhibitor from Royal red kidney bean (*P. vulgaris*) is a mixed noncompetitive type. This finding agrees with mixed noncompetitive type of αAI1 from red kidney bean (*Phaseolus vulgaris*) reported by Le Berre-Anton *et al.* (1997); Santimone *et al*. (2004) and Sitthipong (2005). Kim *et al.* (1999) reported that acarbose, acarviosine-glucose and isiacarbose were compared as inhibitors of α-glucosidase, α-amylase and cyclomaltodextrin glucanosyltransferase. The three inhibitors were found to be competitive inhibitor for α-glucosidase and mixed noncompetitive inhibitors for α-amylase and cyclomaltodextrin glucanosyltransferase. Kim *et al*. (2005) reported the inhibition of pine bark extract against salivary α-amylase as competitive type, yeast α-glucosidase as a combination of mixed noncompetitive. Kandra *et al*. (2005) reported

that the inhibition is therefore of the mixed noncompetitive type for acarbose with K_i 3.7 μM and K'_i 1.08 μM, using amylose as a substrate. Zajácz *et al* (2007) reported that Aleppo tannin showed a mixed noncompetitive against human salivary α-amylase. Yoon and Robyt (2003) reported that acarbose and two acarbose analogues was a mixed noncompetitive inhibitor for porcine pancreatic α-amylase and human salivary α-amylase with K_i value 0.8 and 1.27 μM, respectively. The Lineweaver-Burk plot of them gave straight lines and intercept in the third quadrant at a single point. In mixed inhibition, the inhibitor can bind to either the free enzyme or the enzyme-substrate complex with $K_i \neq K_i'$ the inhibitor binds to a site different from the active site where the substrate binds (below model).



Model of mixed noncompetitive inhibition

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

In this study, it was found that α-amylase inhibitor of proteinaceous and nonproteinaceous types from Royal red kidney bean (*P. vulgaris*) inhibited enzyme pancractic α-amylase (PPA) at IC_{50} = 0.0002, 0.0145 g bean/ml, α-amylase from saliva (HSA) at IC_{50} = 0.0002, 0.0182 g bean/ml but no inhibited sucrase and maltase from yeast. Crude extract inhibited enzyme HSA, PPA and maltase at IC_{50} = 0.0037, 0.0043, 0.0680 g bean/ml, respectively but no inhibited 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. This result suggested that the inhibitor from Royal red kidney bean has the possibility to modulate carbohydrate digestive enzymes which in trun control blood glucose levels.

Kim *et al.* (2004) reported that the pine bark extract can inhibit HSA, PPA, α-glucosidase from (*Saccharomyces cerevisiae*) and α-glucosidase from porcine small intestine at IC_{50} 1.7, 1.69, 0.025 and 155.00μg/ml, respectively. Kim *et al*. (2002) reported that α-D-Glucopyranosyl-α-acarviosinyl-D-glucopyranose (GlcAcvGlc) can inhibit maltase, sucrase from rat intestine and PPA at IC_{50} 4.1, 8.1 and 1.2 μM, respectively. Matsuura *et al*. (2004) found that (7*S*, 8*S*)-syringoylglycerol 9-*O*-β-D-glucopyranoside and (7*S*, 8*S*)-syringoylglycerol-9-*O*-(6′-*O*-cinnamoyl)-β-D-glucopyranoside isolated from extract of hyssop inhibited α-glucosidase, prepared from rat small intestinal acetone powder by 53 and 54% at its concentration of 3×10^{-3} M. McCue *et al*. (2004) reported that 50% ethanol extract of Oregano (*Origanum vulgare*), a rich source of natural phenolic, showed the inhibition against porcine pancreatic amylase by 9-57%. Loizzo *et al.* (2007) reported that the inhibition of α-amylase activity by *Cedrus libani* essential oils obtained from wood exhibited an IC_{50} value of 0.14 mg/ml, whereas the leaves and cones oils were devoid of any significant activity. Sitthipong (2005) reported that the nonproteinaceous α-amylase inhibitor also inhibited maltase and sucrase as acarbose but with lower potency than acarbose. The nonproteinaceous α-amylase inhibitor showed its activity potency on human salivary amylase at 52.2%, porcine pancreatic amylase at 34.7%, maltase from yeast and porcine small intestinal extract at 20.8 and 16.4%, respectively and sucrase from yeast at 13.9%. Bhandari *et al*. (2008) examined the extract of the Nepalese herb Pakhanbhed for its anti-diabetic activity *in vitro* and isolated the active compounds as (-)-3-*O*-galloylepicatechin and (-)-3-*O*-galloylcatechin. These isolated compounds demonstrated significant dose dependent enzyme inhibitory activities against rat intestinal α-glucosidase and porcine pancreatic α-amylase. IC_{50} value for sucrose, maltase and α-amylase were 560, 334 and 739 μM, respectively for [(-)-3-*O*-galloylepicatechin] and 297, 150 and 401 μM, respectively for [(-)-3-*O*-galloylcatechin]. The anti-diabetic potential of Pakhanbhed was postulated to be helpful in developing medicinal preparations or nutraceutical and functional foods for diabetes and related symptoms.

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

In this studies, it was found that the amylase inhibitor from Royal red kidney bean had an effect in decreasing fasting blood glucose level (FBG) in rats after feeding the test samples for 15 days. The test samples that could reduced the FBG significantly ($p<0.05$) were NAI, crude extract at 100% inhibition and solution of NAI, crude extract at IC_{50} against salivary α-amylase. PAI and its solution at IC_{50} against salivary α-amylase did not show any effect in reducing blood glucose level. These test samples were prepared from the extract of 100 g Royal red kidney bean in 500 ml 0.02 M phosphate buffer pH 6.9 containing 0.015 M NaCl. These extract had protein 30.72 mg/ml and 1.63 mg maltose equivalence/ml with 100% inhibition against salivary α-amylase and 50% inhibition against salivary α-amylase at its 54.17 dilution. From the preparation processes the obtained 10 ml solution of NAI contained 15 mg maltose equivalence with 100% inhibition against salivary α-amylase and 50% inhibition against salivary α-amylase at its 2.29 dilution. The obtained 112.5 ml solution of PAI from 3 rounds of purification processes contained 315 mg protein with 100% inhibition against salivary α-amylase and 50 % inhibition against salivary α-amylase at its 85.88 dilution.

The results of this study showed a similar conclusion to the study of Ortiz *et al*. (2007) that the inhibitor of enzyme involves in the liberation of glucose from carbohydrate digestion could reduce postprandial blood glucose level. Methanol extract from *Tournefortia hartwegiana* (METh) at 310 mg per kg body weight can decrease blood glucose level of the Wistar rats weight 200-250 g after 1 hour oral administration to 30 mg/dl significantly at *p*-value< 0.01. This METh was also reported to be more active in reducing blood glucose level than the anti-hyperglycemic reference acarbose at 0.3 mg/kg body weight. In this study a fixed dose of acarbose at 2.5 mg also had a potency in decreasing blood glucose level less than the test inhibitor samples. The 15^{th} blood glucose level of the groups treated with crude extract, extract at IC_{50}, NAI were lower than those treated with 2.5 mg acarbose as follows: 57.83±2.77, 58.33±2.43, 56.83±3.58 and 63.83 ± 2.04, the values were mean ± S.E. of 6 rats in mg/dl.

To explain the effect of the test inhibitor samples on enzymes involve in the liberation of glucose for absorption from carbohydrates, oral tolerance tests fed the

test inhibitors with either maltose, sucrose or starch were performed in comparison to those fed with glucose. Tolerance graph profile from 0-120 min, RGA, ZBG and AUC showed agree results amongst the test samples, control (no treatment) and the anti-hyperglycemic reference group (acarbose 2.5 mg fed); that is no significant difference between the test substances in decreasing blood glucose level in rats loaded with either glucose, maltose, sucrose or starch. In this study the fed carbohydrates were varied according to the rat body weight and the dose used were as described by Ye *et al*. (2002), however, the fed inhibitor samples were the fixed dose (the dose was not vary according to the rat weight). Such of the treatment may be the cause of the opposite result from what is expected. Future studies involve with varying dose of the fed inhibitor samples corresponding to the body weight of the rat shall be applied rather than the fixed dose as that had done in this study. The amount of fed substrates may also required varying of the doses.