CHAPTER 5

DISCUSSION

1. Comparative study of five methods for α-amylase inhibitor extraction efficiency

The aim of this study was to examine the efficiency of extraction method of α- amylase inhibitor from *P. speciosa* powder. In order to accomplish this purpose, five methods were tried and assayed for α-amylase inhibitory activity after the extraction process and after three months storage under dark conditions at -20 °C.

Result of the study revealed that method of Pueyo and Delgado-Salinas (1997) was the best method in the extracting α-amylase inhibitor from the pod powder of *P. speciosa*. The extract had IC_{50} at only 1.1 ± 0.1 mg of the powder/ml while the other four methods required more amount of extract concentration at IC_{50} as follows : Marshall and Lauda (1975), (1.6 ± 0.1) ; Giri and Kachole (1998), (10.7± 0.5) ; Grant *et al*. (1995) without heat (54.6 ± 0.5) and Grant *et al*. (1995) (65.8 ± 3.1). Method of Pueyo and Delgado-Salinas (1997), Marshall and Lauda (1975) and Giri and Kachole (1996) provided more stable inhibitor extracts than the other two methods under three month storage condition since their IC_{50} values were very much close to those obtained after the extraction processes. In consideration of the process details PMSF, a protease inhibitor and β-mercaptoethanol used by Pueyo and Delgado-Salinas (1997), and a defatted process and PVP used by Giri and Kachole (1998) prevented destruction of the inhibitory activity during assay conditions. IC_{50} values and stability of extract from method of Marshall and Lauda, (1975) are close to those by Pueyo and Delgado-Salinas (1997) may be due to 1:3 w/v ratio provided more concentrated extract than the 1:10 w/v ratio by Pueyo and Delgado-Salinas (1997) and the heat treatment caused denaturation of proteases and other interferences. The extract of inhibitor by method of Grant *et al*. (1995) with heat and without heat showed low potency and stability of inhibitory activity in comparison to the other three methods. However, the process contains no harmful

chemicals and also has buffering capacity at pH 6.9 for α-amylase enzyme. For future application in health the method of Grant *et al*. (1995) is thus used in this study especially the process without heat.

2. Partial purification of α-amylase inhibitor

In the first study, to purify amylase inhibitor from crude extract, $AmSO_4$ was chosen for precipitation. The result in Table 4 shows that $AmSO_4$ was not appropriate to precipitate amylase inhibitor from crude extract of *Parkia speciosa* because there was a little quantity of precipitate with a low inhibitory activity. Hence, the attempt on solvent precipitation *i.e*. ethanol and methanol was employed. Several ethanol and methanol concentrations were tried for AI precipitation. These results (Table 5 and 6) showed no precipitation at 0-40% of both solvents whereas at 50-60% showed very minute amount of precipitate with low inhibitory activity. The precipitate obtained from 95% methanol had higher inhibitory activity than that from 95% ethanol. Therefore, precipitation at 95% methanol was used for further purification by Sephadex G-75 column, eluted with 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl. The eluted fractions showed no inhibitory activity value eventhough the concentration of sample was increased (Figure12). Other chromatographic packing materials including CM-cellulose and DEAE-cellulose were tried, but the elution of AI was not possible, indicating amylase inhibitor may bind to the packing materials. Eventually crude extract, 95%MeOH supernatant (Aq 95%MeOH) and the precipitate were each separated by Sephadex G-75 fraction and eluted with 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl. Crude extract and Aq 95%MeOH chromatographic profiles were similar, which contained AI peak and contaminate peaks at OD_{280} without AI activity. Chromatographic profiles of precipitate showed no AI activity and had small peaks at OD_{280} of contaminants. However, the crude extract profile contained higher contaminate peaks at OD_{280} in comparison to Aq 95%MeOH. Therefore, Aq 95%MeOH with high inhibitory activity was considered for further purification. After loading the sample onto Sephadex

G-75 column and eluted with 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl, the elution profile of amylase inhibitory activity peak overlapped with OD_{280} was obtained (Figure 11). The pooled fraction of AI peak was collected for property and other studies.

The inhibitory active fractions were combined and performed electrophoresis analysis on native PAGE and SDS PAGE (native and sodium dodecyl sulphate polyacrylaminde gel electrophoresis), stained with coomassie blue, however, pattern of protein bands were not observed though the fractions gave positive result with Lowry's reagent. This result indicated that the inhibitory active fraction from Sephadex G-75 column was not protein. McCue *et al*. (2004) found that activity of anti-amylase and anti-glucosidase from Asian and American food were associated with total phenolic content more than total protein. Kandra *et al*. (2004) also reported the effect of commercial tannic acid on salivary α-amylase. Therefore, amylase inhibitor in Sephadex G-75 pooled fraction may be a phenolic compound. This puzzle was further investigated by TLC plate for phenolic compound in the last section.

3. Property studies of amylase inhibitor

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 the first and pioneer study on the inhibitor separated from *P. speciosa*, the data obtained would be valuable in the future subsequent studies and better understanding of the inhibitor roles in controlling postprandial plasma glucose levels and insect resistant.

Thermal effect on inhibitory activity and stability of amylase inhibitor in crude extract and Sephadex G-75 fraction at various temperatures were investigated. The optimum temperature for the inhibition of α-amylase by α-amylase inhibitor in the from of crude extract or Sephadex G-75 fraction was at a constant level from 4-37°C.

This inhibition was approximately 53.2 % of the initial α-amylase activity. This finding was in the similar range to that found by Le Berre-Anton *et al*. (1997) that is optimum temperature at 37 °C for the inhibition of human and porcine pancreatic amylase by purified AI from *P. vulgaris* L., cv. Tendergreen, and Marshall and Lauda (1975) for the inhibition of pancreatic amylase by Great Northern white kidney bean.

Keeping inhibitor at various temperature 30 min prior its inhibitory activity assay revealed its stability at temperature range from 4 °C to 40 °C either in the from of crude extract or Sephadex G-75 fraction but its activity gradually decreased at 50-60 °C and further drop seriously at 80-90 °C. This result was 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, but similar to the study of Grant *et al*. (1995) and Giri and Kachole (1998) which reported that proteinaceous α-amylase inhibitor from *P. vulgaris* were no stable at 40-80 °C.

pH effect, Figure 16 shows effect of pH on α-amylase and α-amylase inhibitor in crude extract and Sephadex G-75 fraction. Areas between the line of amylase activity and the amylase activity left after the addition of the inhibitors revealed optimum pH for the inhibition at pH 7. These data were similar to the pH optimum of the proteinaceous α-amylase inhibitors 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 nonproteinaceous α-amylase inhibitors from acarbose with its optimum pH between 4 and 8 (Talamon *et al*., 2002).

Inhibitor either in the from of crude extract or Sephadex G-75 fraction were tolerance under storage conditions at pH range from 4 to 7 for 30 min, since its inhibitory activity were consistence at 0.16 ± 0.0007. These results were similar to α-amylase inhibitor of pine bark extract which was stable in acidic condition (Kim *et al*., 2005). Stability of the inhibitor under acidic condition suggested that it will be easier to handle the inhibitor during processing or manufacturing steps and the inhibitor will remain stable when exposes to the human digestive tract.

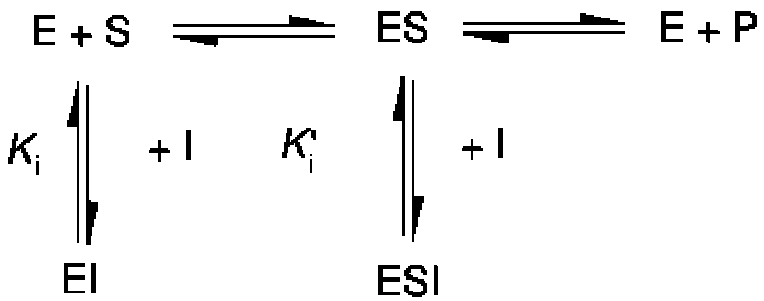
Salt effect, Figure 17 shows no effect of various salts *i.e.* NaCl, KCl, $CaCl_2$ and $MgSO_4$ on inhibitory activity of the α-amylase inhibitors. Sitipong 2005 reported effect of metal ions on the inhibitory activity of proteinaceous amylase inhibitor from *P. vulgaris* from higher to lower as following $CaCl_2$ > NaCl > $MgSO_4$ > KCl > no salt added. Gibbs and Alli (1998) also reported that $CaCl_2$ improved inhibitory activity of proteinaceous amylase inhibitor from *P. vulgaris* against porcine α-amylase followed by NaCl and KCl, accordingly. Their work also reported no effect of $MgSO_4$ on the inhibition. There were no reports the effect of salts on inhibition of a nonproteinaceous amylase inhibitor against amylase activity, so our results could not be able to compare with others.

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

In this study, it was found that α-amylase inhibitor in crude extract and Sephadex G-75 fraction of *P. speciosa* inhibited enzyme from different sources (Salivary α-amylase, porcine pancreatic α-amylase, porcine intestinal maltase, yeast maltase yeast sucrase) to different degrees. The inhibition was high efficiency on luminal enzyme of mammal but less effect on sucrase from yeast. These results suggested that the inhibitor from *P. speciosa* has the possibility to modulate carbohydrate digestive enzyme which in turn control blood glucose level.

Kinetics inhibition, of α-amylase inhibitors in the crude extract and Sephadex G-75 fraction on human salivary α-amylase, yeast sucrase and yeast maltase are a mixed noncompetitive type. This finding agrees with a mixed noncompetitive type of tannins and flavonoid reported by Kandra *et al*. (2004); Kim *et al*. (2000). Kim *et al*. (2005) reported inhibition of pine bark extract against salivary α-amylase as competitive type, yeast α-glucosidase as combination of noncompetitive and uncompetitive type (mixed noncompetitive). The Lineweaver-Burk plot of them gave straight lines and intercept in the either second or third quadrant at single point. The different kinetic

inhibition may be caused by structural differences due to the origin of the enzyme. As reported previously, α-glucosidase broadly consists of type I from yeast *Saccharomyces cerevisiae* and type II from the mammalian species, and there are structural differences between these types (Chiba, 1997). 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

The obtained K_i values demonstrated that the inhibitor can inhibit α-amylase more than maltase and sucrase respectively, its ability to bind (its affinity) to free enzyme is higher than enzyme-substrate complex for α-amylase inhibition (K_i 29.29 mg/ml; K_i'66.36 mg/ml for Sephadex G-75 fraction and K_i 0.24 mg/ml ; K_i' 0.51 mg/ml for crude extract). This result contrasted with maltase (K_i 69.35 mg/ml ; K_i' 47.50 mg/ml for Sephadex G-75 fraction and K_i 0.54 mg/ml; K_i' 0.46 mg/ml for crude extract) and sucrase (K_i 269.94 mg/ml ; K_i' 124.62 mg/ml for Sephadex G-75 fraction and K_i 21.78 mg/ml; K_i' 4.21mg/ml for crude extract) inhibition which the inhibitor can bind to enzyme-substrate complex better than the free enzyme. This finding reveals for the first time a novel attribute of the inhibitor in the regulation of α-amylase and α-glucosidase

activity and may be useful as lead compounds to control the function of these enzymes in controlling postprandial blood sugars levels.

5. Potential application of the inhibitor on pest control

Several (-amylase inhibitors have been reported to inhibit the gut α-amylase from insects (Rekha *et al*., 2004). Our study has shown the specificity of crude extract on amylase of C. chinensis and S. orysea but not with C. maculatus. The specificity was also reported by Chrispeels et al. (1998) that (-AI1 from seeds of P. vulgaris inhibited porcine pancreatic α-amylase and Cowpea weevil (*C. maculatus*) α-amylase but did not inhibit α -amylase of Maxican been weevil (*Zabrotes subfasciatus*) and Bean weevil (*Acanthoscellides obtectus*). For α-AI2 it did not inhibit porcine pancreatic α-amylase, *C. maculatus* α-amylase and *A. obtectus* α-amylase but inhibited the larva of *Z. subfasciatus* α-amylase.

For research and development bioassay for the effect of inhibitor on the growth of *C. chinensis* and *C. maculatus* were preformed in artificial beans and mungbean seeds. This study indicated that the inhibitors had no ability in inhibit the growth of insect under our assay conditions. In addition, the artificial beans may be not convenient for survival of insects due to it stiffness and roughsurface. Effect of amylase inhibitor on the growth of *C. chinensis* were studied intensively by Wang *et al*., (1999) which reported that *C. chinensis* does not growth in artificial beans which was mixed together with amylase inhibitor from *Vigna mungo.* This result contrasted with mungbean seed which were coated by crude extract and Sephadex G-75, the number of adults insect were emerged in equal number to the control group. The result indicated that test inhibitors had no effect in controlling the birth of insect under the assay condition. So, the inhibitor from pod of *P. speciosa* can not be used as a pest controller.

6. Identification of Sephadex G-75 fraction with thin layer chromatography (TLC)

The inhibitory activity of various phenols on digestive enzymes involve in breakdown of starch, such as tannins and flavonoids were reported to inhibit α-amylase and α-glucosidase activity. In this study, it was found that ether extract of the acid hydrolysate of Sephadex G-75 fraction gave a positive blue color with Folin reagent after spraying and after fuming with ammonia vapour. This blue spot had its R_f value equivalent to the R_f value of gallic acid. These results supported that the Sephadex G-75 fraction may be a phenolic compound containing gallic acid in its structure.

According to IR spectrum it could be preliminary concluded that the active amylase inhibitor in Sephadex G-75 fraction had the following groups: hydroxyl, carboxylic acid and aromatic ring in its molecule. Result from TLC of ether extract of the acid hydrolysate of Sephadex G-75 fraction confirmed that basic structure of the molecule comprised of phenolic structure which closed to gallic acid. Positive reaction of Sephadex G-75 fraction to Folin reagent strongly suggested that the active amylase inhibitor was a phenolic compound.