รายงานวิจัยฉบับสมบูรณ์

โครงการ สารยับยั้งอะไมเลสจากผลไม้ไทย

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คณะผู้วิจัย **สังกัด**

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จุฬาลงกรณ์มหาวิทยาลัย

ชุดโครงการ Thai Fruits - Functional Fruits

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

1. Executive summary

Nowadays, peptide/protein drugs are one of the promising for new drug development. There are a lot of drugs in this group are approved by US-FDA during the past few years. The sources of peptide drug are from plant, animal and microorganism. Thailand is rich of natural resource especially medicinal plants which have been reported by many of scientists. At the moments, Thailand and other country around the world are facing with problem of the increasing of aging person due to the more advance of medical knowledge. There are not just at the end we died. There are suffering related to several diseases which came along the aging process such as heart disease cancer, diabetes, kidney disease and hypertension. Consequently, it's necessary to improve our drug to gain better prevention and treatment of these diseases. Diabetes mellitus (DM) is a metabolic disease caused by deficiency in insulin secretion. The disease is developing, along with an increase in both obesity and ageing, in the general population. It is a great challenge now, because about 5% of the global population is affected by DM. Insulin secretion deficiency results in an increase of blood glucose level and causes serious damage to body systems, such as blood vessels and nerves. One of the therapeutic approaches is to decrease the postprandial hyperglycaemia by retarding absorption of glucose by inhibition of carbohydrate-hydrolysing enzymes, such as α-amylase. Certain plant α-amylase inhibitors have been demonstated to have adverse effect in nutrition (antinutrients) due to their inhibition of digestive enzymes in man and animals. The inhibitors have also been proposed for application in obesity and diabetes therapy. In the search for potent α-amylase inhibitors, we focused on traditional local Thai fruits as the sources of α-amylase inhibitors, since they are known to have many pharmaceutical effects and health benefits.

2. Abstract (English)

Diabetes is a syndrome from the disordered control of blood glucose levels and associated metabolism resulting in abnormally high blood sugar levels (hyperglycemia) after feeding. One possibility for lowering postprandial glucose levels, after ingestion of complex carbohydrates anyway, is by the inhibition of α-amylase activity. In this study, aqueous extracts from seven local Thai fruits were tested for their *in vitro* inhibitory effect on α-amylase. The fruits of Kluai Hom Thong; Musa (AAA group) bananas showed a significant reduction in the test α-amylase activity. An α-amylase inhibitor (AI) was purified from this fruit using a sequential combination of ammonium sulfate precipitation, DEAE-cellulose ion exchange and Superdex-75 gel filtration chromatography. The enriched AI protein fraction had a specific activity of 355.7 AI U/mg protein and a yield of 18.5% of the total protein. The molecular weight of this proposed proteinaceous AI, estimated by SDS-15% (w/v) PAGE, was ~20 kDa. Periodic acid staining and the phenol-sulfuric assay showed that the enriched AI protein was a glycoprotein containing 10.74 ± 0.35% by weight carbohydrate, respectively. The AI showed a pH optimum of between pH 4 - 7, with poor tolerance below pH 4 and above pH 9, and was stable up to 40 °C but totally inactivated after exposure to 50 °C for 90 min. The AI activity was stimulated by Ca^{2+}, Mg^{2+}, and Mn^{2+} at less than 25 mM, but not by Fe^{3+}, Hg^{2+}, Co^{2+} and EDTA. Determination of the inhibition kinetics of α-amylase by the enriched AI protein fraction indicated non-competitive inhibition of α-amylase with a K_i of 0.89 mg protein/ml. The sequence of a 16 amino acid internal fragment of this AI protein showed sequence similarity to the plant chitinase family.

3. Abstract (Thai)

โรคเบาหวานเป็นโรคที่เกิดจากความผิดปกติของฮอร์โมนอินซูลิน ซึ่งเป็นฮอร์โมนที่ทำหน้าที่ในการเผาผลาญสารอาหารจำพวกคาร์โบไฮเดรต เป็นผลทำให้กระบวนการเมแทบอลิซึมของคาร์โบไฮเดรตเกิดความผิดปกติส่งผลให้ระดับน้ำตาลในเลือดสูงขึ้น การยับยั้งการทำงานของแอลฟา-อะไมเลสเป็นอีกหนึ่งทางเลือกที่สามารถทำให้ระดับน้ำตาลในเลือดลดลงได้ ในงานวิจัยนี้ได้ทำการคัดเลือกผลไม้ไทยจำนวนมาสกัดในสารละลายบัฟเฟอร์ พบว่าส่วนสกัดหยาบของกล้วยหอมทองมีประสิทธิภาพในการยั้บยั้งแอลฟา-อะไมเลสได้มากที่สุด จึงนำส่วนสกัดหยาบของกล้วยหอมทองมาทำให้สารยั้บยั้งแอลฟา-อะไมเลสบริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวที่ 80 เปอร์เซนต์ และเทคนิคโครมาโทกราฟีแบบแลกเปลี่ยนอิออนด้วยคอลัมน์ ดีอีเออี เซลลูโลส และโครมาโคกราฟีแบบเจลฟิลเตรชันด้วยคอลัมภ์ ซูปเปอร์-เด็กซ์ 75 พบว่ามีกิจกรรมการยั้บยั้งจำเพาะ 355.74 ยูนิตต่อมิลลิกรัมโปรตีน กิจกรรมการยับยั้งคงเหลือ 18.50 เปอร์เซ็นต์ เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพ ซึ่งสารยั้บยั้งแอลฟา-อะไมเลสบริสุทธิ์ที่ได้ มีน้ำหนักโมเลกุลประมาณ 20 กิโลดาลตัน ผลการย้อมสีแบบเพอร์ริออดิก พบว่าสารยั้บยั้งแอลฟา-อะไมเลสชนิดนี้เป็นไกลโคโปรตีน และผลทดสอบหาปริมาณคาร์โบไฮเดรตด้วยวิธีฟีนอล-ซัลฟูริก พบว่ามีสัดส่วนเป็นคาร์โบไฮเดรต 26.30±1.01 เปอร์เซ็นต์โดยน้ำหนัก สารยั้บยั้งแอลฟา-อะไมเลสชนิดนี้มีเสถียรภาพของกิจกรรมของการยับยั้งแอลฟา-อะไมเลส ที่ค่าความเป็นกรด-ด่างเท่ากับ 4 จนถึง 7 และที่อุณหภูมิ 4 จนถึง 40 องศาเซลเซียส และสูงสุดที่ 40 องศาเซลเซียส สารยั้บยั้งแอลฟา-อะไมเลสชนิดนี้ต้องการแคลเซียมไอออน แมกนีเซียมไอออน และแมงกานิสไอออนอย่างน้อย 25 มิลลิโมลาร์ ซึ่งจำเป็นสำหรับเสถียรภาพของโครงสร้าง และของกิจกรรมการยั้บยั้ง เมื่อศึกษาจลนพลศาสตร์ของกิจกรรมยับยั้งแอลฟา-อะไมเลสให้ค่าคงที่ของการยั้บยั้งเท่ากับ 0.89 มิลลิกรัมโปรตีนต่อมิลลิลิตร เมื่อวิเคราะห์ลำดับกรดอะมิโนภายในของสารยั้บยั้งแอลฟา-อะไมเลสชนิดนี้พบว่า ลำดับของกรดอะมิโนที่ได้มีความคล้ายกับเอนโดไคติเนสแฟมิลีจากพืช

4. Introduction

The use of natural drugs, such as plants and herbal remedies, to treat diseases is very common in Asia and developing countries, and is gaining interest in western countries. The chemical diversity and unique biological activities of the compounds found in the natural flora has propelled further discoveries in both the chemical and biological sciences and provided therapeutic agents for many diseases. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. Current ethno-botanical information includes about 800 plants that may possess the potential for diabetes treatment potential (Alarcon-Aguilara, Roman-Ramos, Perez-Gutierrez, Aguilar-Contreras, Contreras-Weber & Flores-Saenz, 1998). Although all plants contain biologically active chemicals, some are of particular interest as potential pesticides, biotechnological and pharmacological agents and they range in structural diversity from glycosides, saponins, alkaloids, triterpenes, steroids, peptides and proteins (Soetan, 2008).

The prevalence of obesity has increased dramatically worldwide with a major impact on public health, since obesity is a known risk factor for metabolic and chronic ailments, including heart disease, cancer, arthritis, obstructive sleep apnea, hypertension, hyperlipidemia and type 2 diabetes mellitus (DM). Current lifestyles with less exercise and the consumption of high caloric foods is the main factor in the increase in the number of overweight people (Giusti, 2007), whilst coupled with the increased proportion of the dietary intake of refined monosaccharide or disaccharide sugars, compounds any DM. DM is a chronic disease that is caused by the inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin. This deficiency results in increased blood glucose levels, which in turn can damage many of the body's systems, including blood vessels and nerves (Matsui *et al*., 2007). DM is currently one of the most costly and burdensome chronic diseases and is a condition that is increasing in epidemic proportions throughout the world (King, Aubert & Herman, 1998). Diabetes affects about 5% of the global population and the management of diabetes without any side effects is still a challenge to the medical system (Chakraborty & Rajagopalan, 2002; Kameswara, Guiri, Kesavulu & Apparao, 2001).

One of the therapeutic approaches for decreasing postprandial hyperglycemia is to retard the absorption rate of glucose by inhibition of the carbohydrate

hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive organs (Deshpande, Venkateswarlu, Babu & Trivedi, 2009). Inhibitors of these enzymes delay carbohydrate digestion prolonging the overall carbohydrate digestion time, and so causing a reduction in the rate of glucose liberation and absorption and consequently blunting the postprandial plasma glucose rise (Rhabasa-Lhoret & Chiasson, 2004). Many natural resources have been investigated with respect to their ability to suppress glucose production from complex carbohydrates in the gut and / or glucose absorption from the intestine (Fernando, Wickramasingle, Thabrew, Ariyananda & Karunanayake, 1991; Welsh, Lachance & Wasserman, 1989).

Pharmacological treatment provides a valid support for the management of obesity when combined with diet or behavioral therapy. Research on the role of various plant extracts or their purified compounds to reduce blood glucose levels because of their anti-digestive enzyme function is fairly widespread. (Bailey & Day, 1989; Ivorra, Paya & Villar, 1988; Marles & Farnsworth, 1995). The α-amylase inhibitors (AIs) are a group of substances which inhibit or inactivate α-amylase (α-1, 4-glucanohydrolase, EC 3.2.1.1, AMS), and are widespread in many different kinds of plants. From the nature of AI, they are divided into two groups, the non-proteinaceous and the proteinaceous types. The non-proteinaceous AI group contains relatively diverse types of organic compounds, such as acarbose (Kim *et al*., 2002) and acarbose analogues (Yoon & Robyt, 2003), as well as phenolic compounds, such as tannin (Kandra, Zajacz, Remenyik, & Gyemant, 2005), luteolin and flavonoid (Kim, Kwon & Son, 2000), which are non-specific inhibitors of the enzyme. The proteinaceous AI group have been isolated and characterized in different plants, notably certain common beans (*Phaseolus vulgaris*) (Gibbs & Alli, 1998; Lee, Gepts & Whitaker, 2002), wheat (*Triticum aestivum*) (Franco, Rigden, Melo, Bloch, Silva & Grossi-de-Sa, 2000), barley (*Hordeum vulgare*) (Abe, Sidenius & Svensson, 1993), and corn seeds (Figueira, Hirooka, Mendiola-Olaya & Blanco-Labra, 2003). The biological role of AI in these plants is not unequivocally resolved, but the possible functions include their involvement in the regulation of starch hydrolysis and protection against microbial attack or insect predation (Marshall & Lauda, 1975). However, because of their ability to inactivate α-amylase *in vivo*, they may be of considerable nutritional significance (Puls & Keup, 1973).

In recent years, there has been growing interest in functional foods that can provide not only the basic nutritional and energy requirements, but also an additional physiological benefit (Goldberg, 1994; Savikin *et al*., 2009). Usually, the functionality of a food is related to some of its ingredients and consumers increasingly prefer ingredients of a natural (non-synthetic) origin, which can be extracted from plants, food byproducts and other natural sources. From this point of view, many efforts have been made to search for more effective and safe inhibitors of α-amylase from natural materials to develop physiologically functional foods to treat DM. The aim of this research was to study the AI activity of seven cultivated local Thai fruits in relation to their proteinaceous content. The seven fruits studied were comprised of the plantains (bananas) (i) Kluai Hom Thong; Musa (AAA group), (ii) Kluai Nam Wa; Musa (ABB group) and (iii) Kluai Khai; Musa (AA group), the (iv) Oak Rong and (v) Num Dok Mai mangoes (*Manaifera indica* L), (vi) mangostein *Garcinia mangostana* L. and (vii) papaya *Carica papaya* L., which were all purchased from a local market in Bangkok, Thailand. The findings from this work will be helpful for understanding these fruits and may well be significant for industrial development.

5. Materials and methods

5.1 Materials

The cultivated local Thai fruits of seven species were periodically (October 2009 - December 2009) purchased from a local market in Bangkok, Thailand, vending food for human consumption. Ammonium sulfate, acrylamide, bis-acrylamide, hydrochloric acid, mercaptoethanol, sodium acetate, TEMED (Tetramethylethylenediamine), Tris (hydroxymethyl) aminomethane, the divalent metal salts and (Ethylenediaminetetraacetic acid) EDTA was purchased from Merck group, Germany. Ammonium persulfate, coomassie Blue G-250, glacial acetic acid, methanol, sodium chloride (NaCl), sodium hydroxide and sodium dodecylsulfate (SDS) were from BDH and purchased from VWR international, USA. Human saliva α-amylase Type XIII-A, as a lyophilized powder at 300 - 1,500 U /mg protein, diethylaminoethyl-cellulose (DEAE-cellulose) and Superdex-75 were purchased from Sigma-Aldrich Co. Ltd, USA. All chemicals were analytical grade.

5.2 Preparation of proteinaceous α-amylase inhibitor

Peeled fruits (400 g wet weight) cut into small pieces and then homogenized in 1 liter of extraction buffer (50 mM sodium acetate (pH 5.0) / 0.15 M NaCl) using a

blender and then left with stirring overnight at 4 ^{o}C. The suspension was then was clarified by filtration through a double-layered cheesecloth followed by centrifugation at 15,000 × g at 4 ^{o}C for 30 min. The supernatant was harvested, agitated at 4 ^{o}C for overnight and then anhydrous ammonium sulfate was gradually added to a final concentration of 80% saturated. After 6 hours at 4 ^{o}C to let the precipitation complete, the insoluble fraction was harvested by centrifugation at 15,000 × g for 30 min at 4 ^{o}C. The pellet was resolubilised in deionized water and then dialyzed (3.5 kDa cut-off tubing) against three changes (2 h each) of 5 liters de-ionized water at 4 ^{o}C. The dialyzed crude protein preparation, referred to as the "*ammonium sulfate cut fraction*", was then freeze-dried and kept at -20 ^{o}C.

5.3 Assay for α-amylase inhibition activity

The assay for α-amylase inhibition was assayed by quantifying the reduction in the level of released reducing sugar (maltose equivalent) liberated under otherwise constant assay conditions, with the AI activity expressed as the decrease in units of maltose liberated. A modified dinitrosalicylic acid (DNS) method of Bernfeld (1955) was used to estimate the maltose equivalent. One ml of plant extract was pre-incubated with human salivary α-amylase (0.5 U/ml) for 30 min and then 1 ml of a 1% (w/v) starch solution was added as substrate and incubated at 37 °C for 10 min. The reaction was stopped by adding 1 ml DNS reagent (12.0 g of sodium potassium tartrate, tetrahydrate in 8 ml of 2 M NaOH and 96 mM 3, 5-dinitrosalicylic acid solution) and heating in a boiling water bath for 5 min. The 100% and 0% α-amylase activity (0% and 100% inhibition, respectively) controls were performed as above except with the test extract / fraction and the α-amylase enzyme omitted, respectively, and replaced with the same volume of the solvent (50 mM sodium acetate (pH 5.0) / 5 mM sodium chloride). The absorbance was measured at 540 nm and the amount of reducing sugar released from the starch was estimated as maltose equivalents from a standard graph. The percentage inhibition was calculated according to the formula:

$$\% \text{ Inhibition} = [(A_0\text{-}A_t)_{control}\text{-}(A_0\text{-}A_t)_{sample} / (A_0\text{-}A_t)_{control}] \times 100\%$$

where A_0 and A_t are the absorbance values at zero time and at the end of the incubation respectively. Each experiment was repeated three times and the derived average values $\pm$ one standard deviation (SD) are reported.

5.4 Enrichment of the AI protein

5.4.1 DEAE-cellulose ion-exchange chromatography

Following the 80% saturation ammonium sulfate cut the next purification step was performed using DEAE-cellulose ion-exchange chromatography. The ammonium sulfate cut fraction, resolvated at 50 mg / ml was applied (5 ml) to the 50 mM sodium acetate pH 5.0 pre-equilibrated column (1.6 cm × 20 cm) and then eluted with the same buffer at a flow rate of 1.0 ml / min collecting 10 ml fractions. After 280 ml a linear gradient of 0 - 1.0 M NaCl in the same buffer was then applied over the next 750 ml. Fractions were assayed for α-amylase inhibitory activity (as per section 5.3), and those found to contain α-amylase inhibitory activity were pooled, dialyzed against 50 mM sodium acetate (pH 5.0) and concentrated by freeze dry to 50 mg / ml ready for further purification by gel filtration chromatography and analysis, and is subsequently referred to as the "*post-DEAE-cellulose AI fraction*".

5.4.2 Superdex-75 gel filtration chromatography

The post-DEAE-cellulose AI fraction (pooled fractions from DEAE-cellulose ion exchange chromatography that displayed α-amylase inhibitory activity) was applied (2 ml at 50 mg / ml) to a preequilibrated (50 mM sodium acetate (pH 5.0) / 100 mM NaCl) Superdex-75 column (1.6 cm × 60 cm) and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5.0 ml were collected and assayed for α-amylase inhibitory activity (as per section 5.3), and contiguous AI positive fractions (from the same peak) were pooled and dialyzed against an excess of same buffer prior to further analysis. This final preparation is referred to as the "*enriched AI protein fraction*".

5.5 Protein content determination

The protein content was determined by Bradford's procedure (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5 - 20 μg/ml to construct the calibration curve. Each sample was serially two-fold diluted with deionized water and then 50 μl aliquots of each dilution were transferred into each well of a microtiter plate and 50 μl of Bradford's reagent added to each well. The plate was shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader. The obtained OD was calculated for the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

5.6 Carbohydrate content determination

The phenol-sulfuric acid technique was slightly modified from the reported procedure (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), by scaling up and using glucose as the standard. The enriched AI protein fraction (post Superdex-75 gel chromatography) was serially diluted and 500 µl aliquots of each dilution was transferred into 15 ml glass tubes, to which 500 µl of a 4% (w/v) phenol solution was added, thoroughly mixed and then left at room temperature for 5 min. Next, 4 ml of conc. H_2SO_4 was added into each tube, carefully mixed using a vortex mixer and 100 µl aliquots transferred into the well of a microtitre plate and the absorbance read at 492 nm. The obtained data was used to calculate the sugar content (glucose equivalent) using the standard curve developed from five different concentrations of glucose (range 10 - 50 µg/ml) analyzed by the same procedure. Glucose (50 µg/ml) in deionized water and deionized water alone were used as the positive and negative controls, respectively, in the assay.

5.7 Determination of the protein pattern by native-PAGE

The protein from each step of the purification procedure (sections 5.2, 5.4.1 and 2.4.2) was analyzed for its native protein pattern according to the method of Bollag, Rozycki and Edelstein (1996), using a 7.5% (w/v) acrylamide separating gel and a 5.0% (w/v) acrylamide stacking gel. Tris-glycine buffer pH 8.3 was used as the electrode buffer, and gels were run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. After electrophoresis, proteins in the gel were visualized by coomassie Blue R-250 staining (0.1% (w/v) coomassie Blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol) and several changes of destaining solution (10% (v/v) acetic acid and 45% (v/v) methanol) until the background was clear.

5.8 Glycoprotein determination

The *in situ* gel periodic acid-Schiff's staining technique was modified from the described method (Trivedi, Frondoza & Humphrey, 1983). After native-PAGE electrophoresis, the gel was submerged into 100 ml of 1% (v/v) periodic acid in 5% (v/v) acetic acid and gently shaken for 5 min. The gel was then rinsed twice with distilled water and replaced with the same volume of Schiff's reagent (prepared 1 day before use) and incubated in the dark at room temperature overnight prior to washing three times in distilled water and soaking in a 5% (w/v) sodium metabisulfite / 5%

(v/v) acetic acid solution for 20 min. A pink-purple color slowly developed in positive samples as the gel was repeatedly washed in distilled water.

5.9 Molecular weight determination by SDS-PAGE

Discontinuous reducing SDS-PAGE gels were prepared with 0.1% (w/v) SDS in 15% and 5% (w/v) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS as the electrode buffer, according to the procedure of Laemmli (1970). Samples to be analyzed were treated with reducing sample buffer and boiled for five min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were coresolved in each gel alongside the samples to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by standard Coomassie blue R-250 staining as detailed in section 5.7.

5.10 Effect of temperature on the AI activity and thermostability

The effect of temperature on the AI activity was determined by incubating the enriched AI protein fraction samples in 50 mM sodium acetate (pH 5.0) at various temperatures (4 - 90 °C at 10 ^{o}C intervals) for 30 min. The thermostability of the AI was investigated by incubating the AI protein fraction sample at 40, 45 and 50 ^{o}C in 50 mM sodium acetate (pH 5.0) for the indicated fixed time intervals (10 - 120 min), cooling to 4 °C and then assaying the residual AI activity with 100% and 0% activity controls, as described in section 5.3.

5.11 The pH-dependence of the AI activity

Incubating the enriched AI protein fraction samples in buffers of broadly similar salinity levels, but varying in pH from 2 - 14 was used to assess the pH stability and the pH optima of the AI. The buffers used were 20 mM glycine-HCl (pH 2 - 4), 20 mM sodium acetate (pH 4 - 6), 20 mM potassium phosphate (pH 6 - 8), 20 mM Tris-HCl (pH 8 - 10) and 20 mM glycine-NaOH (pH 10 - 12). The enriched AI protein fraction was mixed in each of the different buffer-pH compositions, or 50 mM sodium acetate (pH 5.0) for the control, and then left for 1 hour at room temperature. Next, the samples were adjusted back to 50 mM sodium acetate pH 5.0), and assayed for AI activity (section 5.3) and the activities attained were compared with the control which was set as 100% activity.

5.12 Effect of metal ions on the AI activity

The effect of preincubation of the enriched AI protein fraction with six different divalent metal cations and the chelating agent EDTA on the resultant AI activity was evaluated as follows. The enriched AI protein fraction (1 mg / ml) was incubated for 10 h with one of Ca^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} or EDTA, at one of the indicated concentrations (0, 12.5, 25, 50 and 100 mM) in 50 mM sodium acetate (pH 5.0) with continuous shaking and was then tested for AI activity as described in section 2.3 using at least three replicates for each assay.

5.13 Mechanism of the inhibition

To evaluate the inhibition mode of the enriched AI protein fraction sample against the activity of α-amylase, starch solution at one of 0.25, 0.5, 1.0, 2.0 and 2.5% (w/v), as the substrate, was added to the α-amylase (0.5 U/ml) in 50 mM sodium acetate (pH 5.0) in the presence of 0, 0.25 and 0.5 mg /ml of the enriched AI protein fraction sample. The remaining α-amylase activity was determined as outlined in section 2.3. The inhibition type was determined by Lineweaver-Burk plot, where v is the initial velocity and [S] is the substrate concentration used.

5.14 Internal amino acid sequence of lectin by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)

5.14.1 *In situ* (in gel) trypsinization

The sample preparation process followed the published method of Mortz, Vorm, Mann and Roepstorff (1994). Each band in the electrophoretic gel was excised, cut into small pieces (ca. 1 mm^3) and washed with 100 μl deionized water. The gel pieces were destained by adding 200 μl of a 2:1 (v/v) ratio of acetonitrile: 25 mM NH_4HCO_3 for 15 min, and this step was performed several times until the gel pieces were completely destained. The supernatant was removed and gels were then dehydrated by adding 200 μl acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then 50 μl of a 10 mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 h at 56 ^oC. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and the gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile and the solvent evaporated off before adding 10 μl of a trypsin solution (proteomics grade, Sigma) (10 ng/μl in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4 ^oC, 30 μl of 50 mM NH_4HCO_3 was added and the digestion proceeded

at 37 ^{o}C overnight. The supernatant was then harvested following centrifugation at 15,000 × g for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetonitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the supernatant and taken to dryness.

5.14.2 LC-MS/MS and peptide blasting

The likely amino acid sequence of each internal fragment of the trypsinized peptide was analyzed by LC/MS/MS mass spectrometry. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage sites, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ± 0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring (Mortz, Vorm, Mann & Roepstorff, 1994).

6. Result and discussion

DM is one of the more serious chronic diseases that are increasing in frequency, along with an increase in both obesity and ageing, in the general population. One of the therapeutic approaches for decreasing post-pyramidal hyperglycemia is to retard the absorption of glucose by the inhibition of carbohydrate hydrolyzing enzymes, for example α-amylase in the digestive organs. The reported use of herbal extracts as anti-diabetics, which are directly or indirectly used for the preparation of many modern drugs, has not gained in much importance as medicines due to the lack of specific standards being prescribed for herbal medicines and supportive animal/clinical trials (Gupta, 1994). Plants are known to produce a large variety of glucosidase inhibitors that provide protection against insects and microbial pathogens (Ryan, 1989; Lu, Deng, Luo, Han & Gu, 1999). While proteinaceous inhibitors of α-amylase from cereals (Roy & Gupta, 2000; Heidari, Zareae & Heidarizadeh, 2005; Muralikrishna & Nirmala, 2005) and legumes (Giri & Kachole, 1998; Melo, Sales, Pereira, Bloch, Franco & Ary, 1999) have been well characterized, little is known of these inhibitors in local fruits. We therefore investigated the inhibitory effects on purified α-amylase of seven selected local fruits, representative

of four diverse genera. The results of this study are briefly discussed in the following sections.

6.1 Screening of local Thai fruits for AI activity

The aqueous extracts, prepared from seven different local Thai fruits, revealed some AI activity in all seven fruits but these were very weak in the mango and papaya (Table 1). In contrast, the three types of Musa (bananas) evaluated, and to a lesser extent the mangostein, had a higher AI activity. Since the crude protein extract from "Kluai Hom Thong" Musa (AAA group) showed by far the strongest AI activity it was selected for further investigation, involving bioassay guided fractionation, in order to isolate the AI constituent(s) responsible.

Table 1. AI-like activity of the crude aqueous homogenates from seven different fruit types representing four diverse plant genera.

Fruit sample	% Inhibition (mg/ml)[a]
Kluai Hom Thong; Musa (AAA group)	15.5 ± 0.02
Kluai Nam Wa; Musa (ABB group)	7.22 ± 0.41
Kluai Khai; Musa (AA group)	8.31 ± 0.57
Mango (Oak Rong); *Manaifera indica* L.	1.55 ± 0.75
Mango (Num Dok Mai); *Manaifera indica* L.	2.61 ± 0.24
Mangosteen; *Garcinia mangostana* L.	6.86 ± 0.01
Papaya; *Carica papaya* L.	3.77 ± 0.44

[a]Data are shown as the mean ± 1 SD, and are derived from triplicate assays

6.2 Enrichment of the proteinaceous AI from "Kluai Hom Thong" Musa

The occurrence of a proteinaceous AI in the crude extracts of Musa (AAA group) fruits is reported for the first time in this work. The initial ammonium sulfate precipitation reduced the total protein amount by some 55.7%, compared to a loss of AI activity of 30% for a 1.58-fold purification (Table 2). This ammonium sulfate cut fraction was then further fractionated by DEAE-cellulose ion exchange

chromatography with monitoring of all eluted fractions for AI activity. The AI activity remained in the bound fraction, and eluted from the column in the main peak at ~530 - 580 mM NaCl (Figure 1), just after a minor peak (shoulder in figure 1A) and before the other main protein peak (~650 - 730 mM NaCl). The post-DEAE-cellulose AI fraction, after dialysis, had a specific AI activity of 276.2 AI U/mg protein, representing a 43.3-fold purification (Table 2). Note that as well as the loss of ~98.8% of the total protein from the ammonium sulfate cut fraction, this ion exchange chromatography step was also very effective in eliminating pigments, because most of them did not bind to the cellulose matrix. However, other proteins were clearly still abundantly visible on the native PAGE (Figure 2A), and so further enrichment was required.

Thus, based upon the fact that the contaminating proteins may differ in size, and especially be larger, then Superdex-75 gel filtration chromatography was utilized after the DEAE-cellulose ion exchange chromatography. The post-DEAE-cellulose AI fraction was then concentrated to 50 mg / ml and subjected (5 ml loading at a time) to Superdex-75 gel filtration chromatography. The proteins were then eluted from the column, revealing two major peaks (Figure 1B)**.** The first peak could be large molecular weight proteins and contained the AI activity, whilst the second smaller peak was devoid of any AI activity. Harvesting the AI activity positive fractions from the first peak lead to an apparently almost homogenous protein preparation (Figure 2), purified some 55.8-fold at an 18.5% recovery yield to give a specific AI activity of 355.7 AI U/mg proteins (Table 2).

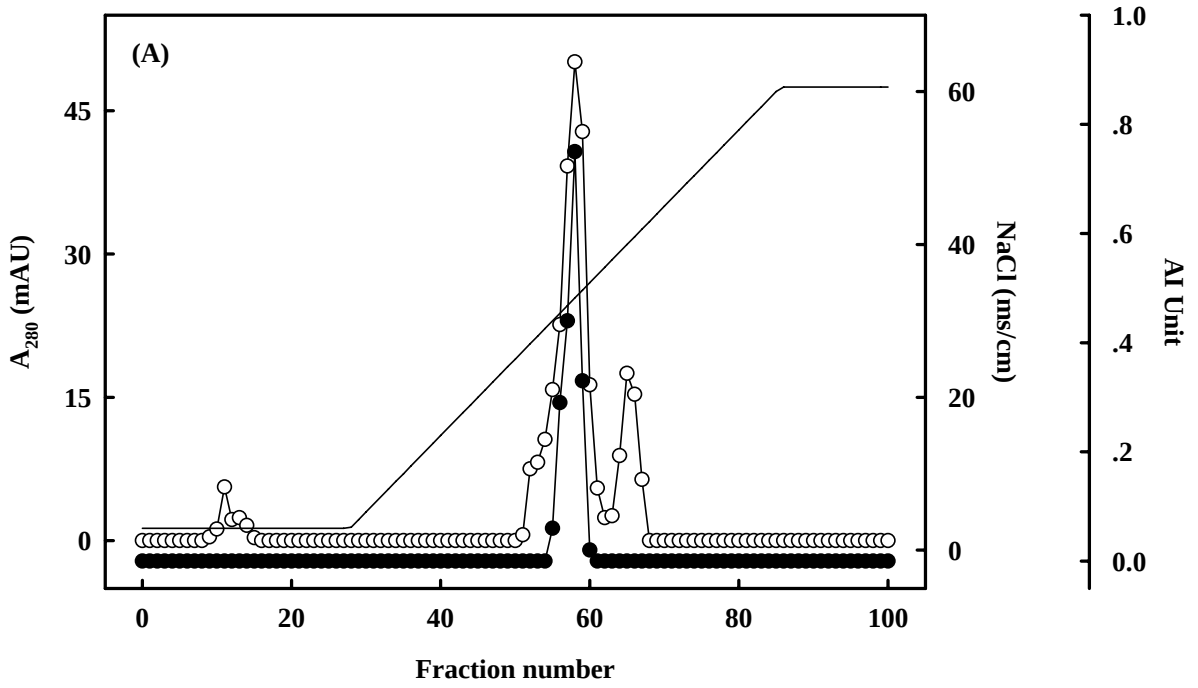

Figure 1(A) DEAE-cellulose ion exchange chromatography of the ammonium sulfate cut fraction solubilized in 50 mM sodium acetate (pH 5.0), and eluted in the same buffer but with a linear gradient of 0 - 1.0 M NaCl at a flow rate of 1 ml/min (section 2.4.1.). (○) Absorbance at 280 nm, (●) α-amylase inhibitory activity.

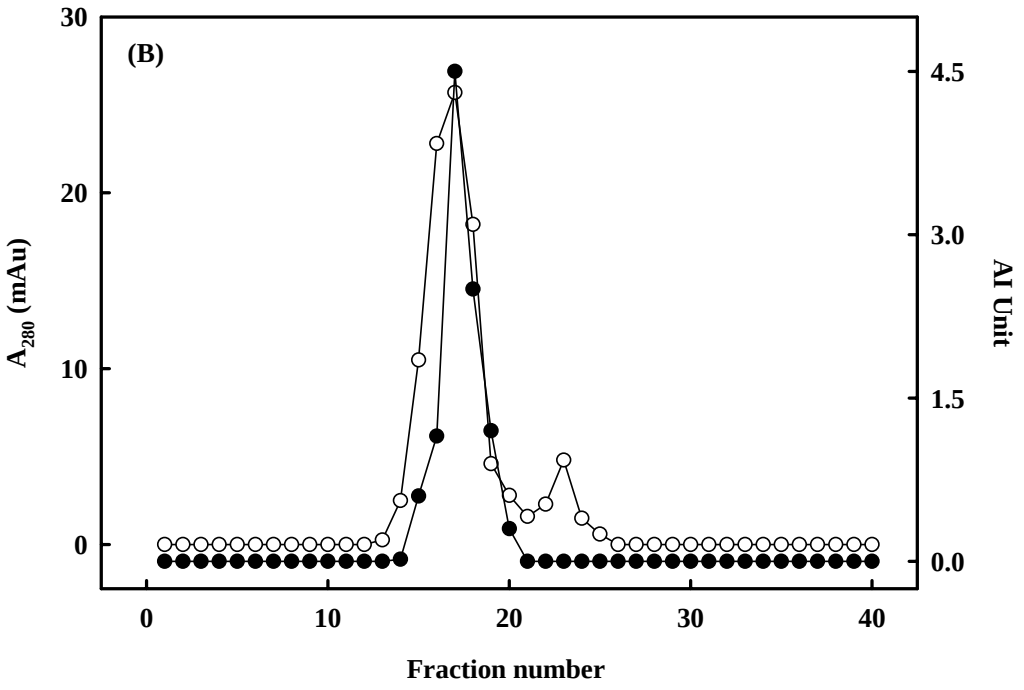

Figure 1(B) Superdex-75 gel chromatography of the post-DEAE-cellulose AI fraction. Fractions (5 ml) were eluted with 50 mM sodium acetate (pH 5.0) /100 mM NaCl at a flow rate of 0.5 ml/min. (○) Absorbance at 280 nm, (●) α-amylase inhibitory activity.

Table 2. Summary of the enrichment of the α-amylase inhibitor (AI)

Purification step	Total protein (mg)	Total inhibitory activity (AI U)	Specific AI activity (AI U / mg)	Yield (%)	Purification (fold)
Crude extract	295.40	1,885.00	6.38	100.00	1.00
80% $(NH_4)_2SO_4$ cut	130.60	1,320.00	10.11	70.03	1.58
DEAE-cellulose	1.64	452.60	275.98	24.01	43.26
Superdex-75	0.98	348.60	355.71	18.49	55.75

6.3 Verification of the proteinaceous AI purity and its molecular weight determination

The selected fractions from each step of the AI enrichment process were analyzed for their apparent purity and protein pattern by native-PAGE, where the enriched AI protein fraction preparation (after Superdex-75 gel filtration chromatography) revealed a single main protein band with a faint lower band on the native-PAGE gel (Figure 2A), but only a single band on the denaturing SDS-PAGE gel (Figure 2B), was observed. Thus, the enriched AI protein fraction obtained after Superdex-75 column chromatography should be relatively pure. The enriched AI protein fraction, and so the likely AI protein, was found to be a glycoprotein containing 10.74 ± 0.35% carbohydrate by weight (as glucose equivalents) by phenol-sulfuric acid determination, which accords to the periodic acid Schiff's staining result of the enriched AI protein fraction after native-PAGE resolution (Figure 2A). SDS-PAGE resolution of the purified α-amylase inhibitor preparations under discontinuous and reducing conditions, revealed an apparent MW of a single protein band of ~20 kDa (Figure 2B). This MW is similar to that reported for the Kunitz-like barley α-amylase / subtilisin inhibitor (BASI), which plays a role in plant defense by inhibiting subtilisin-like serine proteinases of pathogens and pests (Mundy, Hejgaard & Svendsen, 1984; Vallee *et al*., 1998; Nielsen, Bonsager, Fukuda & Svensson, 2004).

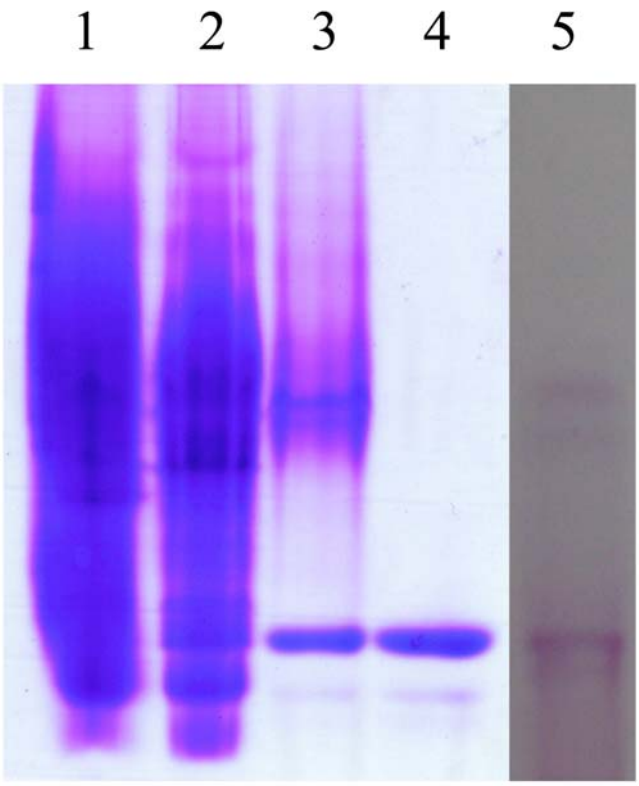

Figure 2. (A) Coomassie blue stained non-denaturing native PAGE of the AI containing fractions from each step of the enrichment procedure; Lane 1, the crude extract (homogenate) (50 μg of protein); Lane 2, ammonium sulfate cut fraction (50 μg of protein); Lane 3, Post- DEAE-cellulose AI fraction (10 μg of protein); Lane 4, enriched AI protein fraction (post-Superdex-75) (7.5 μg of protein), and Lane 5, periodic acid-Schiff's staining of the enriched AI protein fraction.

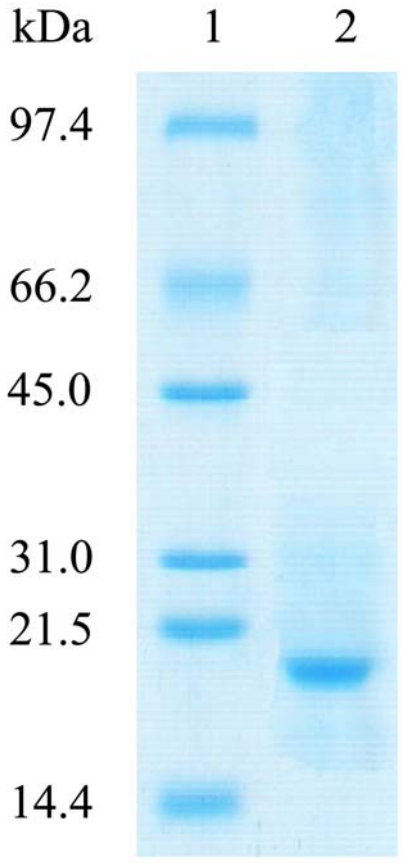

Figure 2. (B) Reducing SDS-PAGE analysis of the enriched AI protein (after superdex-75 gel chromatography). Lane 1, molecular weight standards; Lane 2, enriched AI protein fraction (7.5 μg of protein).

6.4 Property studies of proteinaceous α-amylase inhibitor

Several factors are able to modify the inhibitory activity of AI proteins, such as temperature, pH and salts (Gibbs & Alli, 1998; Giri & Kachole, 1998; Kluh *et al*., 2005). Knowledge of specific nature and unique behavior or properties of enzyme inhibitors is important in the correct assignment of suitable applications or conditions. Since this is the first study on the AIs from Kluai Hom Thong, the data obtained should be a valuable baseline for future studies.

6.4.1 Temperature resistance determination

No significant changes in the inhibition activity of the enriched AI protein fraction was seen when pretreated for 30 min within the temperature range of 4 - 40 °C, but from 45 °C upwards the observed AI activity decreased with increasing

incubation temperature, with essentially no activity being detected after pretreatment at 60 ^{o}C or higher (Figure 3A). This result is similar to that of the proteinaceous AI from *P. vulgaris* which was not stable at 40 - 80 °C (Grant, Edwards & Pusztai, 1995), but opposite to the study on the AI in pine bark extract that was stable at 90 - 100 °C (Kim, Jeong, Wang, Lee & Rhee, 2005). One possible reason is that the higher temperature range caused a change in the structure of AI reducing its ability to bind to the enzyme active site or enzyme-substrate complex.

In this study the optimum temperature for this AI activity was not evaluated, only the pretreatment temperature and stability. However, an optimum temperature of 37 °C was reported for the AI from *P. vulgaris* L., cv. Tendergreen (Berre-Anton, Bompard-Gilles, Payan & Rouge, 1997) and for that from Great Northern white kidney beans (Marshall & Lauda 1975). However, that the AI could be used at a rather high temperature means that α-amylase from other sources that are more thermotolerant should be applied in future cotreatment studies.

The decrease in the subsequent AI activity of the enriched AI protein fraction after pretreatment at 45 °C, and especially at 50 ^{o}C, over that seen at 40 ^{o}C could obviously be explained by degradation or denaturation of the AI away from the active conformational state. The pretreatment temperature for maximum AI activity was thus below 45 ^{o}C, with 75% and 55.8% of the maximal activity being retained after 120 min of incubation at 40 and 45^{o}C, respectively, but was rapidly inactivated at 50 ^{o}C to 50% and 0% activity after 40 and 90 minutes, respectively (Figure 3B).

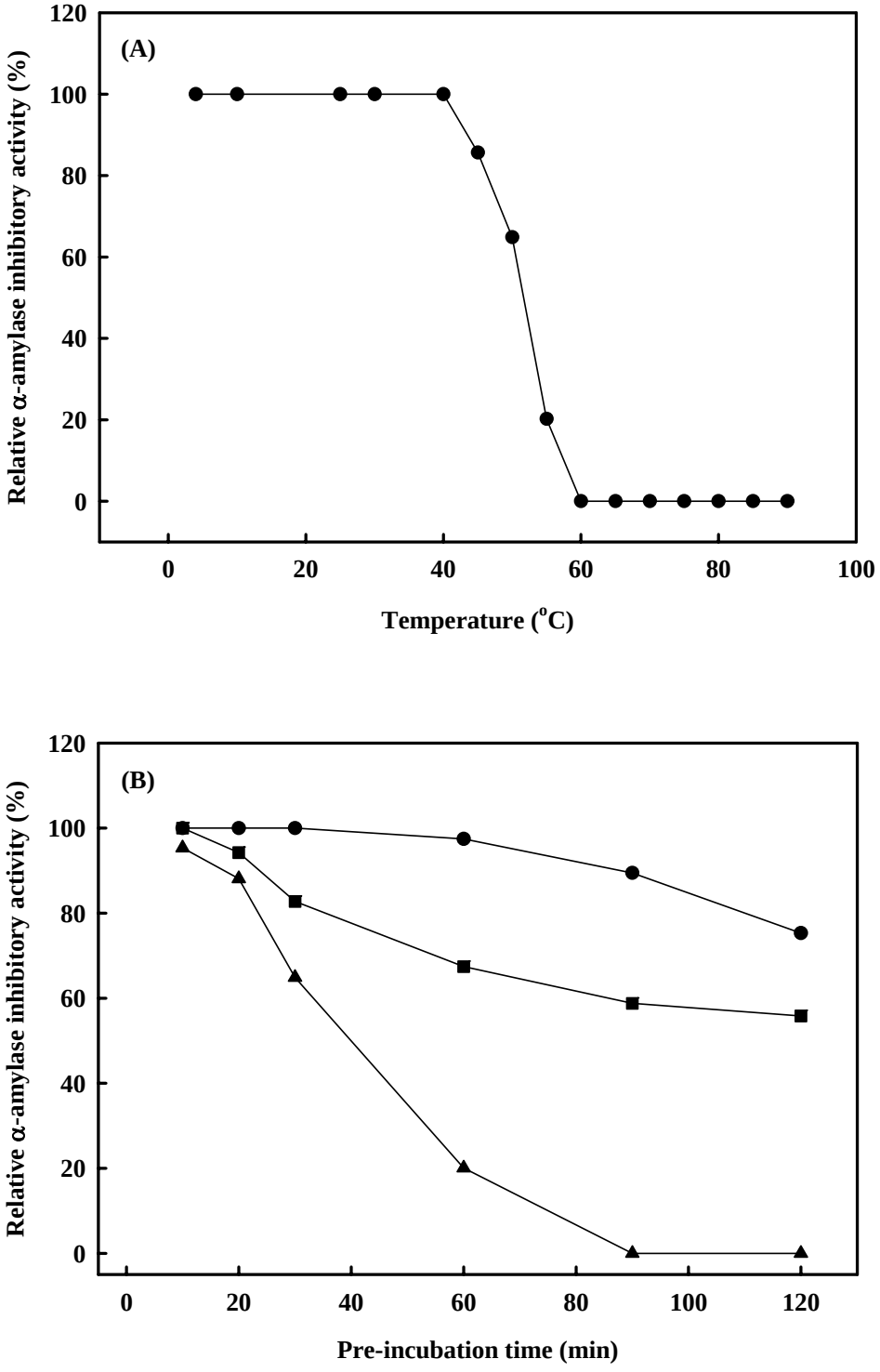

Figure 3. (A) Effect of pretreatment temperature on the AI activity of the enriched AI protein fraction towards α-amylase. (B) Thermostability with increasing pretreatment time of the enriched AI protein fraction at: (●) 40 °C, (■) 45 °C and (▲) 50 °C on the subsequent AI activity against α-amylase. For both panels the data are shown as the mean ±1 SD and are derived from three repeats.

6.4.2 pH resistance determination

The pH sensitivity profile of the AI activity of the enriched AI protein fraction exhibited a broad pH optima between pH 4.0 - 7.0 following a 60 min pretreatment at each pH. The activity, however, declined rapidly with increasing acidity, and less markedly with increasing alkalinity, such that less than 40% AI activity remained at pH 3 and 10, respectively, and no detectable AI activity at pH 2 and 11 (Figure 4). Note that where different buffers overlapped in the pH only a minor buffer-dependent effect was noted, suggesting that the variations seen are indeed largely due to the different pH values and not different buffer effects.These data are somewhat similar to the reported optimal pH (6 - 7) of the proteinaceous AIs from *P. vulgaris* and *Zea mays* (Gibbs & Alli, 1998; Figueira, Hirooka, Mendiola-Olaya, & Blanco-Labra, 2003), but were different from the non proteinaceous AIs from acarbose with a broader optimum pH range of between 4.0 and 8.0 (Talamond, Desseaux, Moreau, Santimone & Marseille-Mouren, 2002).

In addition, the tolerance to weak acid, with the observed AI activity after exposure to a pH of 4.0 or 5.0 for 60 min being essentially the same as the no-pretreatment control is broadly similar to the pine bark extract AI which was stable in weakly acidic conditions (Kim, Jeong, Wang, Lee, & Rhee, 2005). The stability of the enriched AI protein fraction to prior exposure to weak acidic conditions (pH 4 - 5) suggested that it will be easier to handle the AI during processing or manufacturing steps and that the AI activity will remain stable when exposed to the human digestive tract.

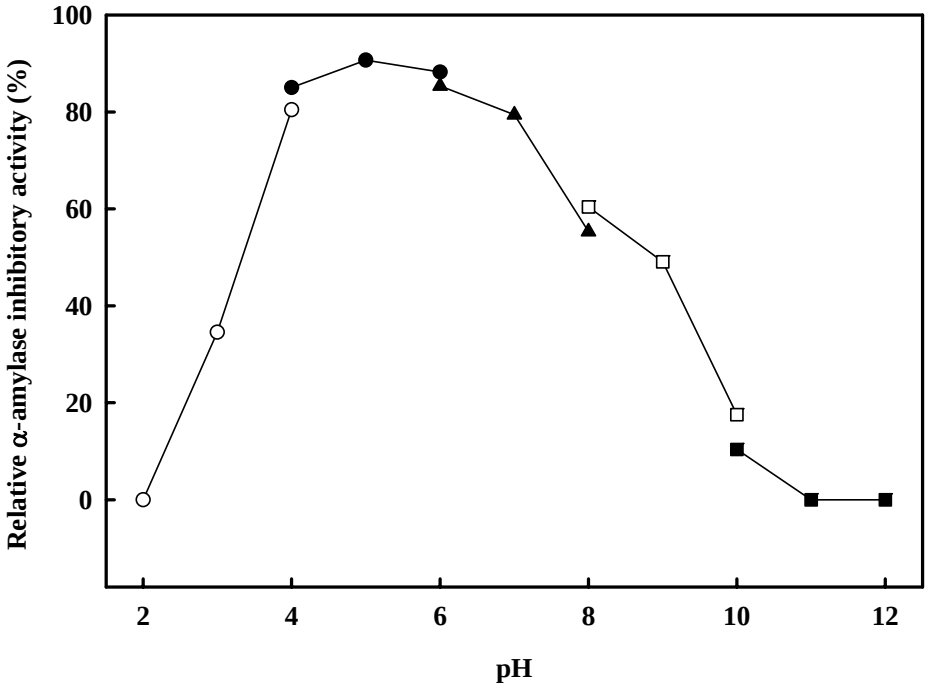

Figure 4. The effect of pH pretreatment on the AI activity of the enriched AI protein fraction against α-amylase. The data are shown as the mean ± 1 SD and are derived from three repeats. The following buffer systems were used: (○) 20 mM glycine-HCl (pH 2.0 - 4.0), (●) 20 mM sodium acetate (pH 4.0 - 6.0), (▲) 20 mM potassium phosphate (pH 6.0 - 8.0), (□) 20 mM Tris-HCl (pH 8.0 - 1 0.0) and (■) 20 mM glycine-NaOH (pH 10.0 - 12.0). For both panels the data are shown as the mean ±1 SD and are derived from three repeats.

6.4.3 Effect of metal ions on the AI activity

Incubating the enriched AI protein fraction preparation (1 mg / ml) with each of the six different divalent metal cation salt solutions, plus EDTA, at five different concentrations (section 2.12) for 24 hr prior to assaying for AI activity, revealed a requirement for Ca^{2+}, Mg^{2+}, and Mn^{2+} at less than 25 mM to be effective for AI activity, suggesting they are essential for the stability of the AI protein structure and activity (Table 3). In contrast, Fe^{2+}, Hg^{2+}, Co^{2+} and EDTA did not support any AI activity. Gibbs and Alli (1998) also reported that $CaCl_2$ improved the activity of the proteinaceous AI from *P. vulgaris*, when assayed against porcine α-amylase, and this was followed by NaCl and KCl, accordingly, but no effect of $MgSO_4$ was observed.

Table 3. The effect of divalent metal cations and EDTA on the α-amylase inhibitory activity

Metal salt	Concentration (mM)			
	12.5	**25**	**50**	**100**
Ca^{2+}	-	0.19 ± 0.004	0.12 ± 0.001	0.08 ± 0.004
Mn^{2+}	-	0.22 ± 0.001	0.15 ± 0.001	0.11 + 0.002
Mg^{2+}	-	0.20 ± 0.001	-	-
Fe^{3+}	-	-	-	-
Hg^{2+}	-	-	-	-
Co^{2+}	-	-	-	-
EDTA	-	-	-	-

6.4.4 Mechanism of inhibition

Catalytic kinetic studies for α-amylase, with different substrate and enriched AI protein fraction concentrations were analyzed using Lineweaver-Burk equations (Figure 5). Both the maximal velocity (V_{max}, y-intercept) and the Michaelis-Menten constant (K_m, slope of the trend lines) decreased with increasing concentrations of the enriched AI protein fraction, and so this AI acted as a non-competitive inhibitor of the tested α-amylase. Non-competitive inhibitors do not compete with the substrate to bind to the active region of the free enzyme, but bind to enzyme-substrate complex, resulting in an enzyme-substrate inhibitor complex. For this reason, inhibition cannot be overcome by increasing the concentration of substrate. When the concentration of the AI was plotted against $1/V_{max}$ (observed), the K_i value was determined as 0.89 mg protein/ml via non-linear regression using the least squares difference method.

In some contrast, the pine bark AI was reported to be a competitive inhibitor of salivary α-amylase but a mixed noncompetitive inhibitor of the yeast α-glucosidase (Kim, Jeong, Wang, Lee, & Rhee, 2005). Likewise, acarbose, acarviosine-glucose and isiacarbose were found to be competitive inhibitors for α-glucosidase and mixed noncompetitive inhibitors for α-amylase and cyclomaltodextrin glucosyltransferase (Kim *et al.*, 1999). The mixed noncompetitive

inhibition by acarbose was also reported against amylase, with K_i and $K_{i'}$ values of 3.7 and 1.08 μM, respectively (Kandara, Zajacz, Remenyik & Gyemant, 2005), and, along with two acarbose analogues, against porcin pancreatic α-amylase and human salivary α-amylase with K_i values of 0.8 and 1.27 μM, respectively (Yoon & Robyt 2003). Finally, aleppo tannin showed a mixed noncompetitive inhibition against human salivary α-amylase (Zajácz, Gyémánt, Vittori, & Kandra, 2007).

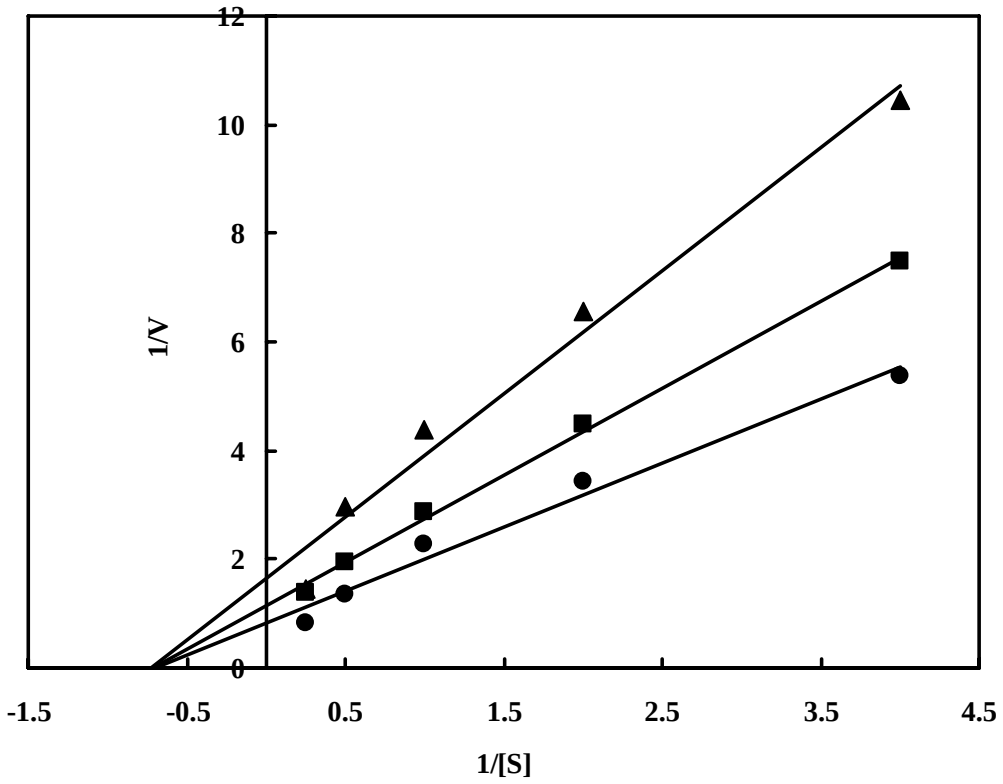

Figure 5. Lineweaver-Burk plots derived from the inhibition of α-amylase by the enriched AI protein fraction from Kluai Hom Thong. α-Amylase was treated with each indicated concentration of starch (0 - 2% (w/v)) in the (●) absence and presence of the enriched AI protein fraction at (■) 0.25 and (▲) 0.5 mg protein/ml. Data are shown as the mean ± 1 SD, derived from 3 repeats.

6.4.5 Internal amino acid sequence of lectin by LC/MS/MS

The internal sequence analysis of the enriched AI protein was obtained by digestion with trypsin and sequence analysis with LC/MS/MS, and found to be

GPIQL SYNYN YGPAGK (m/z of 1742.91) (Figure 6A). Comparisons were then made to all protein sequences in the NCBI and SwissProt database, using the BLASTp and tBLASTn search protocols. A high degree of internal amino acid sequence identity between this 16 amino acid fragment of the AI from Kluai Hom Thong and five other chitinase proteins suggested that this protein could be a member of plant chitinase family (Figure 6B).

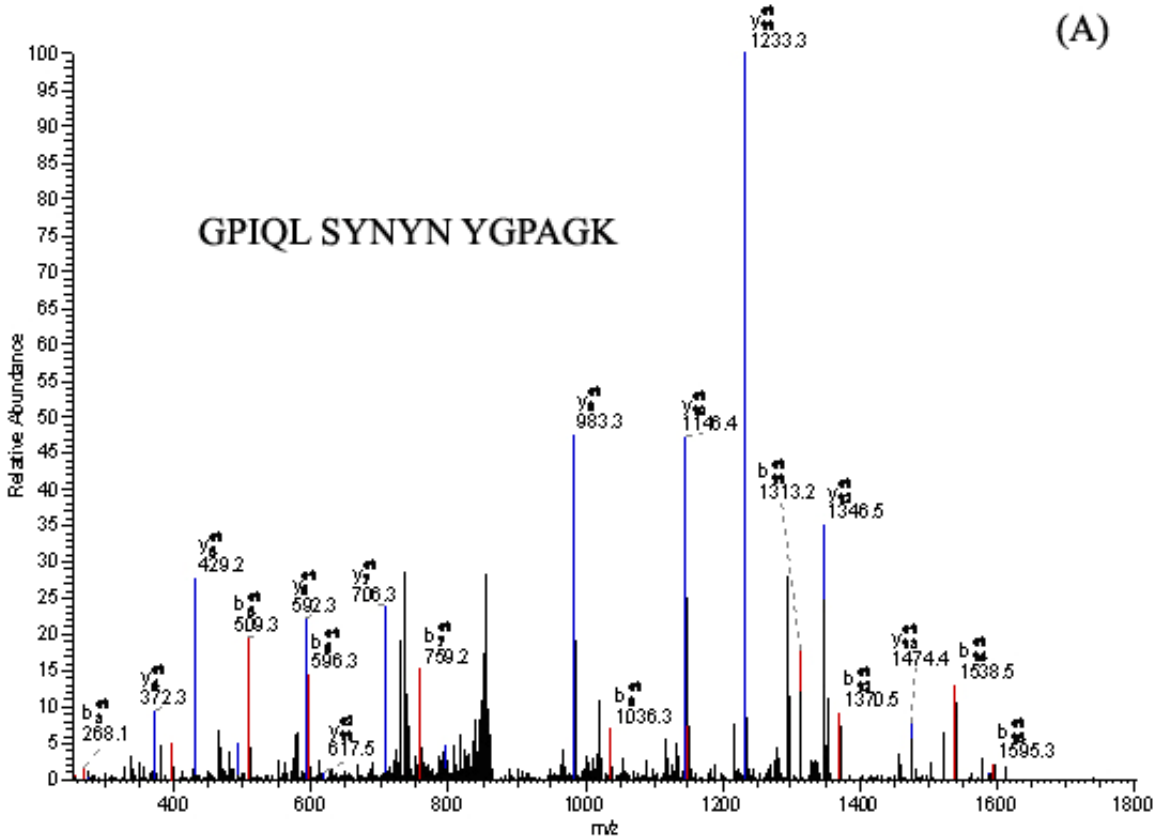

Figure 6. (A) Amino acid sequence from the tryptic fragments of the enriched AI protein fraction. Comparisons are made with other plant proteins that showed the highest sequence homology in BLASTp and tBLASTn searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity.

(B)

		5					10					15						Accession Number
α-amylase inhibitor (Musa AAA group)		G	P	I	Q	L	S	Y	N	Y	N	Y	G	P	A	G	K	
Chitinase II (Musa acuminata AAA group)	73	G	P	I	Q	L	S	Y	N	Y	N	Y	G	P	A	G	K	88	B5TYQ1
Endochitinas (Musa acuminate)	101	G	P	I	Q	L	S	F	N	Y	N	Y	G	P	A	G	-	115	Q93WX9
Putative chitinase (Musa cavendishii)	189	G	P	I	Q	L	S	F	N	Y	N	Y	G	P	A	G	-	203	Q8VXF0
α-amylase inhibitor/endochitinase (*Coix lacryma*)	56	G	P	I	Q	L	S	-	N	Y	N	Y	G	P	A	G	K	70	P15326
Lectin/endochitinase 1 (*Urtica dioica*)	240	G	P	I	Q	L	T	H	N	F	N	Y	G	L	A	G	Q	255	P11218
Lectin precursor (***Clivia miniata***)	99	N	P	I	W	A	S	T	E	G	E	N	G	N	Y	V	C	116	Q39542
Lectin precursor (***Galanthus nivalis***)	92	K	P	I	W	A	S	N	T	G	G	Q	N	G	N	Y	V	107	Q39903
Mannose-specific lectin (***Zephyranthes***	43	R	P	I	W	A	S	N	T	R	G	H	N	-	N	Y	V	59	Q8S332

Figure 6. (B) LC/MS/MS spectra of the tryptic digest of the enriched AI protein used to derive the data in (A) above.

7. Conclusion

An AI of ~20 kDa and a potential member of the plant chitinase family, was purified from the fruits of Kluai Hom Thong, Musa (AAA group), by ammonium sulfate precipitation followed by DEAE-cellulose ion exchange and Superdex-75 gel chromatography, respectively. The AI activity was optimal at a pretreatment pH range of 4 - 7 and relatively stabile, but was markedly reduced by more basic pH values above 8 or acidic pH values below 4. The AI was heat stable below 40 ^{o}C for 30 min, with 75% and 55.8% of its maximal AI activity being retained after 120 min of incubation at 40 ^{o}C and 45 ^{o}C, respectively, but was inactivated at 50 ^{o}C to less than 20 and 0% activity after 60 and 90 minutes, respectively. Divalent cations appeared to be essential for the AI activity, with this requirement being met by some (Ca^{2+}, Mg^{2+}, and Mn^{2+}) but not other (Fe^{2+}, Hg^{2+} and Co^{2+}).

8. Concluding reemarks

This AI has the potential for use in the medical and pharmaceutical fields as a non-competitive inhibitor of digestive enzymes associated with carbohydrate digestion.

9. Acknowledments

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11. Appendix

A Chitinase-Like Protein with α-Amylase Inhibitory Activity from Kluai Hom Thong Banana Fruit: Musa (AAA group). *Food Chemistry* (submitted manuscripts).