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Biochemical Characterization of the α-Amylase Inhibitor in Mungbeans and Its Application in Inhibiting the Growth of *Callosobruchus maculatus*

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The insect *Callosobruchus maculatus* causes considerable damage to harvested mungbean seeds every year, which leads to commercial losses. However, recent studies have revealed that mungbean seeds contain α-amylase inhibitors that can inhibit the protein C. *maculatus,* preventing growth and development of the insect larvae in the seed, thus preventing further damage. For this reason, the use of α-amylase inhibitors to interfere with the pest's digestion process has become an interesting alternative biocontrolling agent. In this study, we have isolated and purified the α-amylase inhibitor from mungbean seeds (KPS1) using ammonium sulfate precipitation, gel filtration chromatography and reversed phase HPLC. We found that the α-amylase inhibitor, isolated as a monomer, had a molecular weight of 27 kDa. The α-amylase inhibitor was purified 750-fold with a final yield of 0.4 mg of protein per 30 g of mungbean seeds. Its specific activity was determined at 14.5 U (mg of protein)$^{-1}$. Interestingly, we found that the isolated α-amylase inhibitor inhibits C. *maculatus* α-amylase but not human salivary α-amylase. After preincubation of the enzyme with the inhibitor, the mungbean α-amylase inhibitor inhibited *C. maculatus* α-amylase activity by decreasing V_{max} while increasing the K_m constant, indicating that the mungbean α-amylase is a mix noncompetitive inhibitor. The *in vivo* effect of α-amylase inhibitor on the mortality of *C. maculatus* shows that the α-amylase inhibitor acts on *C. maculatus* during the development stage, by reducing carbohydrate digestion necessary for growth and development, rather than during the end laying/hatching stage. Our results suggest that mungbean α-amylase inhibitor could be a useful future biocontrolling agent.

KEYWORDS: ***Callosobruchus maculatus*; α-amylase inhibitor; mungbean**

INTRODUCTION

Mungbean (*Vigna radiate* (L.) Wilczek) is an important commercial crop in Thailand and the wider Asian region. However, the productivity and quality of the grain are severely damaged by insect infestation after initial harvesting and during storage (*1*). Two species of weevils, *Callosobruchus chinensis* and *Callosobruchus maculatus*, are the major insect pests of mungbean seeds that are attracted to crops with a high starch content (*2*). These insects lay eggs on the mungbean seeds, and once the eggs hatch, the resulting larvae feed on the seed nutrients. Some farmers solve this problem by coating mungbean seeds with insecticides (*3*); however, this has adverse effects on the environment and human health.

Physical characteristics of mungbean seeds, such as size and thickness, offer a degree of protection from bruchid attack. The thick layer covering mungbean seed acts as an oviposition deterrent and helps protect them from *C. chinensis*, *C. maculatus* and *Callosobruchus phaseoli* (*4*, *5*). In addition, insecticides such as phytohemaglutinin (*6*), tannins (*7*), polysaccharide (*8*), amylase inhibitor (*9*) and proteinase inhibitor (*10*) have also been used as seed protection from bruchid infestation. Additionally, insecticides are effective against *Tenebrio molitor*, *C. chinensis*, *C. maculatus*, *Zabrotes subfaciatus* and *Bruchus pisorum* (*8*−*13*).

Engkagul et al. (*14*) reported that crude protein from the Kamphaengsaen 1 variety of mungbean (KPS1) inhibited C. *maculatus* α-amylase, preventing growth and development of the insect larvae infesting the seed. These α-amylase inhibitors, attractive candidates for seed weevil biocontrol, have been purified and characterized from different varieties of common bean including white kidney bean (*15*), red kidney bean and black kidney bean (*16*). Two isoforms have been identified: α-AI1, which inhibits mammalian α-amylase and insect α-amylase of *C. chinensis* and *C. maculatus,* and α-AI2, which inhibits α-amylase of *Z. subfaciatus* (*17*). A number of structural studies have revealed that the common bean α-amylase inhibitors are oligomeric in structure and composed of either identical (*18*) or different polypeptides (*16*, *19*).

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In addition to common beans, α-amylase inhibitors are also found in other legumes such as *Phaseolus coccineus* (scarlet runner bean), *Phaseolus polyanthus*, *Phaseolus acutifolius* (tepary bean) (*20*, *21*), *Cajanus cajan* (pigeon pea) (*22*) and *Vigna sublobata* (*23*). Inhibitory activity of α-amylase inhibitor from scarlet runner bean and *P. polyanthus* is similar to α-AI1's of common bean (*21*). Tepary bean seed has two α-amylase inhibitor isoforms, designated as αAI-Pa1 and αAI-Pa2, both exhibiting broad specificity toward α-amylase. αAI-Pa1 inhibits larval α-amylase of *C. chinensis* and *C. maculatus* but not that of *Z. subfasciatus*, whereas αAI-Pa2 inhibits larval α-amylase of all three. Both isoforms inhibit larval amylase of cereal storage pests, including the yellow mealworm (*Tenebrio molitor*) and confused flour beetle (*Tribolium confusum*), but do not inhibit mammalian α-amylases (*22*). Like amylase inhibitors from the red kidney bean, the active form of αAI-Pa2 is a heterodimer, whereas αAI-Pa1 is composed of a single glycopolypeptide unit (*24*).

In this study, we are interested in further characterizing α-amylase inhibitors due to their potential use as a relatively abundant low cost biocontrol agent. We put particular emphasis on the α-amylase inhibitor strains obtainable from Thai crops. The objectives of this study are to isolate, to purify and to characterize the α-amylase inhibitor from KPS1 mungbean. We subsequently determined the inhibitory activity of α-amylase from *C. maculatus* using both *in vitro* and *in vivo* inhibition assays. To gain further understanding of the inhibition process, we determined the full kinetics profile of *C. maculatus* α-amylase inhibition of the KPS1 α-amylase inhibitor.

Previous studies suggested that α-amylase inhibitor acts as growth inhibitor of insect as well as plant defense protein (*25*, *26*). This inhibitor can be used to extend the time before the weevil can do damage thereby reducing spraying cost, and removing the need for chemical insecticides during seed storage.

EXPERIMENTAL PROCEDURES

Source of Materials. Mungbean seeds (KPS1) were obtained from the Department of Applied Radiation and Isotopes, Faculty of Science, Kasetsart University, Thailand. *Callosobruchus maculatus* (cowpea weevil) was obtained from the insect section of the Stored Products Laboratory, Division of Entomology and Zoology, Department of Agriculture, Thailand.

Isolation of α-Amylase from *C. maculatus*. Two grams of frozen *C. maculatus* were finely ground in a deep cold mortar with 8 mL of 20 mM sodium phosphate buffer at pH 7 and then centrifuged at 10000*g* for 20 min at 4 °C. The clear supernatant was used as the crude source of α-amylase (20 mg/mL).

Purification of α-Amylase Inhibitor. α-Amylase inhibitor was purified from mungbean seeds using the following methodology. Thirty grams of ground seeds was extracted with 200 mL of 20 mM sodium phosphate buffer at pH 6.7 at 4 °C for 3 h. After centrifugation, the supernatant was subjected to ammonium sulfate precipitation by the addition of salt to 80% saturation. The precipitant was collected by centrifugation at 10000*g* for 20 min at 4 °C and redissolved in 20 mM sodium phosphate buffer at pH 6.7, followed by dialysis overnight at 4 °C. The dialysate was applied to a gel filtration chromatography column using Sephadex G-100 (1× 120 cm, Fluka, Switzerland) which was pre-equilibrated with 20 mM sodium phosphate buffer at pH 6.7 and eluted with the same buffer at a flow rate of 0.2 mL/min. Two milliliter fractions were collected, and absorbance at 280 nm and α-amylase inhibitory activity were measured. The active fractions were applied to a reversed phase HPLC on a Pursuit C18 column (250 × 4.6 mm, 5 mm particle size) at a flow rate of 0.5 mL/min. The two solvents used were 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). The elution was carried out with 0−50% solvent B for 10 min and followed by 50−65% solvent B for 60 min. The protein peaks were monitored at their absorbance at 280 nm. The protein obtained from reversed phase HPLC was dialyzed against 20 mM sodium phosphate buffer at pH 6.7 before inhibitory activity was determined. The target fraction was lyophilized and stored at −20 °C until use.

α-Amylase Inhibitor Assay. The α-amylase inhibitor activity was determined using a modified Bernfeld method (*27*). To begin the reaction, 50 μL of crude *C. maculatus* α-amylase and 100 μL of purified α-amylase inhibitor were preincubated at room temperature for 30 min before the addition of 250 μL of 2% starch solution in 20 mM sodium phosphate buffer at pH 6 which contains 20 mM NaCl and 0.2 mM $CaCl_2$. After incubation at optimum temperature (50 °C) for 10 min, the reaction was terminated by adding 250 μL of 3,5-dinitrosalicylic acid reagent (10% 3,5-dinitrosalicylic acid, 0.4 M sodium hydroxide and 1.06 M potassium sodium tartate), followed by incubation in a boiling water bath for 5 min. The reaction mixture was diluted with 2 mL of distilled water, and the absorbance was measured at 540 nm. One unit of α-amylase inhibitor activity is defined as inhibition of one unit of amylase activity under the given assay conditions. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar in 1 min under the conditions defined.

Molecular Mass Analysis. The molecular weight of α-amylase inhibitor was estimated by filtration on Sephadex G-100 column (1× 140 cm) (Fluka, Switzerland) using 20 mM sodium phosphate buffer at pH 6.7 at a flow rate of 0.2 mL/min. The column was calibrated using standard proteins; aldolase (158 kDa), lipoxidase (108 kDa), creatine phosphokinase (81 kDa) and lysozyme (14.7 kDa). Elution volumes (V_e) were measured for standard proteins and α-amylase inhibitor. The void volume (V_0) and total volume (V_t) were determined using blue dextran and bromophenol blue, respectively. K_{av} values were calculated for each protein using $K_{av} = (V_e - V_0)/(V_t - V_0)$, and the apparent molecular weight of α-amylase inhibitor was calculated from the plot of K_{av} versus log of molecular weight.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). 12% SDS−PAGE was performed at room temperature according to the method of Laemmli using protein molecular weight markers (15−200 kDa) (Fermentas) as standard and bromophenol blue as the tracking dye. After electrophoresis, proteins in the gel were visualized by Coomassie Brilliant Blue R-250 staining.

Kinetic Study. Kinetic experiments were performed at 50 °C in 20 mM sodium phosphate buffer at pH 6 which contains 20 mM NaCl and 0.2 mM $CaCl_2$, 0.2 mM α-amylase and 5 μM α-amylase inhibitor. The mixture was preincubated at room temperature for 30 min before adding substrate to start the reaction. Different concentrations of starch solution (2, 3, 3.25, 3.5, 3.75, 4, and 4.25 mg/mL) were used as substrate. After 10 min, the reaction was terminated by adding 250 μL of 3,5-dinitrosalicylic acid reagent, followed by heating in a boiling water bath for 5 min. The reaction mixture was diluted with 2 mL distilled water, and the absorbance was measured at 540 nm. The kinetic parameters, Michaelis−Menten constant (K_m) and V_{max}, were calculated from a Lineweaver−Burk plot.

Feeding Tests. The effect of the purified α-amylase inhibitor on bruchid growth was evaluated by soaking the mungbean seeds with the α-amylase inhibitor, at concentrations of 0 (control), 0.2, 0.5, and 1.5 mM. After soaking for 1 h, each sample was air-dried for approximately 3 h. Fifty mungbean seeds soaked in each solution were placed in small plastic cups, and five fertilized insect females were then introduced into each cup and allowed to lay eggs. After 24 h, adults were removed. Seven days after the initial oviposition, the numbers of eggs hatched on the surface of the treated seeds were counted. After 30 days, the treated seeds were dissected and the number of adults, surviving larvae, and pupae were recorded.

RESULTS AND DISCUSSION

Purification of α-Amylase Inhibitor from Mungbean. The crude α-amylase inhibitor from mungbean seeds was obtained from the aqueous extraction described above by precipitation using 80% saturation ammonium sulfate precipitate. Protein pellets were resuspended in 20 mM phosphate buffer at pH 6.7 and applied onto a Sephadex G-100 column. The elution profile showed seven peaks, consisting of three major and four minor ones. Only fractions obtained from the fourth peak had inhibitory activity against *C. maculatus* α-amylase (**Figure 1A**). These fractions were pooled and further purified by reversed phase HPLC. The α-amylase inhibitor was found to be eluted around 55% acetonitrile (**Figure 1B**). These data indicate that there was only one isoform of

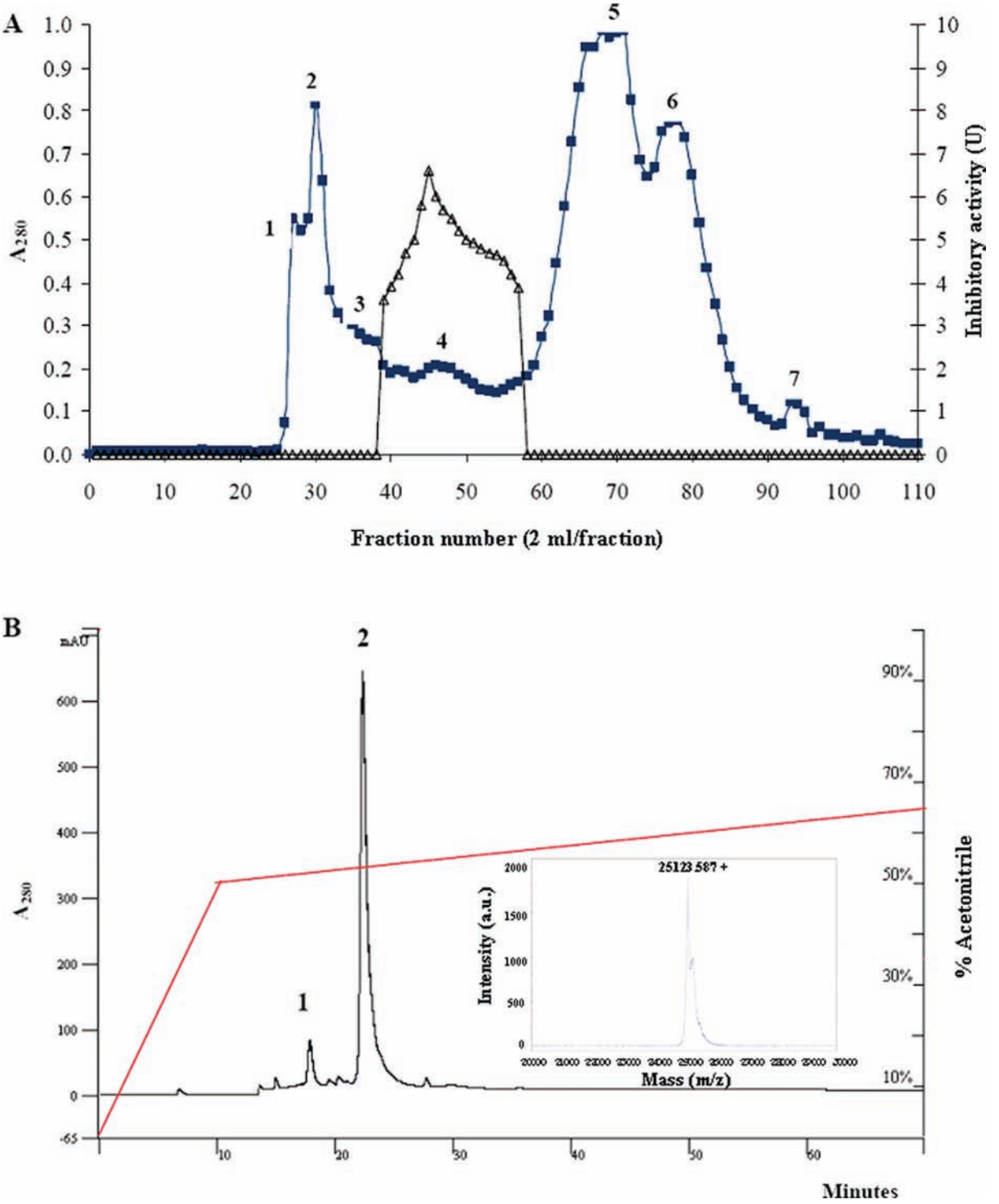

Figure 1. (**A**) Purification of α-amylase inhibitor from mungbeans by gel filtration chromatography using Sephadex G 100. Column chromatography was carried out at a flow rate of 0.2 mL/min. Fractions of 2 mL were collected, and the absorbance at 280 nm (■) and the inhibition percentage of α-amylase inhibitor against C. *maculatus* α-amylase (Δ) were monitored. (**B**) HPLC profile of amylase inhibitor from sephadex G-100 column. Separation was carried out on a Pursuit C18 column at a flow rate of 0.5 mL/min. The two solvents used were 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). Proteins were eluted with 0−50% B 10 min and 50−65% B 60 min. The absorbance was recorded at 280 nm.

amylase inhibitor in mungbean. Purification is confirmed by mass spectrometer. Purification steps of α-amylase inhibitors from mungbeans are summarized in **Table 1**. The purity of α-amylase inhibitor was increased 750-fold with a final yield of 0.4 mg of protein per 30 g of mungbeans. Its specific activity was found to be 14.5 U (mg of protein)$^{-1}$.

The α-amylase inhibitory activity in mungbeans was tested toward *C. maculatus* and human salivary α-amylases under optimum conditions of each enzyme, which were pH 6 at 50 °C for *C. maculatus* α-amylase (*28*) and pH 7 at 37 °C for human salivary α-amylase. The purified mungbean α-amylase inhibitor was found to be effective against *C. maculatus* α-amylase but could not inhibit human salivary α-amylase (**Figure 2**). Our results were similar to those for α-amylase inhibitors from amaranth (*29*), *Phaseolus* and *Vigna* legumes (*8*), *Dipteryx alata* (*30*) and *Pterodon pubescens* seeds (*31*) which are effective against the

α-amylases of their insect enemies but not against mammalian amylases. Nevertheless, other groups of α-amylase inhibitors, for example from cereal, maize and leguminosae seeds, inhibit both insect and mammalian α-amylases (*17*, *32*, *33*).

According to their inhibitory activity against *C. maculatus*, mungbean α-amylase inhibitor is a member of lectin-like inhibitors. This can be confirmed by their hemagglutination activity with rabbit erythrocytes (data not shown). The lectin-like inhibitors have been purified and characterized from leguminosae seed, such as common bean, scarlet runner bean, tepary bean, pigeon pea and *V. sublobata* (*15*, *16*, *20*−*23*). These α-amylase inhibitors have two isoforms called α-AI1 and α-AI2. α-AI1 inhibits mammalian α-amylase and insect α-amylase of *C. chinensis* and *C. maculatus* whereas α-AI2 inhibits insect α-amylase of *Z. subfasciatus* (*17*).

Molecular Mass Analysis. The analysis of purified inhibitor on SDS−PAGE under reducing conditions showed a single protein band with a molecular weight of 27 kDa (**Figure 3**). Furthermore, the purified α-amylase inhibitor had an apparent molecular weight of about 30 kDa as estimated by Sephadex G-100 gel filtration. These data led to the suggestion that the α-amylase inhibitor from mungbeans exists as a monomer.

Kinetic Studies. The inhibitory activity was not observed when the substrate, the *C. maculatus* α-amylase, and the α-amylase inhibitor were not preincubated. This observation was also reported from the α-amylase inhibitor in other beans. Different incubation times were used for the α-amylase inhibitors from Great Northern bean (*34*) and kidney bean (*35*), which reached the maximum inhibition after 40 and 120 min, respectively. Therefore, the preincubation times of the *C. maculatus* α-amylase and α-amylase inhibitor were tested. The inhibiting activity of mungbean amylase inhibitor reached maximum after 20 min (data not shown). So, in our inhibiting experiment, 0.2 mM of *C. maculatus* α-amylase was preincubated with 5 μM of α-amylase inhibitor 30 min before adding the substrate to the reaction mixture.

Table 1. Purification of α-Amylase Inhibitor

procedure	protein (mg)	act. (U)	sp act. (U (mg of protein)$^{-1}$)	yield (%)	purifn (fold)
crude extract	2624.4	50.76	0.02	100	1
80% ammonium sulfate	850.5	408.78	0.48	8.05	25
Sephadex G-100	17.5	93.8	5.36	1.85	277
HPLC	0.4	5.8	14.5	0.11	750

The kinetic parameters, K_m and V_{max}, for starch hydrolysis of amylase were calculated from a Lineweaver−Burk plot. Mungbean α-amylase inhibitor inhibited *C. maculatus* α-amylase activity through the decreasing of V_{max} and increasing of K_m. These data indicated that the inhibition was judged to be a mix noncompetitive inhibitor (**Figure 4A**). Both slope and the vertical axis intercept increased with increasing inhibitor concentration, indicated that only one molecule of inhibitor binds to the free enzyme or to the enzyme−substrate complex (**Figure 4B**,**C**). Similar results have been reported for the α-amylase inhibitor from common bean and porcine pancreatic α-amylase (*34*−*38*).

Most α-amylase inhibitors can cause malnutrition in animals by inhibiting digestive enzymes (*39*−*41*). However in this study, the mungbean α-amylase inhibitor affected only *C. maculatus* and not human α-amylase. Therefore, we further tested its application as a preservative agent for mungbeans against *C. maculatus*.

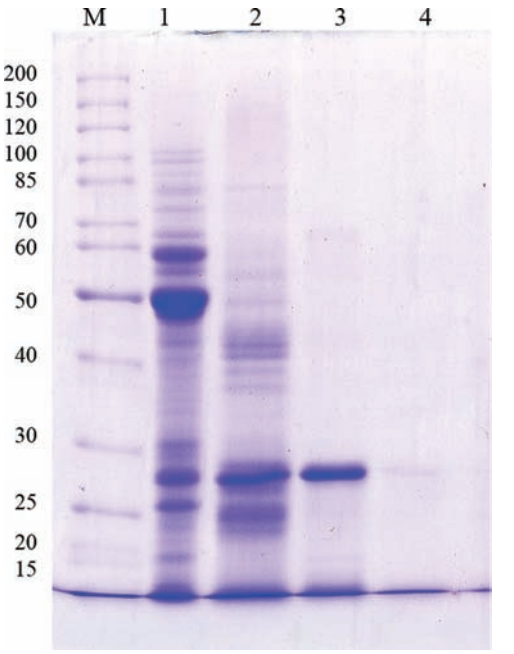

Figure 3. SDS−PAGE analysis of mungbean inhibitor on 12% polyacrylamide gel followed by Coomassie Brilliant Blue R-250 staining: (M) marker protein, (1) crude protein (100 μg), (2) 80% saturation ammonium sulfate precipitate (100 μg), (3) purified α-amylase inhibitor from Sephadex G-100 (100 μg), and (4) after HPLC fractionation (20 μg).

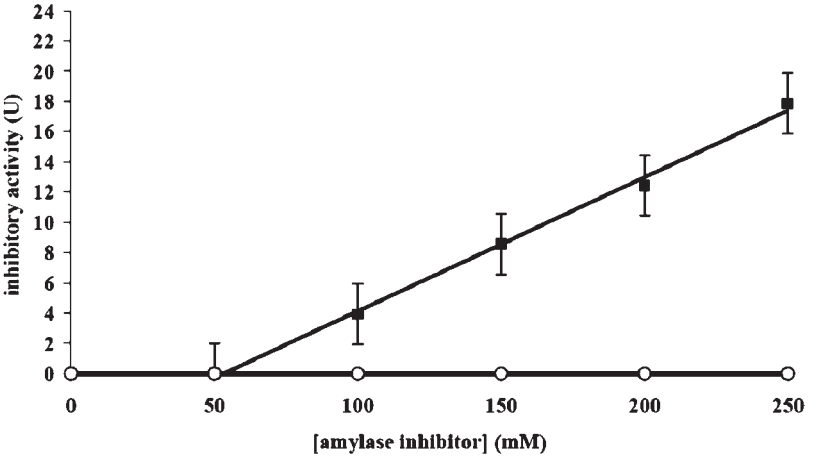

Figure 2. Inhibitory activity of purified α-amylase inhibitor against *C. maculatus* amylase (■) and human salivary amylase (○). *C. maculatus* and human salivary α-amylase (10 μg/μL) activities were measured after preincubation with increasing amounts of purified α-amylase inhibitors.

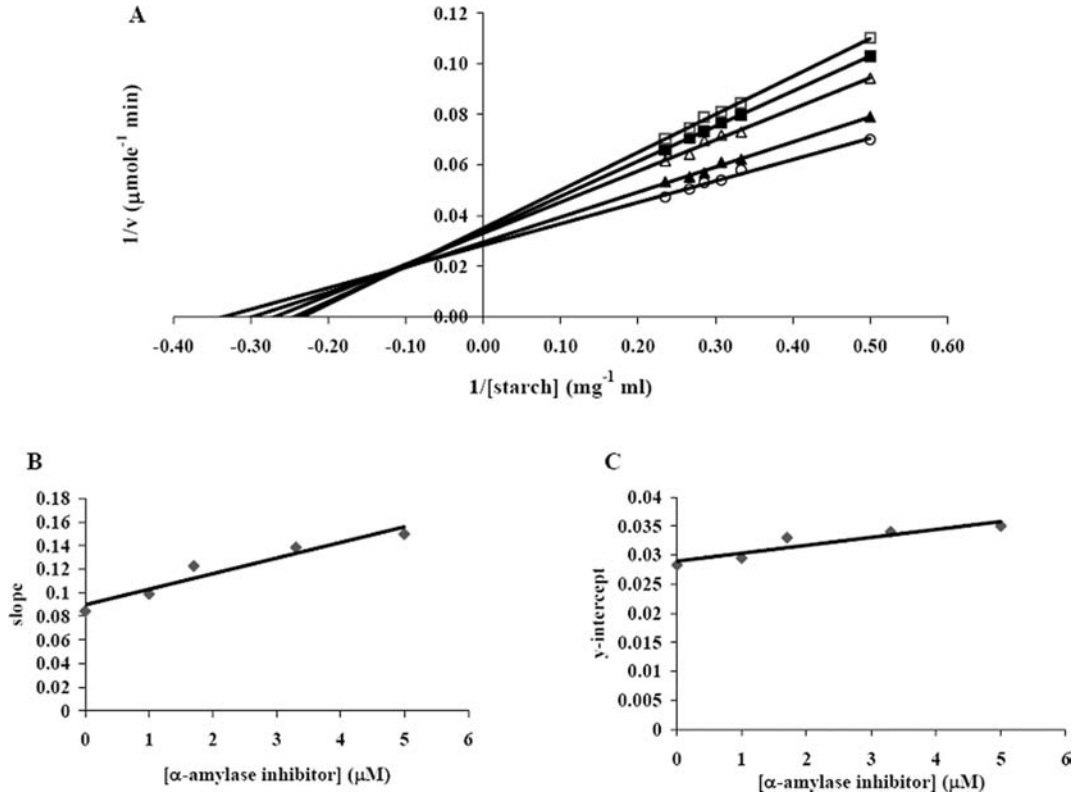

Figure 4. Kinetic analysis of *C. maculatus* α-amylase inhibition by mungbean α-amylase inhibitor. (**A**) Lineweaver−Burk plot using starch as substrate. Reciprocal plots obtained with variable starch concentrations and at fixed α-amylase inhibitor concentration: 0 (○), 1 (▲), 1.7 (△), 3.3 (■) and 5 μM (□). (**B**, **C**) Secondary plots showing the dependence of the slope and of the vertical axis intercept on the concentration of α-amylase inhibitor.

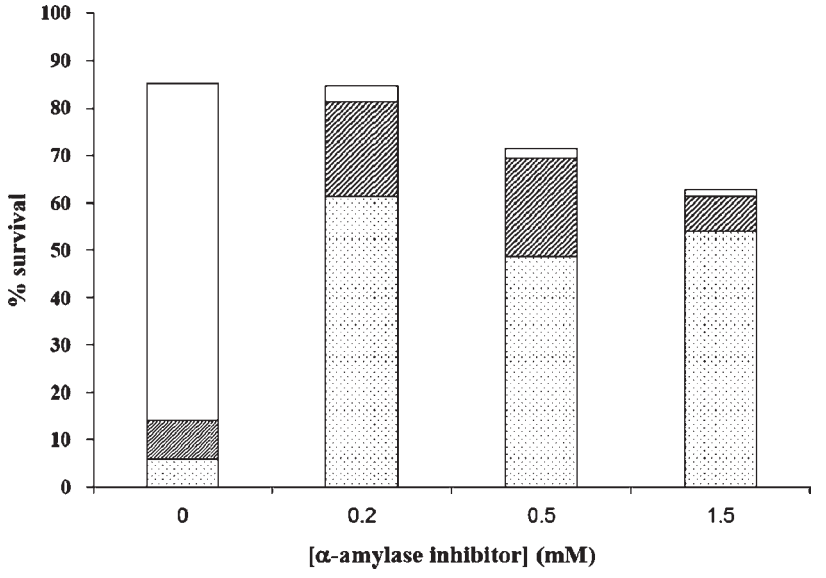

Figure 5. Effect of α-amylase inhibitor on growth and development of *C. maculatus*. Bruchid survival is calculated based on the number of larvae (bars with dot shading), survival pupae (bars with line shading) and adults (open bars) toward hatched eggs on the treated seeds 30 days after first oviposition.

Feeding Test. In order to evaluate the *in vivo* effect of the mungbean α-amylase inhibitor on the growth and development of *C. maculatus*, the mungbean seed was soaked with purified α-amylase inhibitor of various concentrations. Four parameters were analyzed: (a) the number of hatched eggs, (b) the total number of adults, (c) the number of surviving pupae and (d) the number of larvae. Results from the bioassay showed that there were no significant differences in the percentage of hatched eggs from seeds with and without α-amylase inhibitor coating of any concentration (data not shown). These data indicate that the α-amylase inhibitor had no affect on the hatching process. However, 0.2 and 0.5 mM inhibitor caused larval mortality of 14% and 17%, respectively. When the inhibitor concentration was increased to 1.5 mM, larval mortality increased to 35%. Furthermore, the growth of *C. maculatus* larvae was suppressed by the addition of the purified α-amylase inhibitor to the seed. We also observed an increase in larval population and a decrease of surviving adults with the increasing concentration of α-amylase

inhibitor. There were only 1−3% of surviving adults in presence of α-amylase inhibitor. Most of them stopped developing and died at larvae stage. Moreover, percentage of pupae also decreased when the concentration of α-amylase inhibitor increased from 0.2 to 1.5 mM (**Figure 5**). Furthermore, the surviving larvae in the seeds coated with the highest concentration of inhibitor (1.5 mM) were smaller than in other concentrations. These results indicate that the α-amylase inhibitor can inhibit α-amylase activity in the gut of *C. maculatus* larvae, thus lowering carbohydrate digestion which is necessary for growth and development of the insect.

These data were supported by the observation of other groups, showing that several α-amylase inhibitors do not cause acute mortality but may retard pest growth and development (*25*). For example, the α-amylase inhibitor from *Phaseolus vulgaris* seeds suppressed growth and development of *C. maculatus*, *C. chinensis* (*11*, *42*) and *Z. subfaciatus* (*12*). Moreover, wheat α-amylase inhibitor inhibited growth of *Tribolium castaneum* larvae and caused a substantial weight loss of adult insects (*43*). Finally, *Carica papaya* α-amylase inhibitors increased larval mortality (50%) and decreased *C. maculatus* fecundity (*44*).

In conclusion, we identified the α-amylase inhibitor from mungbean seeds that has ability to inhibit the α-amylase of *C. maculatus*. This mungbean α-amylase inhibitor also has no effect on human salivary α-amylase. Mungbean seed already has α-amylase inhibitor in its native strain, but the concentration of the inhibitor in the seed is not enough to efficiently inhibit *C. maculatus*. This could be an opportunity to further use this biotechnological protein as a pest control agent or to develop a strain of mungbean that has a more effective α-amylase inhibitor.

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