

Proteomic and Characterization of α -amylase Inhibitor from Mungbean (*Vigna radiate*)

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ABSTRACT

Callosobruchus maculatus, the insect pest, causes damaging to stored mungbean seeds leading to economical losses. For this reason the use of α -amylase inhibitors to interfere the pest digestion process has been an interesting alternative as a biocontrolling agent. The studies of gamma irradiated mungbean mutant, M5-16, demonstrated that α -amylase inhibitor from the mutant line was more inhibitory effective on *C. maculatus* α -amylase than its wild type, KPS1. To investigate this mechanism, both proteomics and biochemical characterization were employed. The α -amylase inhibitor from mungbean seed, M5-16, was purified in a two-step procedure involving ammonium sulfate precipitation and gel filtration chromatography using a sephadex G-100 column. This α -amylase inhibitor existed as a monomer and had a molecular weight of 27 kDa. Moreover, It was very interesting that α -amylase inhibitor from the mungbean seed inhibited *C. maculatus* α -amylase but did not inhibit human salivary α -amylase. 2D gel electrophoresis suggested that the gamma-induced mutant line has decreased expression of α -amylase inhibitor and other proteins comparing with its wild type.

Key words: mungbean seeds, α -amylase, characterization, *C. maculatus*, purification

INTRODUCTION

Mungbean (*Vigna radiata*) is a self-pollinated legume originated in South and Southeast Asian regions. It is an economically important crop in Thailand, India, Pakistan, Vietnam, Myanmar, and China. There are two major types of mungbeans, Greengram and Blackgram, grown in Thailand with the production of about 155,000 tons of grains per year. Unfortunately, more than 30% of the harvested mungbean seeds are damaged by insects during storage. Two species of weevils, *Callosobruchus chinensis* and *Callosobruchus maculatus*, are

major insect pests of mungbean seeds in Thailand (Tomooka *et al.*, 1992). These insects lay their eggs on mungbean seeds and the eggs-hatched larvae feed on the seed nutrients. Some farmers solve this problem by coating mungbean seeds with insecticides. However, this could lead to the adverse effects on environment and may increase risk for human health.

One self-protecting mechanism from insects of plants is to produce α -amylase inhibitors in the seeds. For example, leguminosae seed contains α -amylase inhibitors which inhibit α -amylases in mammals and insects but not in plants, fungi and bacteria. The plant α -amylase

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inhibitors inhibit α -amylase in a digestive tract of *Tenebrio molitor* (Frels and Rupnow, 1984), *C. chinensis*, *C. maculatus* (Ishimoto and Kitamura, 1989), *Zabrotes subfaciatus* (Suzuki *et al.*, 1993) and *Bruchus pisorum* (Ieluk *et al.*, 2000) resulting in suppression of growth and development of weevil larvae. Using of gamma radiation induced mutation of the standard variety of mungbean KPS1 (known as M5-16) was effective for the inhibition of *C. maculatus* development (Wongpiyasatid *et al.*, 1999). Furthermore, the study also showed that α -amylase inhibitor in the gamma-irradiated mutant was more effective for the inhibition of α -amylase from *C. maculatus* adults and malt than its wild type (Engkagul *et al.*, 2004). These results raised the questions about the mechanism behind the effectiveness of α -amylase inhibitor in the gamma-induced mutant. There were two possible mechanisms may be envisaged. The radiation might cause an increase in the amount of α -amylase inhibitor in the mungbean mutant or a mutation of α -amylase inhibitor to increase their affinity to binding with α -amylase. To investigate these mechanisms underlying the increased inhibitory effect, both proteomics and biochemical characterization were employed. Proteomics techniques using 2D-gel electrophoresis can reveal whether the expression of α -amylase inhibitors are increased in mungbean mutant. Biochemical techniques including protein purification, enzyme activity assay, and kinetic measurement can be used to compare the biochemical properties of purified α -amylase inhibitor from the mutant line with those from the standard variety.

This paper reported the purification and characterization of α -amylase inhibitor from the mutant line, M5-16. The inhibitory activity of α -amylase inhibitor was tested against human salivary α -amylase and α -amylase from *C. maculatus*. This paper also used proteomic tool for investigation of the distribution of spots proteins in standard variety KPS1 and mutant line

M5-16.

MATERIALS AND METHODS

Source of materials

Mungbean seeds (KPS1 and M5-16) were obtained from Assoc. Prof. Arunee Wongpiyasatid, Department of Applied Radiation and Isotopes, Faculty of Science, Kasetsart University, Thailand. *Callosobruchus maculatus* (cowpea weevil) was obtained from Insect Section of Stored Products Laboratory, Division of Entomology and Zoology, Department of Agriculture, Thailand.

Extraction of seed protein from mungbean seeds

The mungbean seeds were ground with mortar and pestle in 6 ml of distilled water, stirred with magnetic stirrer at 4 °C for 3 h. and centrifuged at 10,000xg for 20 min at 4 °C. The supernatant containing mungbean proteins was used for further analysis.

Preparation of *C. maculatus* α -amylase

After 30 min freezing at -20 °C, 2 grams of *C. maculatus* were finely grounded in a deep cold mortar with 8 ml of 20 mM sodium phosphate buffer pH 7 and centrifuged at 10,000xg for 20 min at 4 °C. The clear supernatant was used as crude α -amylase.

Purification of α -amylase inhibitor

The extracted proteins were precipitated by 80% ammonium sulfate. The precipitated protein pellet was collected by centrifugation at 10,000 xg for 20 min at 4 °C and redissolved in 20 mM sodium phosphate buffer pH 6.7, followed by dialysis at 4 °C overnight. The dialysate was applied to gel filtration chromatography column using Sephadex G-100 (Fluka, Switzerland) equilibrated with 20 mM sodium phosphate buffer pH 6.7 and eluted with the same buffer at flow

rate 0.2 ml/min and 3 ml fractions were collected.

Inhibitory activity assay

The 50 µl of the *C. maculatus* or human salivary α -amylases and 100 µl of purified α -amylase inhibitor were preincubated at room temperature for 30 min before addition of 250 µl of 2% starch solution in 20 mM sodium phosphate buffer pH 6 containing 20 mM NaCl and 0.2 mM CaCl_2 as starting reaction. After incubation at 50°C for 10 min, the reaction was terminated by adding 250 µl of 3, 5-dinitrosalicylic acid reagent, followed by heating in boiling water bath for 5 min. The reaction mixture was cooled, diluted in 2 ml distilled water, and the absorbance was measured at 540 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) was performed according to the method of Laemmli (Laemmli, 1970) at room temperature; using protein molecular weight markers (15-200 kDa) (Fermentas) and bromophenol blue as the tracking dye. After electrophoresis, proteins in the gel were visualized by Coomassie Brilliant Blue R-250 staining.

Estimation of protein molecular weight

The purified protein in 20 mM sodium phosphate buffer pH 6.7 was applied onto a Sephadex G-100 gel permeation column (150 ml bed volume, Fluka, Switzerland) and eluted with the same buffer. The elution was performed at flow rate 0.2 ml/min and 3 ml fraction were collected. The column was calibrated using 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 was calculated from the plot of K_{av} versus log molecular weight.

Proteomics of proteins in wild-type and mutant mungbeans

Proteomic was conducted as described by O' Farrell (O' Farrell, 1975). The 50-200 µg of the mungbean proteins were added to isoelectric focusing (IEF) buffer (5 M urea, 2 M Thiourea, 1% 3 [3-cholamidopropyl dimethyl ammonio] propane sulfonate (CHAPS), 10mg/ml dithiothreitol (DTT), and 60 mM Tris-base) to the total volume of 30 µl. The mixture was loaded onto 7 cm immobilized linear pH 3-10 gradient gel. The first dimensional electrophoresis was performed at room temperature for 12 h. After IEF, the gel rods were equilibrated for 10 min in equilibration buffer (1 M tris HCl pH 6.8, 2% SDS and 0.02% bromophenol blue), and then adhered onto the top of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The second dimensional SDS-PAGE was performed in 12% polyacrylamide slab gel at 150 V for approximately 2 h. The protein spots in the gel were visualized by Coomassie Brilliant Blue R-250 staining.

RESULTS AND DISCUSSION

Purification of α -amylase inhibitor from mungbean mutant M5-16

The crude α -amylase inhibitor from mutant mungbean seeds was obtained by an aqueous extraction and 80% ammonium sulfate precipitation. This fraction was applied on the sephadex G-100 gel filtration chromatography to obtain the purified α -amylase inhibitor. The elution profile showed two major peaks and three minor ones. The fractions of peak 2 (fraction 28-34, 3 ml/fraction) contained the inhibitory activity against the *C. maculatus* α -amylase (Figure 1).

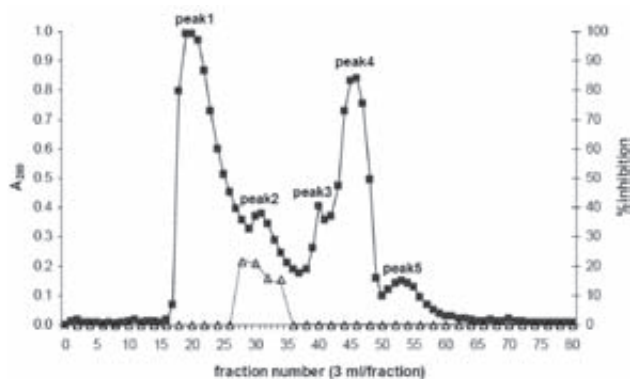


Figure 1 Purification of α -amylase inhibitor from mutant M5-16 by gel filtration chromatography using Sephadex G 100. The column chromatography was done at a flow rate of 0.2 ml/min and the absorbance at 280 nm ($\blacklozenge$) and percentage inhibition of α -amylase inhibitor against *C. maculatus* α -amylase (Δ) were monitored.

The purified α -amylase inhibitor had an apparent molecular weight of 31 kDa as estimated by the Sephadex G-100 gel filtration in relation to the standard proteins (Figure 2) The analysis of purified inhibitor on SDS-PAGE showed a single protein band with a molecular weight of 27 kDa (Figure 3) suggesting that α -amylase inhibitor from mungbean M5-16 existed as a monomer.

Effect of α -amylase inhibitor from mutant mungbean mutant on α -amylase activity

The α -amylase inhibition activity in mungbean mutant was tested toward human salivary and *C. maculatus* α -amylases under

optimum condition (pH 6 and 50 °C). Purified α -amylase inhibitor was found to be more effective against *C. maculatus* α -amylase but could not inhibit human salivary α -amylase (Figure 4)

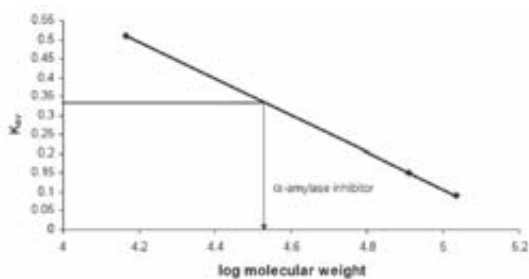


Figure 2 Estimation of molecular mass of purified α -amylase inhibitor by gel filtration chromatography using Sephadex G-100.

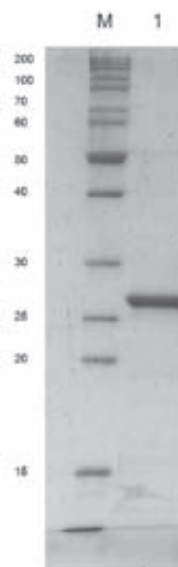


Figure 3 SDS-PAGE analysis of mutant mungbean inhibitor on 12% polyacrylamide gel followed by CBB staining. In the gel, M and 1 indicated marker protein and purified α -amylase inhibitor, respectively.

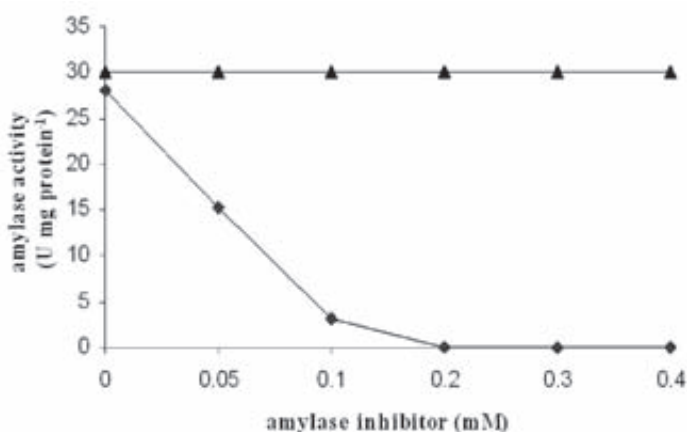


Figure 4 Activity of *C. maculatus* (◆) and human salivary α-amylase (▲) at different concentrations of purified amylase inhibitor.

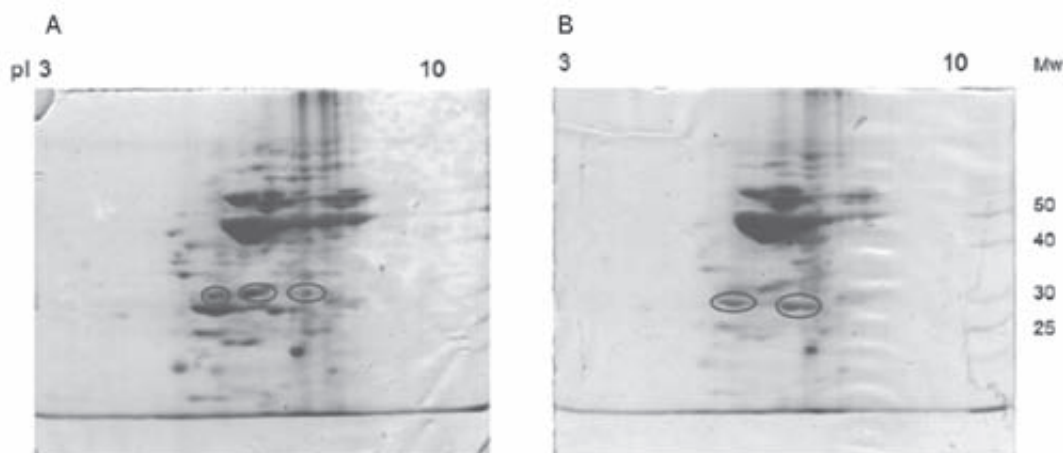


Figure 5 Proteomic comparison of proteins of standard variety, KPS1 (A) and mutant line, M-16 (B).

This was in agreement with the results by Kitamura *et al.* (1990) who reported that the α-amylase inhibitor from *Phaseolus* and *Vigna* legumes inhibited α-amylase from *C. maculatus* and *C. chinensis* but did not inhibit the mammalian α-amylase.

Proteomic analysis of proteins

In this study, 2D gels between the standard variety KPS1 and the mutant line M5-16 were compared for the distribution pattern of proteins. The proteomic analysis wild-type

mungbean seeds showed that the number of protein spots in KPS1 was more abundant than those in M5-16. This indicated that gamma irradiation decreased the amount of protein in mutant line. Furthermore, KPS1 had three spots of α-amylase inhibitor around 27 kDa while M5-16 had only two spots of α-amylase inhibitor indicating that gamma irradiation decreased an expression of α-amylase inhibitor (Figure 5). These results may imply that the α-amylase inhibitor in mutant line have more affinity binding to the α-amylase than the wild-type.

CONCLUSION

The α -amylase inhibitor was purified from the mungbean mutant seeds. The inhibitor existed as a monomer with molecular weight of 27 kDa. It inhibited the *C. maculatus* α -amylase but could not inhibit the human salivary α -amylase. Furthermore, the 2D gel electrophoresis suggested that gamma irradiation decreased the expression of α -amylase inhibitor comparing with the wild type although the inhibition activity was increased. This can be assumed that the radiation decreased amount of the α -amylase inhibitor in the mutant line and it may also cause mutation of the α -amylase inhibitor to increase its affinity for binding α -amylase. To prove this hypothesis, the kinetic studies of binding between α -amylase and α -amylase inhibitor from the mutant will be further employed. If an increase in effectiveness of inhibition results from mutation of α -amylase inhibitor structure, further engineering of this protein can be made to produce more thermostable and effective inhibitor for application on the harvested mungbean as biocontrol agent during storage instead of the currently employed insecticides.

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