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Effects of α -amylase Inhibitor on Mungbean Weevil, *Callosobruchus maculatus, in vivo* and *in vitro* and on Barley Malt α-amylase *in vitro*

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ABSTRACT

Effects of protein part of α-amylase inhibitor crude extracts from mungbean seeds of the recommended varieties, KPS1 and CN36, and mutant lines, M5-16 and M5-29 were evaluated on the mortality of the larval and adult stages of mungbean weevil, *Callosobruchus maculatus.* KPS1 seeds were used as the medium soaked with different extract concentrations of all mungbean varieties/lines and distilled water (the control). Each, with three replicates, was fed to one pair of *C. maculatus* and percent mortality of each stage was recorded. Of all different protein α-amylase inhibitor extracts at each concentration, significant difference was not found in larval mortality percentage between the extract-treated seeds and distilled water-treated seeds. Only seeds treated with 0.2% w/w protein extracts from CN36 and KPS1 seeds significantly differed in percent adult mortality from those of the control, respectively. At 0.4, 0.6 and 1% w/w protein, each extract was significantly different from the control in adult mortality percentage. Each extract of various concentrations, except for KPS1 and M5-29 extracts gave significantly different larval mortality percentages at 0.4 and 0.6 and 1% w/w protein, respectively. There were significant differences in adult mortality percentage of every extract at each concentration from that of the control. An effect of α-amylase inhibitor on α-amylase activity *in vitro* was also studied. One hundred percent inhibition of protein part to α-amylase activity of *C. maculatus* was found in all variety/line mungbean seeds. Similar study was also conducted against α -amylase extracted from barley. The effects were less than those of α-amylase of the weevil.

Key words: α-amylase inhibitor, α-amylase, mungbean weevil, *Callosobruchus maculatus*

INTRODUCTION

Mungbean (*Vigna radiata*) has been grown in Thailand for a long period of time but the yield is still low due to several problems including insect infestation. Tomooka *et al.* (1992) reported that two species of weevils, *Callosobruchus chinensis* and *Callosobruchus maculatus*, were the major insect pests of mungbean seed in Thailand causing low yield and decreased seed quality. They occur all year round. Field damages to pods and grains by *Callosobruchus spp.* were reported by Gujar and Yadav (1978). However, the field's damage to pods and grains by these bruchids is only a minor problem, when the major destination to grain

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occurs during storage. At present, all recommended varieties of mungbean in Thailand are known to be susceptible to these insects. Proteins such as lectin, trypsin inhibitor, amylase inhibitor and high molecular weight hetero-polysaccharides have been reported as bruchid resistance factor in food legumes.

Leguminosae α-amylase inhibitor has been extensively studied in the past since it plays a role of plant resistance to insects. Its potential has already been illustrated by the resistance to *Bruchus pirosum*, *C. maculatus* and *C. chinensis* exhibited in pea seeds. Ishimoto and Kitamura (1988) purified and identified a proteinous α-amylase inhibitor as one of the major inhibitory substances. At levels of 0.2-0.5 %, α-amylase inhibitor was highly toxic to the larvae of *C. maculatus*. Birch *et al.* (1986) also found α-amylase inhibitors to have some detrimental effects upon larval development at concentration occurring naturally in seeds. Numbers of emerging adult of *C. maculatus* were reduced by 30 %. The focus on protein digestion as a target for bruchid control changed to that of starch digestion as a consequence of results showing that α-amylase inhibitors are detrimental to the development of *C. maculatus* and the Azuki bean weevil, *C. chinensis* (Ishimoto and Kitamura, 1989).

Recently, the new mutants, M5-16 and M5-29 of *V. radiata* obtained from gamma irradiation have been shown to confer antibiotic resistance against *C. maculatus* (Wongpiyasatid *et al.*, 1999). Since α-amylase inhibitor has been identified to have detrimental effects to the bruchid, it should be further investigated as the possible source for antibiosis. The objectives of this paper were to extract and purify α-amylase inhibitor from *V. radiata* seeds of recommended varieties, KPS1 and CN36, and the mutant lines, M5-16 and M5-29 and to compare the effects of α-amylase inhibitor, protein extracts of the control varieties with the mutant lines on *C. maculatus in vivo*, and *invitro*, α-amylase extracted from *C. maculatus* adult and barley malt α-amylase (Type VIII-A) *in vitro*.

MATERIALS AND METHODS

1. Insect mass rearing

The bruchids were obtained from Insect Pests of Stored Products Laboratory, Division of Entomology and Zoology, Department of Agriculture. The culture was maintained on healthy, sterilized seeds of mungbean (*V. radiata*) at 27±2°C and 70±10 % R.H. and 10:14 (light: dark) photoperiod for three generations before experimentation to ensure that they were genetically and phenotypically alike. The beetles were cultured under moderately crowded conditions to ensure proper development and equal size of the resultant adults.

2. The recommended varieties and the mutant lines

Seeds of KPS1 and CN36, the recommended varieties and M5-16 and M5-29, the mutant lines were obtained from Mungbean Varietal Screening for Disease and Insect Resistance Project, Department of Applied Radiation and Isotopes, Faculty of Science, Kasetsart University. The mutant seeds (M5-16 and M5-29), derived from gamma irradiation of KPS1 and CN36, respectively, have already been through preliminary resistant screening against *C. maculatus*. Barley malt α -amylase (Type VIII-A) was bought from Sigma to be used in the experiment.

3. Extraction and purification of the proteinaceous α -amylase inhibitor

Mungbean meal (ground to powder with blender and later mortar) of each variety/ line was extracted with 20 mM phosphate buffer, pH 6.7 (PBS), stirred by magnetic stirrer at 4°C for 3 hours, and then centrifuged at 10,000 g for 20 minutes. The supernatant (S_1) was made 80 %

saturated with ammonium sulfate and centrifuged again at 10,000 g for 20 minutes at 4°C to give the protein pellet and the supernatant (S_2). The protein pellet was dissolved in minimum volume of PBS solution to give S_3. Both S_2 and S_3 were dialyzed against PBS and the dialysates from protein (S_3) and non-protein (S_2) parts were tested for the inhibitor activity against α-amylase of mature *C. maculatus*. The levels of concentration of α-amylase inhibitor of each variety/line were compared.

4. Effects of α-amylase inhibitor, protein and non-protein parts, on development of *C.maculatus* by feeding test

The effect of α-amylase inhibitor on insect mortality was examined using seeds of KPS1 as the medium soaking with α–amylase inhibitor, protein part, at the concentration levels of 0.2, 0.4, 0.6 and 1% protein (w/w) with distilled water as the control. The 4 solutions were made from dried powdered α-amylase inhibitor extracted from seeds of the four varieties/lines dissolved in distilled water. Seeds of KPS1 were soaked in distilled water and the inhibitor solutions in plastic cups. After 1 hour soaking, they were air-dried for another hour. Fifty KPS1 seeds soaked in each solution were then put in each small plastic cup. There were 3 replications, 4 varieties/lines per replicate, 5 treatments (solution). One pair of *C. maculatus* (male and female) was introduced in each cup for oviposition. After 24 hours, the adults were removed and the cups kept at room temperature. Seven days after the initial oviposition, the number of eggs hatched on the surface of the seeds of KPS1 was counted. After 30 days, the beans were dissected and the number of dead adults, larvae and pupae were recorded.

5. α-amylase inhibitory activity against α-amylase of mature cowpea weevil

5.1 α-amylase preparation

Adults of mungbean weevil were freezed at -20°C for 30 minutes. After that two grams of the frozen weevils were finely ground in deep cold mortar with 8 ml 20 mM phosphate saline buffer (PBS), pH 7.0, and centrifuged at 10,000 g for 20 minutes at 4°C. The clear supernatant was used as crude α -amylase preparation.

5.2 Assay for α-amylase and α-amylase inhibitor activities

The activity of the crude adult amylase was measured using Bernfeld method (Bernfeld, 1955). The amylase preparation was incubated with 2% soluble starch in 20 mM sodium phosphate buffer containing 20 mM NaCl and 0.2 mM $CaCl_2$ at different pH levels, room temperature. The buffers used were HCl-KCl buffer for pH 1.0 and 2.0, citrate phosphate buffer for the pH range of 3.0-5.0, phosphate buffer for the pH range of 6.0-8.0 and $NaHCO_3$-Na_2CO_3 buffer for the pH range of 9.0-11.0. For pH profile study, the reaction was performed at room temperature at various pHs (1.0-11.0). For temperature profile study, the reaction was performed at various temperatures (20-80°C) at the optimum pH. After 10 minutes the reaction was stopped by adding 250 µl of DNS solution and heated in boiling water bath for 5 minutes. They were cooled down and added with 2.0 ml of distilled water. The amount of reducing sugar produced was determined by measuring the changes in absorbance at 540 nm. Blank was the reaction mixture without the enzyme and the control was prepared by adding the crude enzyme after the DNS reagent. Maltose (0.1-1.0 µmol) was used for preparation of the calibration curve. The amylase specific activity is defined as µmol of maltose produced min^{-1} mg $protein^{-1}$ at the specific reaction condition.

The effects of α-amylase inhibitor on the adult α-amylase preparation and barley malt α -amylase (Type VIII-A) were determined by preincubating the enzyme with varying amounts of α-amylase inhibitor in PBS at room temperature for 15 minutes before the addition of the starch

solution. The protein analysis followed the method of Lowry *et al*. (1951).

RESULTS AND DISCUSSION

1. Extraction and purification of the proteinaceous α-amylase inhibitor

Protein extraction and determination

Quantitative analysis of protein followed the method of Lowry *et al.* (1951) was conducted. The standard curve of BSA (Bovine Serum Albumin) was calculated (y = 0.6771 and R^2 = 9809). Amount of protein (g) in protein part obtained from 30 g of the seeds of recommended (KPS1 and CN36) and mutant lines (M5-16 and M5-29) are shown in Table 1.

Protein determination (Table 1) showed total of the protein part to vary among crude extracts of tested varieties and lines with that of M5-16 seed to be the highest. Inter-varietal variation in the content of protein α-amylase inhibitor in seeds is not uncommon and has been reported in crops such as common bean (Ishimoto *et al.*, 1995). Kokiladevi *et al.* (2005) also reported the protein content of *V. radiata* equalling 15.1 mg/g seed while the inhibitory activity against α-amylase of *C. analis* was only12.01 comparing to *V. umbellata* which had protein content = 14.4 mg/g seed and the inhibitory activity =110.01.

Percent larval mortality

According to the statistical ANOVA analysis in percent larval mortality from KPS1 seeds treated with protein parts of extract, no interaction among means of extract at each concentration was found while there was interaction among means of concentrations of some extracts.

Table 2 shows percent larval mortality from KPS1 seeds treated with distilled water (0% protein) and the 4 protein concentrations of α-amylase inhibitor extracted from the recommended and mutant variety/line seeds. Among the extracts at each concentration, it was

Table 1 Amount of protein parts (g) from 30 grams of the seeds of recommended (KPS1 and CN36) and mutant lines (M5-16 and M5-29).

Extracts from four mungbean variety/line seeds	Protein part in protein extract (g)
KPS1	0.87
CN36	0.86
M5-16	0.90
M5-29	0.62

Table 2 Percent larval mortality of *C. maculatus* on KPS1 seeds treated with distilled water and protein parts extracted from four mungbean variety/line seeds.

KPS1 seeds treated with extracts from four mungbean variety/line seeds	Percent larval mortality [1/2/]				
	% Protein (w/w)				
	0	0.2	0.4	0.6	1.0
Control	0.0	0.0a	0.0a	0.0a	0.0a
KPS1	0.0 B	2.0a AB	3.7a A	1.8a AB	0.0a B
CN36	0.0 A	1.5a A	0.0a A	0.0a A	0.0a A
M5-16	0.0 A	2.0a A	0.0a A	0.0a A	3.3a A
M5-29	0.0 B	0.0a B	0.0a B	4.3a A	3.7a A

[1/] Means followed by the same small letter in the same column are not significantly different as determined by DMRT at p = 0.05

[2/] Means followed by the same capital letter in the same row are not significantly different as determined by DMRT at p = 0.05

found that there were no significant differences either between percent larval mortality from seed treated with extracts of all variety/line seeds at every concentration and that of the control or among one another at every concentration.

In comparison among each extract at various concentrations, percent larval mortality from seeds treated with extract of KPS1 seeds at 0.4% protein (w/w) significantly differed from those of the control and 1% protein (w/w). There were no significant differences in larval mortality percentage at all concentrations of CN36 and M5-16 extracts. At 0.6 and 1.0% protein (w/w) of M5-29, larval mortality percentages were significantly different from those at 0, 0.2 and 0.4% protein (w/w).

Percent adult mortality

According to the ANOVA analysis in percent adult mortality of KPS1 seeds treated with protein parts of extract, there was interaction among means of variety at various concentrations and that of each variety at each concentration.

Table 3 presents the adult mortality percentages from KPS1 seeds treated with distilled water and the 4 protein concentrations of α-amylase inhibitor extracted from the recommended and mutant variety/line seeds. At each protein concentration of different extracts, only the percentage of adult mortality from seeds treated with extract of KPS1 seeds was observed to be significantly different from that of the control and the rest but not from CN36 at 0.2% protein (w/w). At 0.4, 0.6 and 1% protein (w/w), adult mortality percentages of seeds treated with the extracts of all variety/line seeds were not significantly different from that of KPS1 seeds.

As for each extract at various protein concentrations, the percentage of adult mortality from seeds treated with distilled water was significantly different from those of all tested seeds at every protein concentration.

It could be seen according to the results that most protein α-amylase inhibitor extracts of all varieties/lines had detrimental effects causing larval and adult mortalities at high concentrations.

The report of Gatehouse *et al*. (1987) suggested that the heteropolysaccharide fraction was isolated from the resistant line and a susceptible line of *Phaseolus vulgaris* G12935 incorporated into artificial beans over a concentration ranging up to 10% dry wt. At a concentration of 4%, the approximate physiology concentration within the seed, the heteropolysaccharide fraction from the resistant line was very toxic resulting in 80-85% larval mortality of *Acanthoscelides obtectus* with LC_{50} of 2.5%. Furthermore, surviving larvae showed a marked increase in their developmental period. The results were more or less in agreement with

Table 3 Percentages of adult mortality of *C. maculatus* from KPS1 seeds treated with distilled water and protein parts extracted from four mungbean variety/line seeds.

KPS1 seeds treated with extracts from four mungbean variety/line seeds	Percent larval mortality [1/2/]				
	% Protein (w/w)				
	0	0.2	0.4	0.6	1.0
Control	0.0	0.0 b	0.0 b	0.0 b	0.0 b
KPS1	0.0 B	51.5a A	41.7a A	37.6a A	41.9a A
CN36	0.0 B	42.7abA	40.2a A	40.3a A	46.0a A
M5-16	0.0 B	33.5 bA	34.6a A	46.8a A	33.3a A
M5-29	0.0 B	30.9 bA	48.9a A	40.4a A	44.7a A

[1/] Means followed by the same small letter in the same column are not significantly different as determined by DMRT at p = 0.05

[2/] Means followed by the same capital letter in the same row are not significantly different as determined by DMRT at p = 0.05

this study. With less concentration, different legume species, different insect tested and different data analysis, hence, difference in percent larval mortality was obtained.

Ishimoto *et al*. (1999) studied the common bean (*Phaseolus vulgaris* L.) cultivars which had a glycoprotein that reacted with anti–α-AI–1 antibodies. The glycoprotein was purified; the primary structure was identified to be the same as α-amylase inhibitor–like protein (AIL) isolated. AIL was proved to have some inhibitory effect on the growth of *C. maculatus*. The experiment by Farias *et al*. (2006) also stated that several plant defense studies were developed, indicating that α-amylase inhibitors were able to impede and/or reduce bruchid digestive process. Bioassays using artificial seeds containing *Carica papaya* α-amylase inhibitor rich fraction were also conducted showing that α-amylase inhibitors were able to increase larval mortality and also decreased insect fecundity and adult longevity.

In the experiment on the development of *C. maculatus* fed with artificial beans prepared with varying proportions of rice bean (resistant) and azuki bean (susceptible) by Kashiwaba *et al.* (2002), they found that chemical compound(s) contained in the cotyledon of rice bean had an inhibitory growth effect on the growth of the three bruchids, *C. maculatus, C. chinensis and C. analis.* One of such chemicals was α-amylase inhibitor.

Following to these findings whose results were similar to this research study, it was obvious that α-amylase inhibitor affected mortality of the mungbean weevils although different approaches and data collected were employed.

3. Effects of α-amylase inhibitor on α-amylase extracted from *C. maculatus* adults

3.1 Characteristics of α-amylase

Profiles of amylase activity were observed at various pHs and temperatures. The amylase showed optimum pH for the hydrolysis of its substrate at pH 6.0. By varying temperature at pH 6.0 assay condition, amylase expressed the optimum temperature of 50°C.

3.2 Effects of α-amylase inhibitor on activities of *C. maculates* α-amylase *in vitro*

α-amylase inhibitory activities in seed meal of four mungbean varieties/lines were tested against *C. maculatus* amylases obtained at the optimum conditions (pH 6.0 and 50°C). Maximum inhibition of 100% was obtained from protein parts of four mungbean varieties/lines (Figure 1).

This was in similarity to the results of the experiment by Kitamura *et al.* (1990) who reported that the larval midgut α-amylase activity in the crude enzyme preparation of both *C. chinensis* and *C. maculatus* almost completely disappeared when preincubated with 3 to 5 μg of the inhibitor.

Angharad *et al.* (1986) also worked on protein α-amylase inhibitors prepared from wheat and their effects tested against insect storage pests *in vitro*. Fraction B, C and D (0.28) were strong inhibitors of digestive α-amylases from larvae of *Tribolium confusum*, a storage pest of wheat products, and *C. maculatus*, a storage pest of legume seeds. Fraction D, which was a single polypeptide of M, 13000 was the most effective inhibitor *in vitro*.

It was also observed that the percentage inhibition of the crude protein extracts from four mungbean varieties/lines increased with the increasing amounts of the extracts until complete inhibition was obtained whilst the percentage inhibition of the non-protein parts remained fluctuated despite the five times increase in the amount added.

4. Effects of α-amylase inhibitor on activities of barley malt α-amylase *in vitro*

Similar results were obtained when the crude protein extracts were tested against barley malt enzyme at the optimum conditions (pH 4.0, 50°C). The enzymatic inhibitory effects of all four

extracts were slightly less in barley than that of the insect and the percent inhibitions of mutant lines were less than the standard varieties/lines which might reflect different affinities of the inhibitors for different isoforms of the enzyme (Figure 2).

As for the preparation of weevil α-amylase, although the gut contained most of α-amylase, in order to determine the inhibition of the enzyme activity, a whole weevil extract was

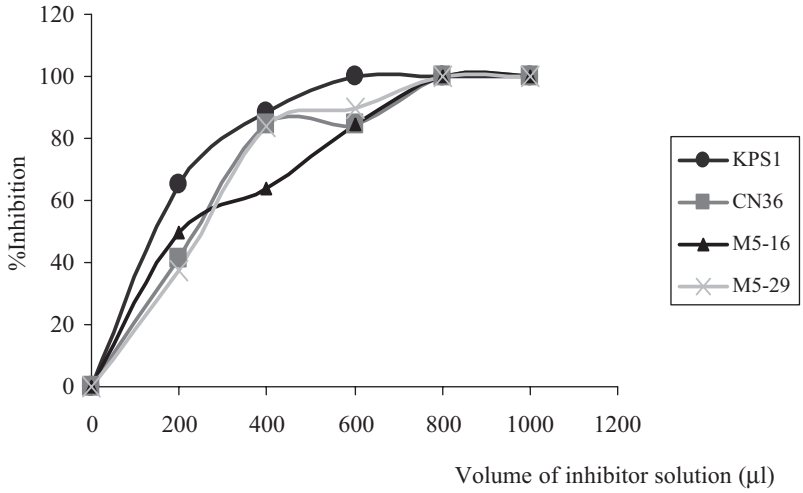

Figure 1 Percent inhibition at different concentrations of four mungbean crude extracts (protein part) against *Callosobruchus maculatus* α-amylase. The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 10 min at the optimum condition for *C. maculatus* enzyme, pH 6.0 and 50°C.

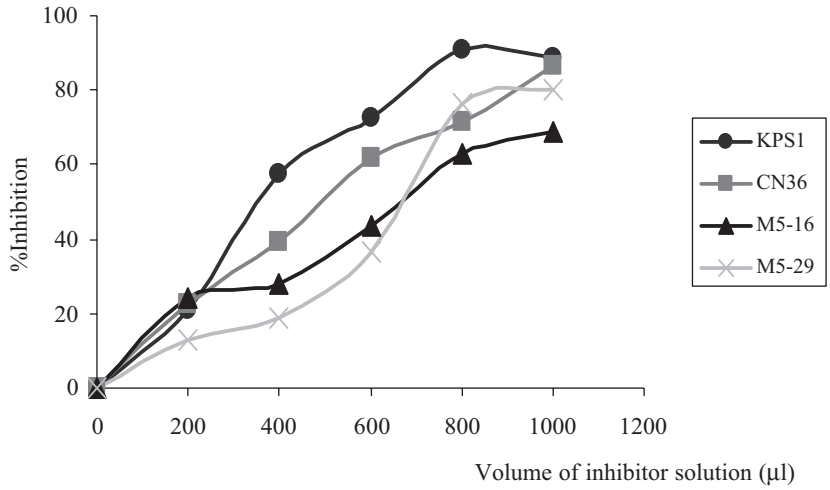

Figure 2 Percent inhibition at different concentrations of four mungbean crude extracts (protein part) against barley malt α-amylase (Type VIII-A). The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 10 min at the optimum condition for barley malt enzyme, pH 4.0 and 50°C.

used because of the difficulty in obtaining sufficient gut α-amylase for several assays. These findings did not agree with the study of Powers and Culberton (1983) which selected *Tenebrio molitor* as the tested insect having its α–amylase purified, characterized and studied with its interaction to wheat α-amylase inhibitors. The rate of combination for the inhibitor and amylase at 30°C and pH 5.4 (optimum for the enzyme) was calculated as a second-order rate constant of 2.7x105 per mole per second. At pH below 3.8, very rapid and irreversible loss of enzyme activity was found which was similar to the observation of the interaction of bean amylase inhibitor and porcine pancreatic α-amylase where an increase in inhibition occurred below what was considered optimal for the enzyme pH. The difference from this research work might lie in the fact that different insect and plant were used resulting in different optimal conditions for α-amylase inhibitor.

However, the results obtained should as well in agreement in α-amylase inhibitor activity as shown by Valencia *et al*. (2000) in the investigation on α-amylase of the coffee borer. The α-amylase activity had a broad pH optimum between 4.0 and 7.0. Using pH indicators, the pH of the midgut was determined to be between 4.5 and 5.2. At pH 5.0, the coffee borer α-amylase activity was inhibited substantially (80%) by relatively low levels of the amylase inhibitor (αAI-1) from the common bean, *Phaseolus vulgaris* L., and much less by the amylase inhibitor from *Amaranthus*.

Although the extracts of all varieties/ lines exhibited varying degrees of inhibitory activities against α-amylase tested, the inhibitors from both standard and mutant lines seemed to be more specific, giving higher maximum inhibition for the insect enzyme than for α-amylase of barley malt and weevil enzymes which belonged to different groups of amylase resulting in differing response to the inhibitor.

According to Bompard-Gilles *et al*. (1986), the proteinaceous enzyme inhibitors showed considerable specificity toward their target enzyme, and a protein that inhibited the activity of one α-amylase might not have the same effect on a different α-amylase. Precise molecular interactions determine whether an amylase inhibitor binds to the active site of a particular α-amylase thereby blocking its enzymatic activity.

Similar investigation to the study was conducted by Yetter *et al.*(1979) who extracted α-amylase inhibitors from five hard winter wheat varieties and assayed against larval α-amylase of both *Sitophilus oryzae* and *Tenebrio molitor*, with correlation in some varieties between *in vivo* inhibition and *in vitro* inhibition of larval α-amylase by extracted inhibitors. As probably with this mungbean amylase inhibitors, it was concluded that α-amylase inhibitor in wheat could be involved in post harvest resistance to grain insects in storage.

CONCLUSION

Effects of α-amylase inhibitor *in vivo* could be concluded that there were no significant differences in terms of larval and adult mortality of *C. maculatus* among most crude extracts of the recommended varieties, KPS1 and CN36, and the mutant lines, M5-16 and M5-29 at each concentration.

Effects of α-amylase inhibitor on activities of *C. maculatus* α-amylase *in vitro* showed the 800 and 1000 volumes of protein part of all mungbean variety/line inhibitor extracts to have 100% inhibition

Effects of α-amylase inhibitor on activities of barley α-amylase *in vitro* presented the 1000 volume of the protein part of KPS1 and CN36 inhibitor extracts to give the highest 80% inhibition.

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