Kasetsart J. (Nat. Sci.) 38 : 207 - 215 (2004)

Gamma-Irradiation Induced Alterations in Mungbean α-amylase Inhibitory Activities : Effects on α-amylase and Development of Mungbean Weevil (*Callosobruchus maculatus*)

Arunee Engkagul[1], Praparat Hormchan[2] and Arunee Wongpiyasatid[3]

ABSTRACT

Crude α-amylase inhibitors, the protein and the non-protein parts, were extracted from seeds of standard mungbean varieties KPS1 and CN36 and the mutant lines M5-16 and M5-29, derived from CN36 by gamma-irradiation. Only the protein part was found to produce substantial inhibition when tested against α-amylase from adult mungbean weevil (*Callosobruchus maculatus*) and barley malt (Type VIII-A) with 4-5 times greater percentage inhibition by the extracts from the mutants than those from the standard varieties. In the *in vivo* study, feeding larval *C. maculatus* with seeds of KPS1 and M5-29 treated with 0.5% solution of the proteinaceous inhibitor extracted from M5-16 resulted in a significant reduction in the average number of emerging adults. The effects of the two standard varieties and the two mutant lines seeds, with and without seed coats, on two parameters related to the development of the weevil ie., the average number of eggs laid and the number of emerging adults, were also investigated. No significant differences were found in the average numbers of egg laid on seeds, with and without seed coats, of all varieties/lines. However, significant reduction in the average number of emerging adults were observed in mutant seeds, with or without seed coats, as compared with the standards.

Key words: α-amylase, gamma-irradiation, *Callosobruchus maculatus*, mungbean

INTRODUCTION

Mungbean 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. Field damage to pods and grain by *Callosobruchus* spp. were reported by Raina (1971) and by Gujar and Yadav (1978). However, the field damage to pods and grain by these bruchids is only a minor problem, when the major infestation to grain occurs during storage. At present, all recommended varieties of mungbean in Thailand are known to be susceptible to these insects.

Proteinaceous enzyme inhibitors are present in multi forms in plant tissues. They have been extensively studied because of their possible function in the prevention of unwanted hydrolysis

1 Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
3 Department of Applied Radiation and Isotopes, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

Received date : 10/05/04 Accepted date : 30/06/04

and because they have a role of host plant resistance to insects (Farmer and Ryan, 1990; Gatehouse *et al.*, 1986). α-amylase inhibitors are found in many legumes and cereal including sweet potato and they are known to inhibit the α-amylases that are essential for insect development.

According to Franco *et al.* (2000), the common bean contains two allelic variants of α-amylase inhibitors called α-AI1 and α-AI2, differing in their specificity towards α-amylases. While α-AI1 inhibits porcine pancreatic α-amylase as well as α-amylase of *C. maculatus* and *C. chinensis*, α-AI2 inhibits only the α-amylase of *Zabrotes subfasciatus*.

Yetter *et al.* (1979) extracted α-amylase inhibitors from the hard winter wheat varieties and assayed against larval α-amylases of *Sitophilus oryzae* and *Tenebrio molitor*, with correlation in some varieties between *in vivo* inhibition (resistance) and *in vitro* inhibition of larval α-amylase by extracted inhibitor. Ishimoto and Kitamura (1989) later purified and identified a proteinaceous α-amylase inhibitor from kidney bean as one of the major inhibitory substances. At levels of 0.2 – 0.5%, α-amylase inhibitors were highly toxic to *C. maculatus* larvae. However, Lemos *et al*. (1990) showed that a total extract of cowpea seeds did not inhibit the development of *C. maculatus* and *Z. subfasciatus* that infested these seeds. Probably the concentrations of α-amylase inhibitors were too low to allow the inhibition of the α-amylase of such bruchid species. In the early 1990's it was definitely demonstrated that an α-amylase inhibitor and not a lectin, was indeed the factor involved in the antibiosis to *C. maculatus* (Huesing *et al.*, 1991).

By using gamma-irradiation to induce mutation of the standard varieties of mungbean, KPS1 and CN36, Wongpiyasatid *et al*. (1999) reported the evaluation of resistant lines to *C. maculatus* to find M5-16 and M5-29 to affect the development of this insects.

This paper reports the isolation of α-amylase inhibitor from control and mutant mungbean seeds, with and without seed coat, and α-amylase-from *C. maculatus.* The activities of α-amylase inhibitors were tested against α-amylase from *C. maculatus* adults, *in vitro* and *in vivo*, compared with the effect on barley malt enzyme. The development of the weevil on control and mutant seeds, with and without seed coat, was also determined.

MATERIALS AND METHODS

1. Extraction of α-amylase inhibitor, the protein and non-protein parts, from mungbean seeds

Mungbean meals of the recommended varieties, KPS1 and CN36 (the controls), and the mutant lines, M5-16 and M5-29, obtained from γ-irradiation, were extracted with 20 mM phosphate buffer saline (PBS), pH 6.7, by stirring with magnetic stirrer at 4°C for 3 hours, and centrifuged at 10,000 xg for 20 min. While discarding the undissolved precipitate, the supernatant obtained was made 80% saturated with $(NH_4)_2SO_4$ and centrifuged again at the same speed and time,after left standing at 4°C for 30 min for optimum protein precipitation, to give the supernatant (S_2) and the protein pellet. This protein pellet was dissolved in minimum volume of PBS 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 inhibitory activities against α-amylase extracted from mature *C. maculatus* adults and from barley malt (purchased from the Sigma Chemical Company). The levels of α-amylase inhibition of each variety/line were compared.

The effects of α-amylase inhibitor, the protein and non-protein parts, on α-amylases from *C. maculatus* and barley malt adults were determined by preincubating the enzyme with varying amounts of α-amylase inhibitor at room temperature for 15 minutes before 1% starch solution was added. The protein analysis followed the protein-dye binding method of Bradford (1976).

2. Extraction of α-amylase inhibitor, the protein part, from mungbean seeds without seed coats

Seeds without seed coats (cotyledon) of four varieties/lines were used for α-amylase inhibitor extraction employing the same procedure as described above. The seed coats were removed by spreading 20 g seeds of each variety/line in plastic boxes. PBS, 20 mM pH 6.7, was added into each box in sufficient volume to soak all seeds. Cleaning tissues were used to cover on top and water was sprayed on tissues. The boxes were then closed and were maintained at 4°C until the seeds were soft. The soaked seeds were gently pressed to separate the coats from the cotyledon.

3. Extraction of α-amylase inhibitor, the protein part, from mungbean seed coats

The removal of seed coats of the mutant lines M5-16 and M5-29 and the extraction steps followed the same procedure as above.

4. Preparation of α-amylase from *C. maculatus*

After 30 min freezing at –20°C, 2 grams of frozen adult weevils were finely ground in deep cold mortar with 8 ml of 20 mM PBS, pH 7.0 and centrifuged at 10,000 xg for 20 minutes at 4° C. The clear supernatant was used as crude α-amylase preparation.

5. Assays for *α-amylase* and *α-amylase* inhibitor activities *in vitro*

The activities of the crude adult amylase and barley malt amylase were measured following the method of Bernfeld (Bernfeld, 1955). The assays were performed at optimum conditions of each enzyme ie., pH 5.8, 55°C for *C. maculatus* enzyme and pH 6.9, 37°C for barley malt enzyme.

To determine the inhibitory effect of the inhibitor, the α-amylase preparation and α-amylase inhibitor were preincubated at room temperature for 15 minutes before addition of 1% soluble starch in 20 mM PBS containing 20 mM NaCl and 0.2 mM $CaCl_2$ and incubated at optimum condition for each enzyme. After 5 minutes the reducing sugar produced was determined by the method of Bernfeld (Bernfeld, 1955). The absorbance of the solution was measured at 540 nm and the α-amylase activity was expressed in μg maltose liberated per minute.

6. Effects of α-amylase inhibitor on the number of emerging *C. maculatus* adults *in vivo*

The effect of α-amylase inhibitor, the protein part, on the number of emerging weevil was examined using seeds of KPS1 and M5-29 as the medium soaking in 0.2 and 0.5% solutions of α-amylase inhibitor with distilled water as the control. The 2 solutions were made from dried powdered α-amylase inhibitor extracted from M5-16 in distilled water.

The seeds of each variety/line were soaked in distilled water and the inhibitor solutions in separate vessels. After 1 hour soaking, they were air-dried for another hour. Fifty seeds of each variety/line soaked in each solution were then put in each small plastic cup. There were 3 replications, 2 varieties/lines per replicate, 3 treatments (solutions) per each variety/line. One pair of *C. maculatus*, female and male, was introduced in each cup for oviposition. After 7 days, they were removed from the cups which were kept at room temperature. When the first weevil emerged, daily counts of emerging adult were made. Observations were discontinued 5 days after the emergence of the last adult from each treatment of variety/line.

7. Egg laying of *C. maculatus* on mungbean seeds with and without seed coat

In the experiment, the standard varieties and the mutant lines were used. One set of seeds of all varieties/lines had their seed coats removed while the others were left intact. The seed coats were removed after 3 – hour soaking in tap water by gently pressing the soaked seeds to separate the coat from the cotyledon. The seeds were allowed to dry on tissue paper. The test was conducted

under no-choice condition. Fifty seeds, with and without seed coats, were randomly selected from each variety/line and placed in the small plastic cup in four replications, 4 varieties/lines per replicate. Two pairs (female and male) of newly emerged weevil were introduced into each cup and allowed to lay eggs. The adults were removed 5 days later. The number of eggs laid on the seeds were determined using a dissecting microscope.

8. Emerging adult of *C. maculatus* on mungbean seeds with and without seed coat

The same procedure as of the egg – laying test was employed. After the first bruchid emerged, daily counts of the emerging adults were made. Observations were discontinued 5 days after the emergence of the last adult from each variety/line.

RESULTS AND DISCUSSION

Effects of α-amylase inhibitor on activities of α-amylase *in vitro*

α-amylase inhibitory activities in seed meal of four mungbean varieties/lines were tested toward *C. maculatus* amylases at optimum condition (pH 5.8 and 55°C) with the result as shown in Figure 1. Crude inhibitor extracts, the protein, from M5-16 and M5-29 were found to be more effective against *C. maculatus* α-amylase than those from KPS1 and CN36. Maximum inhibition of nearly 100% was obtained from both mutant lines while the standard varieties KPS1 and CN36 gave not more than 25% inhibition.

This was in agreement with 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.

It was also observed that the percentage inhibition of the crude protein extracts from the mutant lines M5-16 and M5-29 increased with increasing amounts of the extracts until complete inhibition was obtained whilst the percentage inhibition of the extracts from the standard varieties KPS1 and CN36 remained quite constant despite five times increase in the amount added. Similar results were obtained when the crude protein extracts were tested against barley malt enzyme at optimum condition (pH 6.9 and 37°C) (Figure 2). The inhibitory effects of all four extracts were slightly less than those seen with the insect enzyme (not more than 13% for standard varieties and 92% for mutant lines) which might reflect different affinities of the inhibitors for different isoforms of the enzyme.

The limited ability of the extracts from standard varieties to inhibit not more than about 10-25% of the total amylase activity of both the insect and barley malt enzymes, even when the amount of the inhibitor has been increased 5 times, as compared to almost complete inhibition of the extracts from the mutant lines supported the view that the structure of α-amylase inhibitor might have been altered by irradiation process. Gamma irradiation might have induced changes in the structural gene of α-amylase inhibitor resulting in the production of α-amylase inhibitor with altered specificity towards different forms of α-amylases. This was confirmed by Frylink *et al*. (1987) who stated that massive doses of ionizing radiation had been shown to induce physicochemical changes in plants such as induction of enzyme activities. Song *et al*. (2000) also put it that radiation could cause the irreversible change of protein conformation at the molecular level. Further study is required to characterize the nature of the α-amylase inhibitor molecule of the mutant, gamma irradiated lines.

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 used because of the difficulty in obtaining sufficient gut α-amylase for several assays. However, the results obtained should as well reflect α-amylase activity

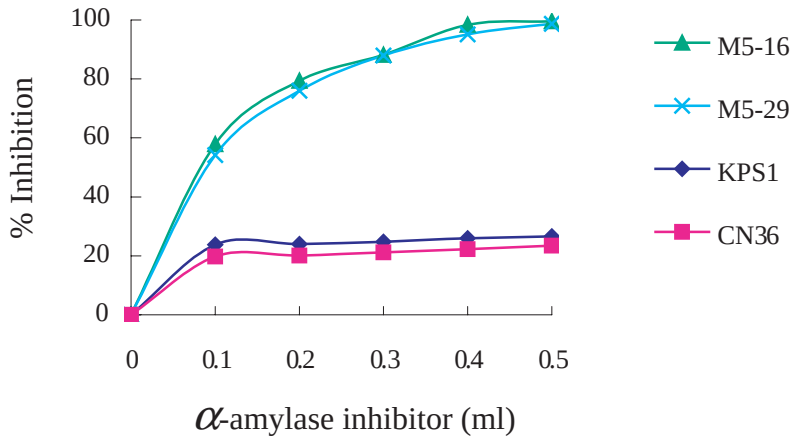

Figure 1 Percentage inhibition at different concentrations of mungbean crude protein extracts against *Callosobruchus maculatus* α-amylase. The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 5 min at the optimum condition for *C. maculatus* enzyme, pH 5.8 and 55°C.

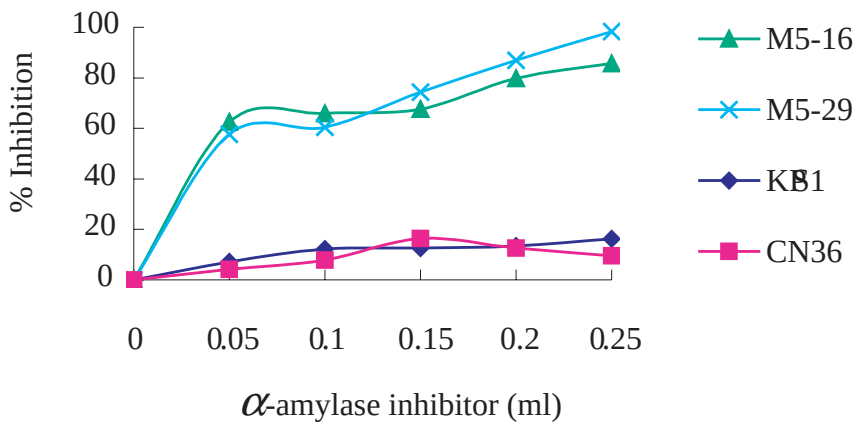

Figure 2 Percentage inhibition at different concentrations of mungbean crude protein extracts against barley malt a-amylase. The assays were performed at the optimum condition for barley malt enzyme, pH 6.9 and 37°C.

as shown by Valencia *et al*. (2000) in his investigation on α-amylase of the coffee borer. Although the extracts of all varieties/lines exhibited varying degrees of inhibitory activities against α-amylases tested, the inhibitors from both standard and mutant lines seemed to be more specific, giving higher maximum inhibition for the insect enzyme than for that of the barley malt. The fact could be due to different structures (isomer) of α-amylase between barley malt and weevil enzymes which belong to the different groups of amylase (http://www.biochem.ucl.ac.uk) resulting in differing responses 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 inhibits 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.

Since the inhibitions against both barley malt and weevil α-amylases of the non-protein part of the crude extracts from all varieties/lines were quite low (not more than 15% for *C. maculatus* enzyme and not more than 5% for barley malt enzyme), the results hence, were not presented. This could only be emphasized that the inhibitory activity of the inhibitor reside in the protein part of the extracts.

Similar results to that in Figure 1 was obtained with the α-amylase inhibitor, the protein part, extracted from the mutant lines M5-16 and M5-29 seeds without the seed coat. Maximum inhibition of more than 98% of the adult weevil α-amylase activity was observed indicating the presence of α-amylase inhibitor in the cotyledon as against the report of Lale and Makoshi (1999) suggesting the presence of biochemical factors in the seed coat affecting resistance to the bruchid attach in cowpea.

Effects of α-amylase inhibitor, the protein, on the number of emerging weevils *in vivo*

Table 1 presents the average number of *C. maculatus* emerging adults after treating seeds of KPS1 and M5-29 with 2 concentrations of α-amylase inhibitor solution, the protein, extracted from M5-16. There were no significant differences in the number of adult weevil emerging from the control seeds of both the recommended and the mutant line, and those seeds from both groups treated with 0.2% inhibitor solution. Whereas in each variety/line, while the number of emerging adults from the control and those seeds treated with 0.2% inhibitor solution were not significantly different, they were significantly different from those treated with 0.5% inhibitor solution. Treatment with higher concentration of inhibitor solution resulted in the reduction in the number of emerging adults from both variety/line tested. The results were similar with the work of Kitamura *et al.* (1990) where azuki bean meals were used as artificial medium in the feeding test of *C. maculatus*, *C. chinensis* and *Z. subfasciatus*. Increased concentrations of the inhibitor applied to the azuki bean meal resulted in a decreased number of total adults of *C. maculatus* and *C. chinensis*. Assuming that the amount of α-amylase inhibitor absorbed into KPS1 and M5-29 seeds was proportional to the concentration of the inhibitor solution, the results reflected the same effects as in the *in vitro* assay, ie. the development of *C. maculatus* decreased with increasing concentrations of the inhibitor. In other words, the increasing concentrations of α-amylase inhibitor resulted in higher percentage inhibition of α-amylase activity in the gut of *C. maculatus* thus reducing carbohydrate digestion necessary for normal

Table 1 Average number of *C. maculatus* emerging adults after seed treatment with different concentrations of α-amylase inhibitor solution.

Concentration (%)	Average number of weevil		T-value
	KPS1	M5-29	
0	32.3 ± 7.51 a[1/]	26.0 ± 22.5 a	0.487[2/]
0.2	31.7 ± 5.69 a	28.0 ± 20.3 a	0.274
0.5	11.7 ± 6.51 b	14.3 ± 5.03 b	0.561

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

[2/] None of T-value was significantly different (p = 0.05)

growth and development of the insect pest.

Egg laying and emerging adults of *C. maculatus* on mungbean seeds with and without seed coats

Table 2 and 3 show the average numbers of eggs and emerging adults from the recommended variety and the mutant line seeds with and without seed coat. It was found that the number of eggs laid on the intact seeds of CN36 was significantly higher than those on the other varieties/lines while in seeds without seed coat, KPS1 was revealed to be significantly higher than the others (Table 2). Table 3 also presents the significant difference of the emerging weevil adults in both seeds with and without seed coat between the two standard varieties and the two mutant lines. Statistical analysis, hence, indicated no significant differences between the presence or absence of seed coat for any of the 2 parameters of each variety/line except the number of eggs in CN36. This supported the *in vitro* experiment of α-amylase inhibitors against α-amylases which showed inhibition of the inhibitor mostly coming from cotyledon of M5-16 and M5-29 (data not shown). It could be explained that the seed coat might have no specific growth deterrent properties. The study was more or less similar to that of Eddie and Amatobi (2003) who reported that in the cowpea resistant and the susceptible varieties, the number of emerging adults from the decorticated and the intact seeds was not significantly different. Even though the experiment was conducted *in vivo* and no chemicals had bean identified, it still indicated the lack of substance (s) that could inhibit *C. maculatus* growth in the seed coat.

The results from Table 2 also indicated that

Table 2 Average number of eggs of *C. maculatus* on mungbean seeds with and without seed coat.

Var/Line	Number of eggs		T-value
	Seeds with seed coat	Seed without seed coat	
KPS1	16.5 ± 1.29 b[1/]	15.5 ± 1.00 a	1.22
CN36	20.5 ± 2.08 a	13.5 ± 2.06 b	4.61*
M5-16	14.5 ± 0.577 bc	13.8 ± 1.73 b	1.10
M5-29	16.0 ± 1.83 b	12 ± 2.83 b	2.38

* The difference between means is significant at p = 0.05

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

Table 3 Average emerging adults of *C. maculatus* from mungbean varieties/lines seeds with and without seed coat.

Var/Line	Emerging adults		T-value
	Seeds with seed coat	Seed without seed coat	
KPS1	20.8 ± 2.22 a[1/]	19.8 ± 1.5 a	0.747[2/]
CN36	22.2 ± 2.22 a	21.5 ± 1.73 a	0.533
M5-16	11.8 ± 2.22 bc	8.25 ± 1.71 bc	2.50
M5-29	14.5 ± 2.38 b	10.5 ± 1.29 b	2.95

1/ Means followed by the same letters in the same column were not significantly different as determined by DMRT at p = 0.05

2/ None of the T-values was significantly different (p = 0.05)

the higher level of α-amylase inhibitory activities in the mutant lines did not affect the egg laying ability of *C. maculatus* while that from Table 3 confirmed that the level of α-amylase inhibitory activities correlated inversely with growth and development of the insect.

In summary the results indicated that increasing level of α-amylase inhibitory activities in the cotyledon of the gamma-irradiated mutant lines, M5-16 and M5-29, was responsible for increased resistance to *C. maculatus* through inhibition of carbohydrate digestion necessary for normal growth and development of the insect. The nature of the mutant inhibitor molecules need be determined further. Mutant lines with high level of α-amylase inhibitory activities against specific insect species will be valuable for breeding program for improvement of resistance to specific pest.

CONCLUSION

1. α-amylase inhibitor crude extracts of M5-16 and M5-29, the mutant seeds had greater percentage inhibition against *C. maculatus* and barley malt α-amylases than those of KPS1 and CN36, the standard seeds.

2. The inhibition came from the protein rather than the non-protein parts and from the cotyledon rather than the seed coat.

3. The *in vitro* and *in vivo* tests gave supportive results to one another.

ACKNOWLEDGEMENTS

This work was supported by the research grant from the Kasetsart University Research and Development Institute (KURDI)

LITERATURE CITED

Bernfeld, P. 1955. Amylases α- and β^{-}, pp. 149 – 158. *In* S.P. Colowich and N.O. Kaplan (eds.). **Methods in Enzymology, vol 1**. Academic Press, New York.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein – dye binding. **Analyt. Biochem.** 72: 248 – 254.

Bompard – Gilles, C., P. Rousseau, P. Rouge and F. Payan. 1986. Substrate mimicry in the active center of a mammalian α-amylase from midgut of larvae of bruchid beetle *Callosobruchus maculatus* (F.) **Comp. Biochem. Physiol.** 92B: 51-57.

Eddie, P.A. and C.I. Amatobi. 2003. Seed coat has no value in protecting cowpea seed against attach by *Callosobruchus maculatus* (F.). **Journal of Stored Products Research** 39: 1 – 10.

Enzyme : Alpha-amylase. http://www.biochem.ucl.ac.uk/bsm/enzymes/ec3/ec02/ec01/ec0001/ .11oct.2003.

Farmer, E.E. and C.A. Ryan. 1990. Interplant communication : air borne methyl jasmonate induces synthesis of defense inhibitors in plant leaves. **Proc. Natl. Acad. Sci. USA** 87: 7713 – 7716.

Franco, O.L., D.J. Rigden, R.M. Francislete, C. Block Jr., C.P. Silva and M.F. Grossi de Sa. 2000. Activity of wheat α-amylase inhibitors towards bruchid α-amylase and structural explanation of observed specificities. **Eur. J. Biochem.** 267: 2166 – 2173.

Frylink, L., I.A. Dubery and J.C. Schabort. 1987. Biochemical changes involved in stress response and ripening behaviour of gamma-irradiated mango fruit. **Phytochemistry** 26: 681 – 686.

Gatehouse, A.M.R., K.A. Fenton, I. Jepson and D.J. Pavey. 1986. The effects of α-amylase inhibitors on insect storage pests : inhibitor of α-amylases *in vitro* and effects or development *in vivo*. **J. Sci. Food Agri.** 37: 727 – 734.

Gujar, G.T. and T.D. Yadav. 1978. Feeding of *Callosobruchus maculatus* (Fab.) and

Callosobruchus chinensis (Linn.) in green gram. **Indian J. Entomol.** 40: 108 – 112.

Huesing, J.E., R.E. Shade, M.J. Chrispeels and L.L. Murdoch. 1991. α-amylase inhibitor, not phytohemagglutinin, explain resistance of common bean seeds to cowpea weevil. **Plant Physiology** 96: 993 – 996.

Ishimoto, M. and K. Kitamura. 1989. Growth inhibitory effects of an α-amylase inhibitor from kidney bean *Phaseolus vulgaris* (L.), on three species of bruchids (Coleoptera : Bruchidae). **Appl Entomol. Zool.** 24: 281 – 286.

Kitamura, K., M. Ishimoto and S. Ishii. 1990. Bruchid resistance factors in *Phaseolus* and *Vigna* legumes, pp. 229 – 239. *In* K. Fujii *et al*. (eds.). **Bruchids and Legumes : Economics, Ecology and Convolution.** Kluwer Academic Publishers, Netherlands.

Lale, N.E.S. and M.S. Makoshi. 1999. Role of chemical characteristics of the seed coat in the resistance of selected cowpea varieties to *Callosobruchus maculatus* (F) (Coleoptera : Bruchidae) in Nigeria. **Int. J. of Pest Management** 46: 97-102.

Lemos, F.J.A., F.A.P. Campos, C.P. Silva and J. Xavier-Filho. 1990. Proteinases and amylases of larval midgut of *Zabrotes subfasciatus* reared on cowpea (*Vigna unguiculata*). **Entomol. Exp. Appl.** 56: 219-227.

Preston, K.R. and S.J. Symons. 1993. Measurement of heat damage in wheat by assessment of protein fibril formation. **J. Cereal. Sci.** 18: 53-59.

Raina, A.K. 1971. Observation on bruchids as field pests of pulses. **Indian J. Entomol.** 33: 194 – 197.

Song, K.B., S. Lee, S. Lee and N. Sun. 2000. Effect of γ-irradiation on the physicochemical properties of blood plasma protein. ...http://ift.contex.com/ift/202/techprogram/paper_10694.htm

Tomooka, N., C. Lairungreang, P. Nakeeraks, Y. Egawa and C. Thavaraksook. 1992. Development of bruchid resistant mungbean line using wild mungbean germplasm in Thailand. **Plant Breeding** 109: 60 – 66.

Valencia, A., A.E. Bustillo, G.E. Ossa and M.J. Chrispeels. 2000. α-Amylases of the coffee berry borer (*Hypothenesums hampei*) and their inhibition by two plant amylase inhibitors. **Insect Biochemistry and Molecular Biology** 30: 207 – 213.

Wongpiyasatid, A., S. Chotechuen, P. Hormchan and M. Srihuttagum. 1999. Evaluation of yield and resistance to powdery mildew, cercospora leaf spot and cowpea weevil in mugbean mutant lines. **Kasetsart J. (Nat. Sci.)** 33: 204-215.

Yetter, M.A., R.M. Saunders and H.P. Boles. 1979. Amylase inhibitor from wheat kernels as factors in resistance to posthavest insects. **Cereal Chem.** 56: 243 – 244.