EFFECTS OF α-AMYLASE INHIBITOR ON THE DEVELOPMENT OF *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae)

INTRODUCTION

Grain legumes, also termed as pulse crops, are major sources of dietary protein in many parts of the world, particularly in the countries situated along the tropical and subtropical belts where the availability and consumption of animal protein are rather low because of social and/or economic constraints. Pulses are much cheaper compared to meat, fish and egg and contain about 25 % protein rich in lysine and tryptophan. This makes them a good supplement to cereal and root crop based diets which are usually very low in protein and high in carbohydrates. Different grain legumes are grown in different regions because of the differential agro-climatic requirements. For example chickpea (*Cicer arietinum*), green gram or mungbean (*Vigna radiata*) are more widely grown in Asia; beans *(Phaseolus vulgaris*) in South America and East Africa whereas cowpea (*Vigna unguiculata*) is principal grain legume in West Africa and a secondary legume in parts of East Africa, Central and Southern America and Asia (Singh and Singh, 1990).

Mungbean (*V. 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 Raina (1971) and 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 occurs during storage. At present, all recommended varieties of mungbean in Thailand are known to be susceptible to these insects.

Most food crops do not contain substances toxic to insects, and if they are naturally present, they probably exist in concentration that would not significantly affect insect or man. However, the plants containing components that may be in toxic levels to insects and render

harmless to man by preparation and cooking are known in legumes or pulses. Leguminous plants, especially wild legumes, have evolved to produce antibruchid chemicals such as alkaloids, non-protein amino acids, and saponin in their seeds, all of which are shown to be detrimental to the larval growth of bruchids. However these compounds are not actually responsible for the pest resistance in the food legume seeds since they have been eliminated or reduced from the seeds because of their toxicity and tastelessness to human and animals. So far 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 they play 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* (1989) also found α-amylase inhibitors to have some detrimental effect upon larval development at concentration occurring naturally in seeds. Numbers of emerging adults 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.

OBJECTIVES

In order to study the possible effects of α-amylase inhibitor to physiology of *C. maculatus* that could be used in the resistance control strategy of this bruchid, the following objectives were set:

1. To extract and purify α-amylase inhibitor from the mutant lines and the controls of *V. radiata* seeds.
2. To compare the effects of α-amylase inhibitor, protein and non-protein extracts of the control varieties with the mutant lines on each developmental stage of *C. maculatus*.
3. To compare the effects of α-amylase inhibitor, protein and non-protein extracts of the control varieties with the mutant lines on α-amylase extracted from *C. maculatus* adult.
4. To compare the effects of α-amylase inhibitor, protein and non-protein extracts of the control varieties with the mutant lines on barley malt α-amylase (Type VIII-A).

LITERATURE REVIEWS

Several workers have described an 'active' or 'flight' form of *C. maculatus*, the cowpea or mungbean weevil which is apparently, more active and is more strongly marked, with a white pygidium. The function of this form, which appears in populations as a result of genetic and environmental factors, is not understood. Infestation can begin in the field where eggs are laid on maturing pods. As the pods dry, the pest's ability to infest them decreases. Thus dry peas stored in pods are quite resistant to attack, whereas threshed peas are susceptible to attack throughout storage (Haines, 1991).

Insect pests of bruchid and the control methods

Among the most important pests of stored grains of common beans and cowpea are the bruchids *Zabrotes subfasciatus*, the Mexican bean weevil, and the cowpea weevil *C. maculatus.* The former can infest seeds of both legume species, but *C. maculatus* does not survive on *P. vulgaris* seeds. An alternative method of reducing losses, which has the potential to overcome the drawbacks of chemical pesticides, is through development of crop varieties showing significant resistance to specific insect pests. Host plant resistance can take a variety of forms but typically, for stored grain, it prolongs or prevents larval development within the seed. Another method such as using oil and botanical chemicals, Ofuya (1986) noted that onion scales and dried chilli pepper fruits conferred some degrees of protection against *C. maculatus*. As for sealed container storage, co-storage with ash and abiotic materials, Wolfson *et al* (1991) revealed that ash storage did not provide complete protection against a buildup of cowpea bruchid, *C. maculatus*, unless the ratio of ash to grain is 3 or more parts ash to 4 parts grain. Use of resistant cultivars, seed resistance was a valuable tool against *C. maculatus* but must be carefully deployed to avoid the rapid development. Although all pods provide a mechanical barrier, which increases bruchid mortality compared to development in seeds alone, certain varieties can reduce bruchid survival on infested pods to 1 % (Kirch *et al*, 1991). Solar and other heat disinfestations technique, insects die when exposed to high temperatures because of limited physiological capacity to thermoregulate.

Cowpea bruchid eggs, larvae, and pupae do not thermoregulate and being immobile are unable to escape from a hot environment.

The search for possible targets in bruchid physiology that could be used in control strategies made an important advance in late 1970's when Gatehouse and co-workers published result suggesting that trypsin inhibitors were involved in resistance of cultivar of *V. unguiculata* of the cowpea weevil (Gatehouse *et al*, 1979). Although these results were not verified by other workers (Xavier-Filho *et al*, 1989: Zhu *et al*, 1994), the study of Gatehouse and co-workers stimulated research on digestive proteinases in bruchid beetle, leading to a greater understanding of these enzymes (Kitch and Murdock, 1986).

Enzyme

An enzyme is a protein that allows the digest to distribute throughout the entire body. Enzymes are catalysts that allow biochemical changes to occur in any biological system. An enzyme speeds up a specific reaction. In the most general sense, a chemical reaction proceeds by 1) collision of the reactants; 2) reorganization of bonds (orbital electron) into an "activated intermediate state" at a higher energy level; and 3) decay (further rearrangement of orbital electron) to the final products.

The seed is a remarkable structure that enables seed plants to survive unfavorable conditions. Each seed contains an embryo that can grow into a mature plant. In addition, the seeds of flowering plants have structures called cotyledons. The cotyledons store food for use by the embryo in the form of starch. Starch is long chains of glucose molecules. The embryo needs to break these chains, forming sugars it can use for providing energy. It does this by releasing the enzymes α-amylase and α-amylase. Cereal α-amylases are enzymes that cleave the α-(1→4) D-glucosidic linkages in starch components. The cleavage is believed to be restricted by terminal or α-(1→6) interchain linkages. It keeps dividing the chains until they are one, two, or three glucose molecules long. Glucose being the smallest molecule, maltose is a chain of two glucose

molecules, and maltriose is a chain of three glucose molecules. β-amylase works by nibbling at the ends of the starch chains to make them into chains of one, two, or three glucose molecules.

Digestive enzyme of *Callosobruchus maculatus*

Amylase

Amylases are glycosidases that catalyze the hydrolysis of α-D-1, 4-glucosidic linkages of starch, glycogen and related α-D-1-4 glucan consisting of two types of polymers, amylose and amylopectin. Amylases catalyze hydrolysis of amylose to disaccharides and monosaccharides (maltose and glucose). The enzymes requiring calcium as a cofactor are stimulated by chloride, bromide and fluoride and are inhibited by cadmium, copper, zinc and lead. The optimum pH range is 6.5-8.0. Amylases are classified into two groups according to site of hydrolysis:

1. Endoamylase is an α-amylase (EC 3.2.1.1, α-1, 4-D-glucanohydrolase) which hydrolyzes the α-1-4 glucosidic linkages in polysaccharides apparently in random manner. It attacks linkages in the middle or in the interior of large molecules. Salivary amylase and pancreatic amylase are endoamylases found in several animals.

2. Exoamylase hydrolyzes the α-1-4-glucosidic linkage at the non-reducing end of polysaccharides such as starch. There are two types of exoamylases.

A. β-amylase (EC 3.2.1.2, α-1, 4-D-glucan maltohydrolase) seems to occur only in higher plant tissues such as barley malt, wheat, sweet potatoes and soybeans. The enzyme removes maltose units from the non-reducing end of the polysaccharide chain by breaking alternate glycosidic linkages to maltose in β-configuration. The enzyme does not require calcium for activity.

B. γ-amylase (EC 3.2.1.3, α-1, 4-D-glucan glycohydrolase) is a microbial enzyme found only in molds, yeast and bacteria. The enzyme hydrolyzes both α-D 1, 4 and α-1, 6-glucoasidic linkages at the branch point and removes glucose units from the non-reducing end of the substrate. The end product is exclusively glucose in the β-configuration (Wong, 1995; Reed, 1966).

Insect α -amylases

Several insects, especially those similar to the seed weevils that feed on starchy seeds during larval and/or adult stages, depend on their α-amylases for survival. Research on starch digestion as a target for control of starch-dependent insects has been stimulated in recent years after results showing that α-amylase inhibitors from *P. vulgaris* seeds are detrimental to the development of cowpea weevil *C. maculatus* and Azuki bean weevils *C. chinensis* (Ishimoto and Kitamura, 1989; Shade *et al*, 1994).

The carbohydrate digestion of bruchid weevils, such as the Mexican bean weevil, *Z. subfasciatus* and the cowpea weevil *C. maculatus*, occurs mainly in the lumen of the midgut. High enzymatic activities against starch, maltose, maltodextrins and galactosyl oligosaccharides were found in the luminal fluid, while only aminopeptidase activity was predominantly associated with gut membrane (Silva *et al*, 1999).

To validate insect α-amylases as targets for crop protection, it is important to research their varieties and understand how the expression of different forms is controlled. Studies in this area are at an early stage, although some important observations have been made. The presence of different forms of α-amylases in the insect midgut lumen has been observed in *C. maculatus* and Z. *subfasciatus*. Patterns of α-amylase expression vary in *Z. subfasciatus* fed on different diets, apparently in response to the presence of antimetabolic proteins such as α-amylase inhibitors, rather than as a response to structural differences in the starch granules. Bean bruchids, such as the Mexican bean weevil larvae, also have the ability to modulate the concentration of glucosidases and α-amylases when reared on different diets.

Enzyme inhibitor

Just as it is important for enzymes to catalyze biological reactions, so is the ability to control and regulate enzymatic activity. This is the role of small, specific molecules and ions known as enzyme inhibitors. Inhibitors are often molecules that are similar in shape to a certain

substrate and can thus fit the active site of the enzyme that was intended to fit the substrate. Once the inhibitor occupies the active site, however, it does not act to catalyze the reaction as the enzyme would. Instead, it binds up the active site and does not allow any activity there; thus, the reaction is inhibited.

An enzyme inhibitor is an ingredient found in all grains, seeds, tree nuts, and beans and these stop enzyme activity from happening. Their main purpose is to preserve these foods until the right condition exists where they can now grow into a parent plant. It is nature's way of preserving the life force for the purpose of future plant reproduction. These enzyme inhibitors are waiting for the right signal when the seed may start growing.

The enzyme inhibitors act on key insect gut digestive hydrolases, the α-amylases and proteinases. Several kinds of α-amylase and proteinase inhibitors, present in the seeds and vegetative organs, act to regulate numbers of phytophagus insects. α-amylase inhibitors are attractive candidates for the control of seed weevils as insects are highly dependent on starch as an energy source (Chrispeels *et al*, 1998).

Alpha-amylase inhibitor

Non-proteinaceous inhibitors

The class of non-proteinaceous inhibitors contains diverse types of organic compounds such as acarbose, isoacarbose, acarviosine-glucose, hibiscus acid and the cyclodextrins. The two hibiscus acid forms, purified from Roselle tea (*Hibiscus sabdariffa*), the acarviosine-glucose, the isoacarbose and α-, β- and γ-cyclodextrins are highly active against porcine and human pancreatic α-amylase (PPA and HPA). The inhibitory activity of these compounds against α-amylases is due in part to their cyclic structures, which resemble α-amylase substrates and therefore bind to α-amylase catalytic sites.

Proteinaceous inhibitors

Proteinaceous α-amylase inhibitors are found in microorganisms, plants and animals. In plants, proteinaceous inhibitors are mainly present in cereals such as wheat (*Triticum aestivum)*, barley (*Hordeum vulgareum)*, sorghum (*Sorghum bicolor)*, rye (*Secale cereale)* and rice (*Oryza sativa)* and also in leguminosae such as pigeonpea (*Cajanus cajan)*, cowpea (*Vigna unguiculata)* and bean (*P. vulgaris)*. Different plant α-amylase inhibitors exhibit different specificities against α-amylases from diverse sources. Determination of specificity of inhibition is the important first step towards the discovery of an inhibitor that could be useful for generating insect-resistant transgenic plants. In some cases, the α-amylase inhibitors act only against mammalian α-amylases or, on the contrary, just against insect α-amylases. In the latter case, this provides a highly specific potential weapon in plant defense. α-AI 1, α-AI 2 and some wheat inhibitors are among those naturally possessing favorable inhibition profiles. However, in general, α-amylase inhibitors inhibit several α-amylases from different sources. In these cases, an improved understanding of the structural bases for inhibition profiles is needed.

A number of substances capable of reducing the activity of one or more α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) of different origins have been described in the literature. These substances include drugs, several polyanions, and some end products of α-amylase action on starch, and low molecular-weight compounds or macromolecules either produced by microorganisms or occurring naturally in plants. For example, aflatoxin B is another type of α-amylase inhibitor of microbial origin (Uwaifo, 1980).

The favored hypothesis about physiological roles of the enzyme inhibitor in seeds is that they act as storage or reserved proteins, as regulators on endogeneous enzyme or as defensive agents against the attacks of animal predators or an insect or microbial pest. It seems likely that in certain species, these proteins may fulfill a combination of these functins (Octavio and Rigdon, 2002; Octavio and Rigdon, 2000; Richardson, 1991). Also plant α-amylase inhibition shows great potentials as tools to engineer resistance of crop plant against pests (Octavio and Rihdon, 2000)

α-Amylase inhibitors that occur naturally in plants include low-molecular-weight compounds such as salicylic acid and abscisic acid (Hemberg, 1967, 1975) and high-molecular-weight substances such as enzyme activators and protein inhibitors. Several short reviews focusing particularly on α-amylase inhibitors from beans and wheat have been published (Marshall, 1975).

The common bean contains two allelic variants of α-amylase inhibitors call α-AI 1 and α-AI 2, differing in their specificity towards α-amylases. While α-AI 1 inhibits porcine pancreatic α-amylase (PPA) as well as the α-amylases of the cowpea weevil, *C.maculatus* and the azuki bean weevil, *C.chinensis*, α-AI 2 inhibits only the α-amylase of the Mexican bean weevil, *Zabrotes subfasciatus.* None of the inhibitors present in bean seeds have any effect against the α-amylase of the bean weevil, *Acanthoscelides obtectus.*

Seeds with α-amylase inhibitor (α-AI) in several cultivars of the common beans play a protective role against bruchid pests. The α-AI strongly inhibited the larval midgut α-amylase activities of *C.chinensis* and *C. maculatus* and non pest species of the common beans. Bean α-AI 1 in transgenic peas provided complete protection from pea weevil (*Bruchus pisorum*) under field conditions (Morton *et al*, 2000). Earlier work reported that the presence of α-AI is one among the possible strong factors for bruchid resistance in the wild relatives of mungbean, *Vigna sublobata* (Sahu, 1996).

Jaffé *et al* (1973) found α-amylase inhibitors in 79 of 95 legume cultivars tested. The greatest inhibitory activity was found in kidney beans. Lima beans (*Phaseolus lunatus*), runner beans (*P. coccineus*), wild beans (*P. arborigineus*), mungbeans (*P. aureus*) displayed moderate amylase inhibitor activity. Lentils (*Lens culinaris*), cowpeas (*Vigna sinensis*) exhibited very low inhibitory activity. According to Powers and Whitaker (1977), red kidney beans contained more amylase inhibitor than California white beans or cowpeas, whereas garbanzo beans and Wistan and Wesley lima beans did not contain inhibitors. α-Amylase-inhibiting activity was absent in black gram (*P. mungo*) seeds of two different origins (India and Thailand). Singh *et al* (1982) also studied α-amylase inhibitors in chickpeas.

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 from *P. vulgaris* seeds were detrimental to the development of *C. maculatus* and the azuki bean weevil, *C. chinensis* (Ishimoto and Kitamura, 1989), in spite of earlier suggestions that lectins from *P. vulgaris* were the factor active against *C. maculatus* (Janzen *et al*, 1976; Gatehouse *et al*, 1984). They found this inhibitor to be extremely toxic to the larvae, all of which died before the second instar when fed artificial bean containing 0.2-0.5 % of the protein. In the earlier 1990's it was definitively 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). Birch *et al* (1989) also found a commercially available preparation of *P. vulgaris* α-amylase inhibitors to have some detrimental effect upon larval development at concentration occurring naturally in seeds. The numbers of emerging adults of a non-pest species, *C. maculatus*, were reduced by 30 % whilst those of pest species, *Z. subfasciatus*, were reduced by 10 %.

Crude protein extracts from the seeds of ten *Vigna* genotypes were assayed for inhibitory activity against the larval amylase of *Callosobruchus analis. V. umbellata, V. Sublobata* and *V. glabracens* showed high levels of inhibitory activity while the others showed moderate to low inhibitory activities. *Vigna radiata* var CO5 had protein content of 15.1 units/g and the inhibitory activity of 12.01. Intervarietal variation in the content of α-AIs in seeds is not uncommon and has been reported in crops such as cowpea (Prasad *et al,* 1996) and barley (Jarret *et al,* 1997). The α-AI content in chickpeas varieties ranged from 11.6-84.4 units/g seed (Mullimani *et al,* 1994)

Knowledge of legume seed defense protein has progressed significantly in the last few decades. However the same is not true of the digestive process of bruchid, in which α-amylase and proteinases have been far more intensively investigated than enzyme involved in the intermediate and final step of the digestive process. Only crude midgut preparations were used as enzyme sources in the studies of α-amylase inhibitors.

Other chemical factors affecting bruchid development

Insect attack on mature seeds of legumes is primarily limited to a specialized family of insects, the Bruchidae, and differences within this family show varying degrees of specialization with respect to host species. In considering the biochemical defenses employed in legume seeds, two levels of resistance mechanisms can be identified. First, general defensive substances are present conferring protection against the non-pest species, and, secondly, there are targeted resistance mechanisms, often showing marked varietal differences within a host species, which give resistance to the host's specific pests. The former constitutes by far the largest category of resistance mechanisms so far investigated (Gatehouse *et al*, 1990).

The mechanism underlying the growth inhibition seems likely to be ascribed to the direct inhibition of starch digestion by the inhibitor causing a large reduction in carbohydrate assimilation in the larvae. Most larval digestive enzyme activities were found in the luminal contents. Activities against starch, maltose and maltodextrins were found to show the highest level of activities followed by enzymes active against galactosyl oligosaccharides. The data suggested that the majority of carbohydrate digestion occurred in the midgut lumen, whereas protein digestion should take place partly in the lumen and partly at the cell surface (Silva *et al*, 1999). The resistant lines are characterized by delayed, staggered, and slow adult emergence while in susceptible lines like Ife Brown, the adult emergence is relatively early and extremely rapid. Thus, the resistant lines are not immuned to bruchids but suffer considerably less damage compared to the susceptible lines (Singh *et al*, 1985).

Seed cotyledon attributes of a chemical nature may affect insect development; these include enzyme inhibitors and other antimetabolites. Proteinase inhibitors (PIs) are major constituents of seeds and are considered likely to have a role in defense because they are present at levels far higher than necessary for intracellular proteolysis. PIs are subdivided into proteinases (endo-peptidases) and exo-peptidases (Jongsma and Bolter, 1997). It has been shown that the midgut of *C. maculatus* contains a thiol-dependant proteinase (Barlett, 1986; Kitch and Murdock, 1986) and that the activity of this proteinase was powerfully inhibited by natural occurring and

synthetic specific cysteine proteinase inhibitors (CPIs) (Gatehouse *et al*, 1979; Xavier-Filho *et al*, 1989). Levels of PIs in legume seeds attacked by *C. maculatus* ranged from 610 to 13,000 µm, and caused 55–100 % mortality in the pest (Jongsma and Bolter, 1997). It is evident, however, that populations of *C. maculatus* are variable in terms of virulence, and so host plant resistance may not on its own provide a sustainable means of defense against the bruchid (Shade *et al*, 1999).

Gatehouse *et al* (1979) reported a higher level of trypsin inhibitor in Tvu 2027 compared to the susceptible varities and attributed the bruchid resistance in cowpea to this factor. They also showed that trypsin inhibitor isolated from cowpea and mixed in ground cotyledons of a susceptible cowpea variety Tvu 57 reduced the survival of the bruchid eggs. Osborn *et al* (1988) identified 'arcelin', a major seed in wild *P. vulgaris* as the factor responsible for resistance to bean bruchid *Z. subfasciatus.* Similarly, para-aminophenylalanine in several wild *Vigna* species was shown to be toxic to *Z. subfasciatus* as well as to *C. maculatus* (Birch *et al*, 1986). Ishimoto and Kitamura (1988) showed that a water-soluble substance present in kidney beans strongly inhibits the larval growth of *C. chinensis.* All these indicated a chemical factor to be responsible for bruchid resistance.

Chemicals in seed and seed testa

Chemical factors in cowpea testa and cotyledons are known to have antixenotic and antibiotic effects on egg and developing larvae and in most cases these lead to impaired larval eclosion, high larval mortality and prolonged development (Janzen, 1977; Gatehouse and Boulter, 1983). It is now well established that resistance of these cowpea varieties is attributable to the physical and/or chemical characteristics of the pod, seed coat or cotyledons.

Although various studies have been carried out indicating the presence of toxic or antifeedant chemicals in the testa of certain leguminous seeds (Janzen, 1977; Birch *et al*, 1989) this aspect is only considered in brief. Despite the tissue itself having been shown to be toxic, their role as an effective defense mechanism remains unproven since many bruchid larvae are

thought to tunnel through the intact testa without actually ingesting any tissue (Southgate, 1984). Stamopoulous and Huignard (1980) demonstrated that when milled testa from the seeds of *P. vulgaris* was incorporated into a diet at a level of 10 % and fed to *Acanthoscelides obtectus*, larval mortality of 98 % occurred; they subsequently demonstrated toxicity of the lignin fraction towards these larvae. In certain legume species, such as *Vicia faba* (Griffiths, 1981) there is evidence for the localized accumulation of polyphenolic compounds, including condensed tannins, in the seed testa. It shows that the condensed tannins from these seeds have an adverse effect upon the development of *C. maculatus* (Boughdad *et al*, 1986). Lale and Makoshi (2000) also reported that the resistance observed in some selected cowpea varieties combined antixenosis and antibiosis in the seed coat manifested in reduced oviposition and egg-hatch with antibiosis in the cotyledons manifested in prolongation of larvae development and high larval mortality. The analysis provided strong support for the conferment of resistance against bruchid infestation by chemical factors contained in the seed coats of cowpea.

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 the mutant lines, M5-16 and M5-29 were obtained from Mungbean Varietal Screening for Diseases 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 development was examined using seeds of KPS1 as the medium soaking with α–amylase inhibitor, protein and non-protein parts 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 hour, the adults were removed and the dishes 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 cowpea 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 (Fasman, 1984), 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).

6. Place and duration

The studies were conducted in the laboratories at Department of Entomology, Faculty of Agriculture and Department of Biochemistry, Faculty of Science, Kasetsart University.

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) is shown in Appendix Figure 1. Amount of protein (g) in protein and non-protein parts obtained from 30 g of the recommended (KPS1 and CN36) and mutant lines (M5-16 and M5-29) seeds are shown in Table1.

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

Extracts from four mungbean variety/line seeds	Protein Extract (g)	
	protein part	non-protein part
KPS1	0.87	0.10
CN36	0.86	0.00
M5-16	0.90	0.04
M5-29	0.62	0.01

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 and Kitamura, 1991; Ishimoto *et al*, 1995). Kokiladevi *et al.* (2005) 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. It could also be

seen that the amount of protein in the protein parts of all extracts were higher than non-protein parts. This was due to the fact that in precipitation at 80 % NHSO4, the protein part was quite completely eliminated. Even though the non-protein part was devoid of protein, it might contain diverse types of organic compounds such as acarbose, isoacarbose, hibiscus acid, etc. which likely affected the development of the insects.

2. Effects of α-amylase inhibitor, protein and non-protein parts on the development of *C. maculatus*

Egg laying and emerging adults of *C. maculatus* on seeds of KPS1

Average number of eggs

ANOVA analysis showed no interaction in the number of eggs on KPS1 seeds treated with protein and non protein parts of extract among means of extracts at each concentration or those of each extract at various concentrations.

Table 2 presents the average number of eggs after treating KPS1 seeds with distilled water and the 4 protein concentrations (0.2-1.0 % (w/w)) of α-amylase inhibitor extracted from the recommended (KPS1 and CN36) and mutant variety/line (M5-16 and M5-29) seeds. In comparison among each protein extract at different concentrations, there were no significant differences found between the average number of eggs in seeds treated with distilled water and the 4 concentrations of extracts. No significant differences in the number of egg-laying were noticed among extracts as well.

At each protein concentration of each extract, significant difference in the amount of eggs was also not observed between seeds treated with distilled water and those treated with the other extracts. The egg numbers of eggs from seeds treated with various extracts at each concentration were also not significantly different from one another.

The average number of eggs after treating KPS1 seeds with distilled water and the 4 concentrations of non-protein extracts from the recommended and mutant variety/line seeds are shown in Table 3. Similar results to those of the protein part experiment were also observed in both seeds treated with extracts of all variety/line seeds at each concentration as well as those treated with various concentrations of each extract.

Table 2 Average number of eggs of *C. maculatus* on KPS1 seeds treated with distilled water and protein part extracts of four mungbean variety/line seeds

KPS1 seeds treated with extracts from four mungbean variety/line seeds	**Number of eggs** [1/2/]				
	% Protein (w/w)				
	0	**0.2**	**0.4**	**0.6**	**1.0**
Control	41.5	41.5a	41.5a	41.5a	41.5a
KPS1	41.5 A	36.0a A	37.0a A	49.3a A	44.3a A
CN36	41.5 A	41.0a A	36.0a A	38.7a A	31.3a A
M5-16	41.5 A	39.7a A	37.0a A	38.0a A	51.0a A
M5-29	41.5 A	58.0a A	41.0a A	44.7a A	46.0a A

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

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

Table 3 Average number of eggs of *C.maculatus* on KPS1 seeds treated with distilled water and non-protein part extracts of four mungbean variety/line seeds.

KPS1 seeds treated with extracts from four mungbean variety/line seeds	**Number of eggs** [1/2/]				
	% Non-protein (w/w)				
	0	**0.2**	**0.4**	**0.6**	**1.0**
Control	41.5	41.5a	41.5a	41.5a	41.5a
KPS1	41.5 A	48.3a A	41.7a A	34.0a A	42.3a A
CN36	41.5 A	36.3a A	47.0a A	46.7a A	36.3a A
M5-16	41.5 A	44.0a A	42.0a A	36.0a A	31.0a A
M5-29	41.5 A	37.3a A	48.0a A	24.7a A	51.0a A

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

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

According to the results in Tables 2 and 3, the non significant difference in the number of eggs between KPS1 seeds treated with distilled water and the extracts of all varieties/lines, both protein and non-protein parts, indicated either the same response to all chemicals or to KPS1 seed characters affecting egg-laying of the weevil.. As for the chemicals, Singer (1992) reported that oviposition was stimulated by chemical cues on the seed surface and discrimination between host species was mediated by sensory receptors on the maxillary palps. Resistance of certain cultivar of cowpea to *C. maculatus* did not seem to be dependent on the levels of proteinase inhibitors or on tannin content. In this work, it was clear that the chemicals contained in all vaiety/line seed extracts, protein and non-protein parts, were not oviposition-deterrent and/or ovicidal against *C. maculatus* since the numbers of eggs laid at every concentration were not significantly different from one another.

Generally, the cowpea weevil prefers seed types with a smooth testa over rough ones to oviposition. A smooth testa allows a better attachment of the eggs to seed, resulting in a higher chance of successful development (Nwanze and Horber, 1976). Color preference is ambiguous but in no-choice situations, no differences were found (Shazali, 1990). In addition to the effect of preferred host seeds, the other specific stimuli, such as the seed's surface curvature, chemical constituents of seed coat, oviposition marking substance and seed size can all serve associating reinforces and therefore influence the response (Szentesi and Jermy, 1990). Wasserman (1986) also showed the female *C. maculatus* preference for large and smooth seeds in ovipositiion.

Following the works of these authors, it might be concluded that in this study where there were no significant differences among the extracts or among the various concentrations in the number of eggs laid, therefore, the weevils might lay eggs on KPS1 seeds owing to a no-choice test rather than the specific preference to the characters of KPS1 seeds or the attraction from the chemicals of the extracts. Further investigation should then be conducted in preference test of egg-laying on mungbean seeds of different varieties.

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 4 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 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).

ANOVA analysis in percent larval mortality from KPS1 seeds treated with non-protein parts of extract also showed no interaction among means of extract at each concentration or among those of each extract at various concentrations.

Percentages of larval mortality from KPS1 seeds treated with distilled water (0 % non-protein (w/w)) and the 4 concentrations of non-protein α-amylase inhibitor extracted from the recommended and mutant variety/line seeds are shown in Table 5. There were no significant differences in percent larval mortality found between the control and seeds treated with various concentrations of the 4 non-protein concentrations from extracts of the recommended and mutant

variety/line seeds, both among extracts at each concentration and among different concentrations of each extract.

Table 4 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 letter in the same column are not significantly different as determined by DMRT at p = 0.05

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

Table 5 Percent larval mortality of *C. maculatus* on KPS1 seeds treated with distilled water and non-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/]				
	% Non-protein part (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 A	0.0a A	1.5a A	0.7a A	0.8a A
CN36	0.0 A	0.0a A	0.7a A	2.0a A	1.8a A
M5-16	0.0 A	0.0a A	0.8a A	0.0a A	0.9a A
M5-29	0.0 A	1.9a A	2.0a A	0.0a A	2.2a A

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

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

Percent pupal mortality

From the ANOVA in percent pupal mortality of KPS1 seeds treated with protein parts of extract, statistical interaction was found among means of extract at some concentrations while there was no interaction among concentrations of each extract. The same results were shown in the non protein parts.

Table 6 presents percent mortality of pupae from KPS1 seeds treated with distilled water and 4 protein concentrations of α-amylase inhibitor extracted from the recommended and mutant variety/line seeds. Significant differences in percent pupal mortality were found between seeds treated with extracts of all variety/line seeds at 1.0 % protein (w/w) but not from one another. At 1.0 % protein (w/w) protein, pupal mortality percentages from seeds treated with extracts of M5-16 and M5-29 seeds were noticed to be significantly higher than that of CN36 seeds whereas all were significantly different from the control.

At for the extracts at different protein concentrations, no significant differences were noticed in percent of pupal mortality between seeds treated with distilled water and extracts of all variety/line seeds at each concentration.

Table 7 presents the percent pupal mortalities from KPS1 seeds treated with distilled water and the 4 concentrations of non-protein α-amylase extracted from the recommended and mutant variety/line seeds. Among extracts at each concentration, percent pupal mortality from the extract of M5-16 seeds was observed to be significantly different from that of KPS1 and M5-29 seeds at 0.6 % non-protein (w/w). At the 1.0 % non-protein (w/w) concentration, while seeds treated with extract from of M5-16 seeds showed percentage of pupal mortality not to be significantly different from that of KPS1 seeds, it was found to be significantly different from those of the others.

At each extract at various non-protein concentrations, no significant differences were noticed in percentage of pupal mortality between seeds treated with distilled water and extracts of all variety/line seeds at each concentration.

Table 6 Percent pupal 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 pupal mortality** [1/2/]				
	% Protein (w/w)				
	0	**0.2**	**0.4**	**0.6**	**1.0**
Control	0.0	0.0a	0.0a	0.0a	0.0 a
KPS1	0.0 A	2.0a A	17.3a A	15.3a A	16.6abA
CN36	0.0 A	12.4a A	11.4a A	12.2a A	9.2 bA
M5-16	0.0 A	14.2a A	6.5a A	10.2a A	23.2abA
M5-29	0.0 A	12.3a A	17.0a A	15.8a A	29.9a A

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

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

Table 7 Percent pupal mortality of *C. maculatus* on KPS1 seeds treated with distilled water and non-protein parts extracted from four mungbean variety/line seeds

KPS1 seeds treated with extracts from four mungbean variety/line seeds	**Percent pupal mortaliy** [1/2/]				
	% Non-protein (w/w)				
	0	**0.2**	**0.4**	**0.6**	**1.0**
Control	0.0	0.0 a	0.0 a	0.0 b	0.0 b
KPS1	0.0 A	20.0a A	18.7a A	12.5 bA	16.6 abA
CN36	0.0 A	16.2a A	16.3a A	21.6abA	7.7 bA
M5-16	0.0 A	6.20a A	18.2a A	31.1a A	26.3 a A
M5-29	0.0 A	13.4a A	17.9a A	7.1 bA	9.6 bA

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

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

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. Similar results were expressed in the non protein part of extracts.

Table 8 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 were 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 percentage 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.

Adult mortality percentages from KPS1 seeds treated with distilled water and the 4 concentrations of non-protein extracts of α-amylase inhibitor from the recommended and mutant variety/line seeds are presented in Table 9. Among extracts at each non-protein concentration, percent adult mortality of seeds treated with extracts of KPS1 and M5-16 significantly differed from that of the control at 0.2 % protein (w/w). It was also observed that at 0.2 % protein (w/w) protein, seeds treated with extract of KPS1 and M5-16 seeds had percentages of dead adult not significantly different from those of CN36 and M5-29 respectively. Percent adult mortality of both varieties was found to be significantly different from the two lines at the same concentration. Similar results to those of the protein parts at 0.4, 0.6 and 1 % protein (w/w) where adult mortality percentages of all variety/line extracts that gave significant differences from that at 0 % protein (w/w) protein were obtained.

At each non-protein concentration of each extract, it was found that at each non-protein concentration of every variety/lines extract, percent adult mortality of the treated seeds significantly differed from that of the control. The results were also in resemblance with the protein parts.

Table 8 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 adult 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 letter in the same column are not significantly different as determined by DMRT at p = 0.05

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

Table 9 Percentages of adult mortality of *C. maculatus* from KPS1 seeds treated with distilled water and non-protein parts extracted from four mungbean variety/line seeds

KPS1 seeds treated with extracts from four mungbean variety/line seeds	**Percent adult mortality** [1/2/]				
	% Non-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	52.7a A	48.9a A	40.5a A	28.2a A
CN36	0.0 B	43.4ab A	44.5a A	36.6a A	27.5a A
M5-16	0.0 B	19.3 cA	39.4a A	34.2a A	33.4a A
M5-29	0.0 B	27.9 bcA	37.5a A	25.2a A	33.9a A

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

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

In the preliminary of *in vivo* test, the extraction of both protein and non-protein α-amylase inhibitors of all variety/line seeds including the bioassay on *C. maculatus* developmental stages was as well conducted. However, the acquired results from all extracts at every concentration and each extract at various concentrations were not in consistency which was difficult to give the explanation. Perhaps it could be because the weevils fed only little on seed coats, just to get through the cotyledon; or so little time was spent in soaking resulting in too small amount of extracts absorbed, which was never proved in this study.

However, Griffiths (1981) showed that there was evidence for the localized accumulation of polyphenolic compounds including condensed tannins in the seed testa of some legume species and that condensed tannins from these seeds had an adverse effect upon the development of *C. maculatus* (Boughdad *et al*, 1986). Lale and Makoshi (1999) also suggested the presence of biochemical factor in the seed coat affecting resistance to the bruchid, *C maculatus* in cowpea. The egg-hatch was significantly reduced in seeds with intact seed coats by 88.6 %, while the proportion of eggs that failed to hatch in de-coated seeds was 31.9 %. Treatment of Borno brown seeds especially with 32 and 64 mg of extracts from Kanannado and IT89KD-391 seed coats reduced oviposition by 61.9 % and 95.2 %, respectively. Identical dosages (32 and 64 mg) of these seed coat extracts also significantly reduced susceptibility of Borno brown to *C. maculatus* (SI values 6.7 and 1.5, respectively). Comparable SI values for Borno brown treated with 16 mg of the seed coat extracts or extract-free acetone were 14.9 and 14.0, respectively.

On the contrary, according to the report of Engkakul *et al.* (2004), the statistical analysis showed no significant difference between the presence or absence of seed coat of KPS1, CN36, M5-16 and M5-29 in affecting the number of eggs laid or adult emergence. Eddie and Amatobi (2003) also found that in the cowpea resistant and the susceptible varieties, the number of emerging adults from the decorticated and the intact seeds were not significantly different. Similarly, Kashiwaba *et al*. (2003) reported the rice bean with seed coats removed showed complete resistance to *C. maculatus, C chinensis and C. analis.* Results indicated that physical attributes and/or chemical(s) present in the seed coat of rice bean were not the main factors responsible for resistance.

Yet, no work in identifying chemicals in *V. radiata* has so far been known, hence, *in vivo* and *in vitro* tests of extracts from seeds without seed coats on the development of *C. maculatus* would not be reported here owing to the mentioned explanation.

It could be seen according to the results that most protein α-amylase inhibitor extracts of all varieties/lines had detrimental effects on larval, pupal 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. This was more or less in agreement with this study. With less concentration used, different legume species and different insect tested, hence, difference in percent larval mortality was obtained. The similar part might be the same detrimental effect on the larval development.

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 decrease 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.* (2003), 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.

According to these findings whose results were similar to this research study, it was obvious that α-amylase inhibitor gave inhibitory effects to development of the mungbean weevils although different approaches and data collected were employed.

The results obtained should as well reflect the non-protein part as shown by Janzen *et al.* (1976) in the investigation on the non-protein amino acids which was more toxic than protein amino acids. The latter could be toxic at 1 and 5 % incorporation in the diet. A variety of other secondary compounds found in seeds were toxic at various level representatives. At those levels found in seeds in nature, and for all secondary compounds tested, at 0.1 – 5 % in corporation in the diet, a detrimental effect on production of adult beetles was encountered. This was also more or less in similarity to the study. Because of less concentrations were used in the experiment, the results were not the same. Even though at most concentrations of each mungbean variety/line extract, no effect was observed on inhibitory activity on most developmental stages; yet, at higher concentration of some extracts, the detrimental effects could still be seen as in the adult mortality. Further investigation should then be carried out using the higher concentrations as employed by these authors.

The above reports partly supported these acquired results of α-amylase inhibitor extract of all mungbean variety/line effects on the *C. maculatus* developmental stages since there were also other chemicals conferring physiological effects on the weevils as reviewed in the Literature Review. The difference in extract concentration might also cause various degrees of detrimental effects to different *C. maculatus* stages.

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 (Appendix Figure 2). 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) with the results as shown in Appendix Figures 2 and 3. From crude inhibitor extracts, the protein parts of four mungbean varieties/lines were found to be more effective than the non-protein parts against *C. maculates* α-amylase. Maximum inhibition of 100 % was obtained from protein parts of four mungbean varieties/lines while the non-protein part gave no more than 10 % inhibition (Figures 1 and 2).

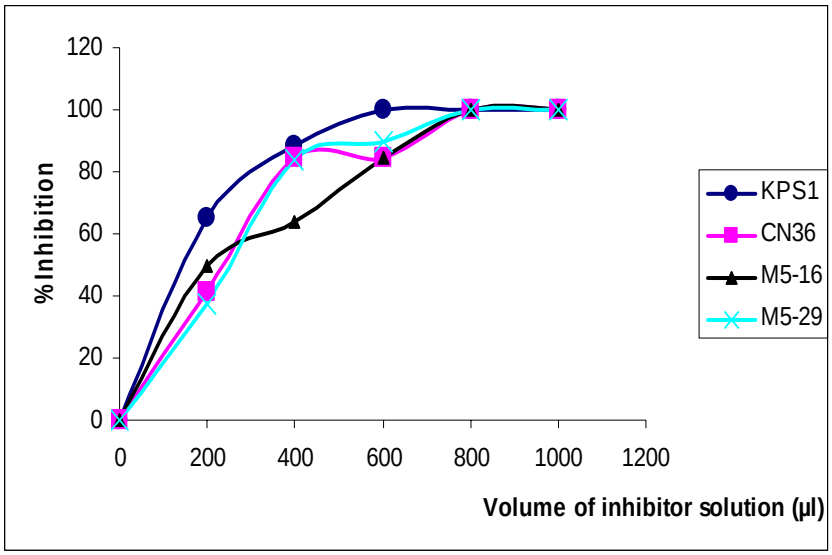

Figure 1 Percent inhibition at different concentrations of four mungbean crude extracts **(protein part)** against *Callosobruchus maculatus* α-amylase. The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 10 min at the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

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.

Angharad *et al.* (1986) also worked on protein α-amylases inhibitors prepared from wheat and their effects tested against insect storage pests *in vitro* against the insect α-amylases. 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*.

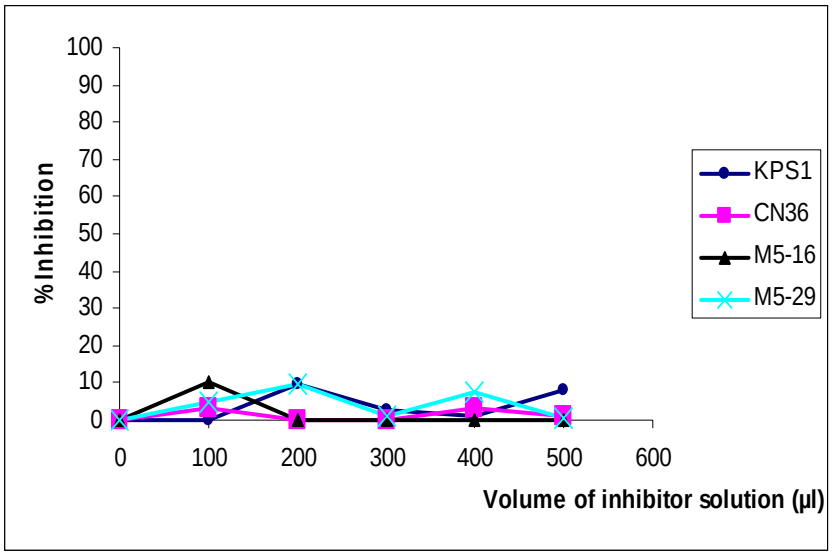

Figure 2 Percent inhibition at different concentrations of four mungbean crude extracts **(non-protein part)** against *Callosobruchus maculatus* α-amylase. The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 10 min at the optimum condition for *C. maculatus* enzyme, pH 6.0 and 50°C.

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) in Appendix Figures 4 and 5. 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 3).

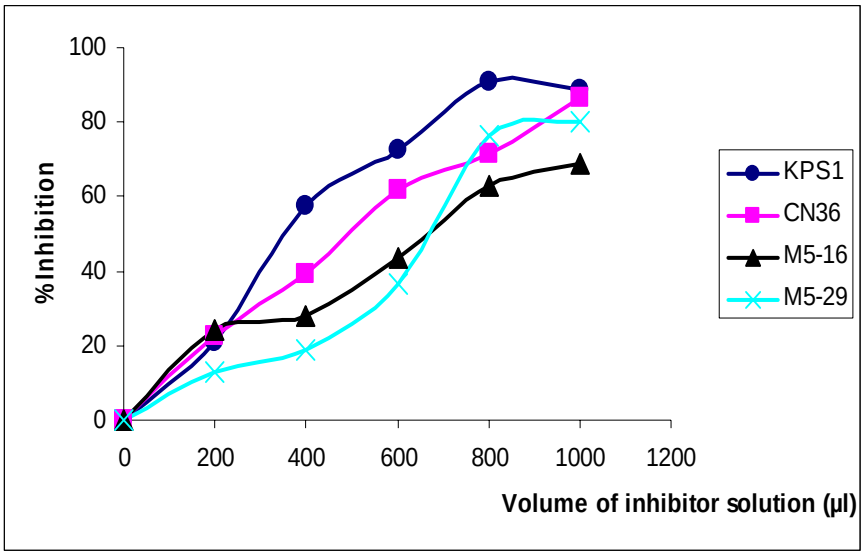

Figure 3 Percent inhibition at different concentrations of four mungbean crude extracts **(protein part)** against barley malt α-amylase (Type VIII-A). The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 10 min at the optimum condition for barley malt enzyme, pH 4.0 and 50°C.

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. These findings were not similar to 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 (http://www.biochem.ucl.ac.uk) 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 *J.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.

Since the inhibitions against both barley malt and weevil α-amylase of the non-protein part of the crude extracts from all varieties/lines was quite low (not more than 10 % for *C. maculatus* and not more than 7 % for barley malt enzyme) (Figure 4), this could only emphasize that inhibitory activities of the inhibitor resided in the protein part of the extracts.

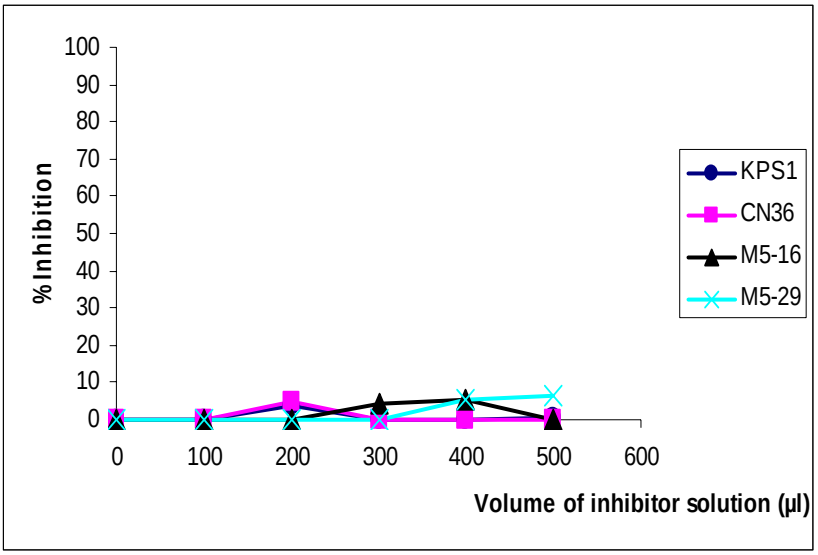

Figure 4 Percentage inhibition at different concentrations of four mungbean crude extracts **(non-protein part)** against barley malt α-amylase (Type VIII-A). The inhibitor and enzyme extracts were preincubated at room temperature for 15 min before addition of the substrate. Incubation was for 10 min at the optimum condition for barley malt enzyme, pH 4.0 and 50°C.

CONCLUSION

Effects of α-amylase inhibitor *in vivo* could be concluded that there was no significant difference in terms of the number of egg laid and antibiosis on the developmental stages of *C. maculatus* between the crude extracts of the control varieties, KPS1 and CN36, and the mutant lines, M5-16 and M5-29. Yet, the protein part showed more detrimental effect to some stages of mungbean weevil than the non-protein part. The difference in antibiotic resistance in the mutant lines and the control varieties obtained from the previous study might be caused by the other chemicals which needed further investigation, both in preference and antibiosis.

. 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 while in non-protein extracts the highest percent inhibition of 10 % was from100 volume of M5-16 inhibitor extract.

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 whereas 100 % inhibition in non-protein part were from 1000 volume KPS1 and M5-16 inhibitor extract.

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APPENDICE

Protein Determination

Lowry's method

1. Diluting BSA (Bovine Serum Albumin) 0.1 – 1 mg/ml for standard curve and 2 ml sample in 98 ml distilled water (final volume of 100 ml)

2. Adding A:B:C (100:1:1) 3 ml, incubated at room temperature for 10 minutes

3. Adding Folin Ciocaltue's reagent (diluted in distilled water 1:1 before use) of 3 ml, incubated in the dark for 30 minutes

4. Recording absorbance at 750 nm

Protein Color Reagent

A : Sodium Carbonate 10 g was dissolved in 960 ml distilled water with 3 N NaOH 35 ml

B : Copper Sulfate 1 g was dissolved in 100 ml distilled water

C : Potassium Sodium Tartrate 2 g was dissolved in 100 ml distilled water

- Each 1 ml of B and C solution was mixed well before adding 100 ml Solution A

Optimum pH of α-amylase from cowpea weevil and barley malt

1. Preparing buffer of pH 2-12
2. Preparing 2 % starch in each buffer of pH 2-12
   - 2.1 0.2 M buffer 0.5 ml
   - 2.2 0.1 M $CaCl_2$ 0.1 ml
   - 2.3 1 M NaCl 0.1 ml
   - 2.4 10 % starch 1.0 ml
   - 2.5 distiller water 3.3 ml
3. Add 50 ml α-amylase enzyme in each buffer pH 125 ml 2 % starch, incubated at room temperature 30 minutes
5. Add 125 ml DNS boiled in hot water 5 minutes

6. Add 2 ml distilled water
7. Record absorbance at 540 nm

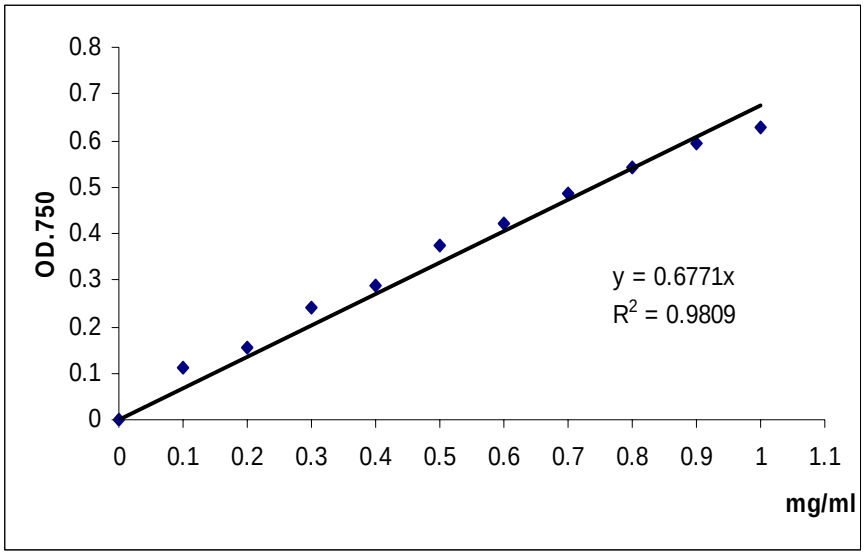
Optimum Temperature

1. Preparing 2 % starch boiled in each temperature (20-80 °c) before use for 5 minutes
2. Adding 50 ml α-amylase enzyme in 125 ml 2 % starch, incubated at each temperature (20-80 °C) for 30 minutes
3. Adding 125 ml DNS boiled in hot water 5 minutes
4. Adding 2 ml distilled water
5. Recording absorbance at 540 nm

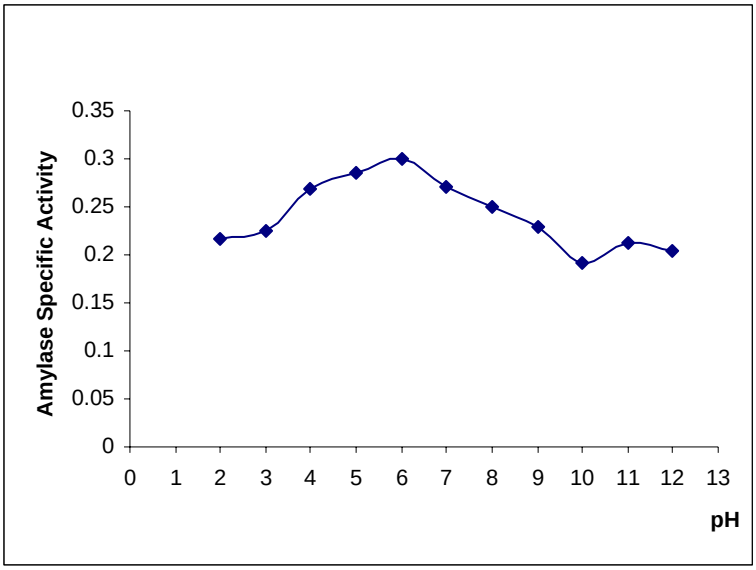
DNS Color Reagent

1. Dissolving3, 5 – dinitrosalicyclic acid 1 g in 20 ml 2 M NaOH
2. Adding 30 g Potassium Sodium Tartrate, mixed well
3. Adjusting final volume of 100 ml

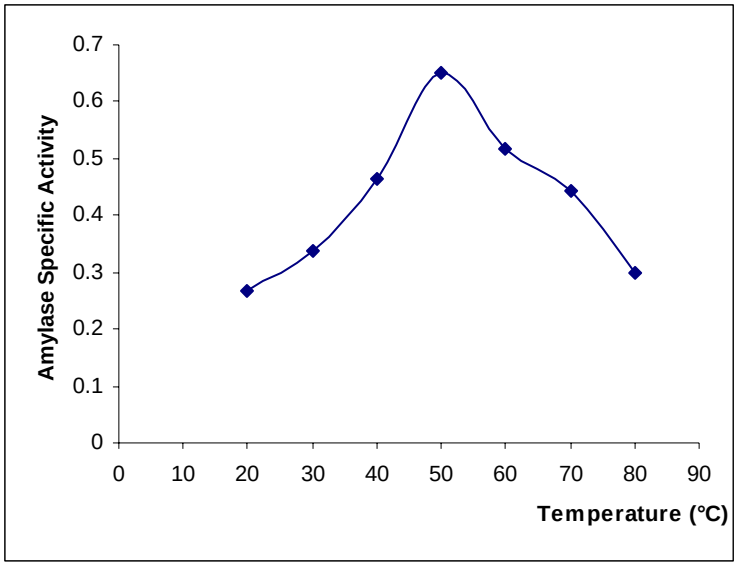
Protein extraction analysis



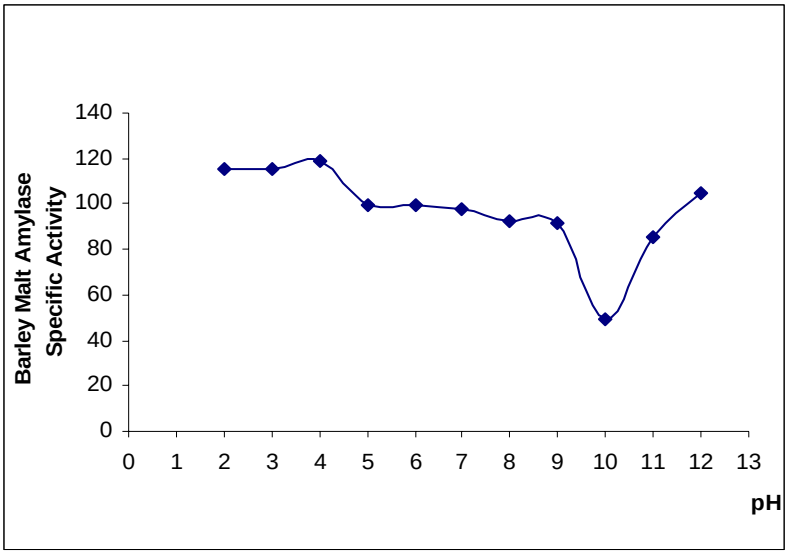
Appendix Figure 1 The standard curve of Bovine Serum Albumin (BSA)



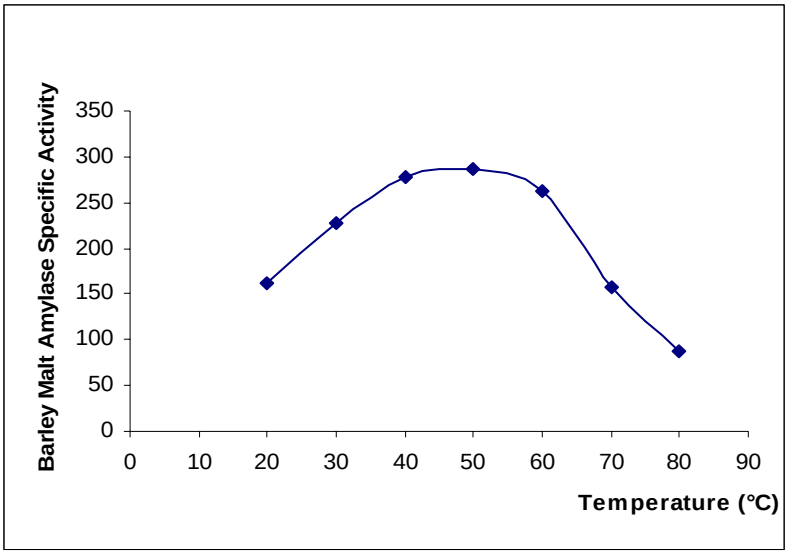
Appendix Figure 2 Amylase specific activity (µmol maltose min^{-1} mg $protein^{-1}$) in the crude extracts of cowpea weevil *C. maculatus* performed at room temperature showing pH activity profiles at pH 2-12.



Appendix Figure 3 Amylase specific activity (μmol maltose min^{-1} mg $protein^{-1}$) in the crude extracts of cowpea weevil *C. maculatus* performed at 20-80°C.



Appendix Figure 4 Amylase specific activity (µmol maltose min^{-1} mg $protein^{-1}$) in the barley malt amylase (Type VIII-A) performed at room temperature showing pH activity profile at pH 2-12.



Appendix Figure 5 Amylase specific activity (μmol maltose min^{-1} mg $protein^{-1}$) in the barley malt amylase (Type VIII-A) performed at 20-80°C.