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TITLE: The Effect of Whole Bean Flour and Protein-Enriched Bean Flour as Deterrent and Protectant Against Penetration into Packaging by *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae)

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THESIS

THE EFFECT OF WHOLE BEAN FLOUR AND PROTEIN-ENRICHED BEAN FLOUR AS DETERRENT AND PROTECTANT AGAINST PENETRATION INTO PACKAGING BY *SITOPHILUS ZEAMAIS* (MOTSCHULSKY) (COLEOPTERA: CURCULIONIDAE)

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Pornsarun Leagamnuy 2010: The Effect of Whole Bean Flour and Protein-Enriched Bean Flour as Deterrent and Protectant Against Penetration into Packaging by *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae). Master of Science (Zoology), Major Field: Zoology, Department of Zoology. Thesis Advisor: Associate Professor Boongeua Vajarasathiara, Ph.D. 85 pages.

Sitophilus zeamais caused deterioration of whole stock of maize and cereals in storage. A laboratory experiment was conducted to investigate the deterrent effect of whole flour and protein flour of red kidney bean, mung bean and navy bean against *S. zeamais.* The deterrent effect of whole bean flour was detected at 48 and 72-hour. The average number of *S. zeamais* moving out of rice grains treated with whole flour of navy bean, after 48-hour, was significantly different among concentrations. Similar result was observed for all kind of beans after 72-hour. The deterrent effect of rice grains treated with 1, 10 and 20% whole flour of navy bean against *S. zeamais*, after 48 hours, was significantly higher than that of the untreated control. The deterrent effect of rice treated with 20% whole bean flour was significantly different among bean types. Polyethylene sheets coated with protein flour solution for all kind of beans cannot prevent insect penetration.

Inhibitory effect of crude bean protein on activity of *S. zeamais* α-amylase, *C. maculatus* α-amylase, human salivary α-amylase and barley malt α-amylase was tested. Inhibitory effect of three beans was more effective against *S. zeamais* α-amylase, *C. maculatus* α-amylase and barley malt α-amylase but less effective against human salivary α-amylase. α-Amylase inhibitors from all three beans were classified as αAI-2 type because they inhibited α-amylase of *S. zeamais*, *C. maculatus* and barley malt but not human salivary. α-Amylase inhibitors from three beans may play an important role as antifeedant against *S. zeamais*.

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Pornsarun Leagamnuy

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LIST OF ABBREVIATIONS

µl	=	microliter
µmol	=	micromole
αAI	=	alpha amylase inhibitor
°C	=	celcius degree
$CaCl_2$	=	calcium chloride
cm	=	centimeter
DNS	=	3, 5 dinitrosalicylic acid
kDa	=	kilo Dalton
KPS2	=	Kamphaengsaen 2
LT_{50}	=	lethal time 50
M	=	molar
ml	=	milliliter
mM	=	millimolar
NaOH	=	sodium hydroxide
nm	=	nanometer
R.H.	=	relative humidity

THE EFFECT OF WHOLE BEAN FLOUR AND PROTEIN-ENRICHED BEAN FLOUR AS REPELLENT AND PROTECTANT AGAINST PENETRATION INTO PACKAGING BY *SITOPHILUS ZEAMAIS* (MOTSCHULSKY) (COLEOPTERA: CURCULIONIDAE)

INTRODUCTION

Milled rice and cereal products are commonly subjected to attack by a group of insect pests which have adapted themselves to feed on finished products (Kengkarnpanich, 2003). Many insect pests are able to feed, develop and multiply on whole seeds. These insects multiply rapidly when they are in granaries at 25-35°C (Sinha and Watter, 1985). They cause both quantitative and qualitative loss to harvested grains during storage, with higher losses in developing countries (Hall, 1970; Madrid *et al*., 1990). Thus, prevention and control of insects in stored products are important to farmers, grain handlers and consumers. Synthetic pesticides are currently the method of choice to control insect pests from causing insect damage (Mohan and Field, 2002). Although synthetic insecticides are the main means to control stored-product insects (Mohan *et al.*, 2007), application of chemical insecticides can result in harmful residues in foodstuff (Subramanyam and Hagstrum, 1995) and development of resistant strains in the target insect populations (Zetter and Cuperus, 1990).

Repellent and packaging have potential for controlling stored-product pests and have been used to prevent insects from feeding and oviposition (Schmutterer, 1990). Many plants are rich sources of effective insecticidal activity (Weaver and Subramanyam, 2000). The admixture of plant products with grain provides promising alternatives for packaging and repelling insect pests. Legume seeds comprise a wide range of secondary metabolites with toxic and deterrent effects against insect pests (Harborne *et al*., 1971; Bell, 1978). Recently, research on protein-enriched pea flour showed that it had both toxic and repellent properties against stored-product insects (Bodnaryk *et al*., 1999; Fields *et al*., 2001; Mohan and Fields, 2002; Hou and Fields, 2003; Pretheep-kumar *et al*., 2004b). Fields *et al*. (2001) and Hou *et al*. (2004b) demonstrated that the deterrent compounds of protein-enriched pea flour are

detrimental through defatting procedures. At present, the information on repellent effects of Thai legume seeds is limited. Thus, the purpose of this study was to evaluate the deterrent of whole bean flour and protein-enriched bean flour against *Sitophilus zeamais* (Motschulsky).

OBJECTIVES

Overall objectives

To find novel botanical pesticides with deterrent effect that is not harmful to human health and the environment.

Specific objectives

1. To study the effectiveness of whole bean flour and protein-enriched bean flour of three legume seeds as deterrent against maize weevil, *S. zeamais*.

2. To find the appropriate concentration of whole bean flour and protein-enriched bean flour for controlling *S. zeamais*.

3. To test whether the protein-enriched bean flour could prevent insects from penetrating food packages.

4. To find the appropriate temperature and pH of α-amylase activities for inhibitory effect testing.

5. To test inhibitory effect of red kidney bean, mung bean and navy bean α-amylase inhibitors against α-amylase of *S. zeamais, Callosobruchus maculatus*, human salivary and barley malt.

LITERATURE REVIEW

Stored Product Insects

Stored products are vulnerable to attack by various insect pests during storage. These insects have adapted themselves to dried food crop. They are able to thrive on cereal and grains. Due to their small size in nature, these insects smartly conceal themselves particularly in grains. They primarily are in the order Coleoptera. Sukprakarn (1985) reported that many species of beetle have been recorded in association with grain and other agricultural products in Thailand.

Importance and Destruction

Maize weevil, *S. zeamais*, is a pest of stored maize and of maize cob prior to harvest by internal feeding (Longstaff, 1981; Anonymous, 2008). This insect is related primarily with maize although it is capable of developing on all cereal grains such as sorghum, rice and cereal products (Walgenback and Burkholder, 1986; Tipping *et al*., 1987). Initial infestations of maize grain occur in the field just before harvest and insects are carried into the store where the population builds up rapidly (Appert, 1987; Adedire and Lajide, 2003). The enormous post harvest losses and quality downfall caused by this pest is a major barrier to achieve food security in developing countries (Rouanet, 1992). Infestations initiated in the standing crop may further develop in storage as the grain dried whether stored as cobs or bulk grain. It may also infest other cereals if the moisture content is moderate or high. The major effect of infestation by the maize weevil is the damage to grain by feeding activities of the adults and the development of immature stages within the grain. This not only reduces the grain quality but also produces a considerable amount of grain dust mixed with frass (Longstaff, 1981; Anonymous, 2008). The most destructive strains are able to cause losses of up to 90% of the stock after 5 months of storage (Nukenine *et al*., 2002).

Sitophilus zeamais (Motschulsky)

Classification of *Sitophilus zeamais* (Motschulsky)

Phylum: Arthopoda
Subphylum Uniramia
Class: Insecta
Order: Coleoptera
Suborder Polyphaga
Family: Curculionidae
Genus: *Sitophilus*
Species: *Sitophilus zeamais* (Motschulsky)

Common name: maize weevil, corn weevil

Maize weevil and rice weevil are almost indistinguishable from each other externally but maize weevil is always larger and darker than rice weevil (Sukprakarn *et al*., 1983). Maize weevil is distinguished from rice weevil on the basis of the shape of male aedeagus and sclerite in the female genitalia (Hill, 1990). The aedeagus of maize weevil is curve and sharp but rice weevil is blunt (Chumram, 1978). Dobie *et al*. (1986) concluded that maize weevil and rice weevil could be separated easily by dissecting male specimen. The differences between two species were mentioned in Chaisaeng (2007).

Biology

The female drills a hole into the kernel, deposits the egg, and then secretes a mucilaginous plug to enclose the egg as the ovipositor is withdrawn. The plug rapidly hardens, leaving a small raised area above the seed surface, which provides the only external evidence that the kernel is infested. Eggs may be laid anywhere in the kernel, but few are laid in the embryo. In wheat, most eggs are deposited at the end farthest from the embryo. Sometimes, more than one egg may be laid in a single grain but it

is rare for more than one larva to develop to maturity because of cannibalism (Longstaff, 1981). Not all excavated holes are used for oviposition; some are abandoned and others are expanded into feeding holes (Campbell, 2002). There are four larval instars all of which remain within the grain. Immediately after hatching, the first instar feeds by burrowing through the tissues of the grain. At the end of the fourth instar the larva uses a mixture of frass and larval secretion to close off the end of the burrow to form a pupal cell. Under normal developmental conditions, weevil larvae allow their frass to accumulate around themselve inside the grain in which they are feeding. However, if the carbon dioxide level exceeds 5%, the fourth instar larva makes a small hole in the grain and ejects much of the frass. The larva then assumes a prepupal form for a short period before transforming into the pupa (Longstaff, 1981).

Description of life stage

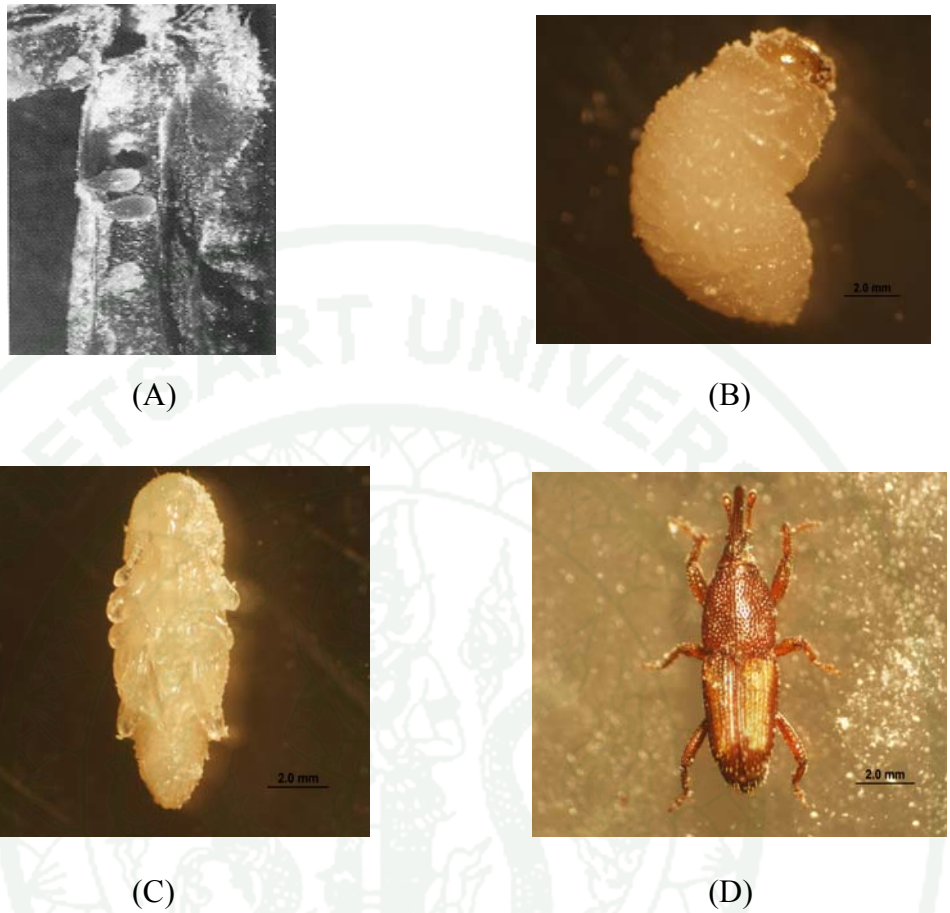
Egg: The egg is opaque, shining white; ovoid to pear shaped, widest below middle; bottom broadly rounded, neck narrowing gradually toward top, which is somewhat flattened and has a small rounded protuberance that fits into a cap or plug cementing the egg in place (Fig. 1A). It is small in size and cannot be seen by naked eyes. The averages of egg length and width were 0.65±0.04 mm and 0.27± 0.02 mm, respectively (Nualvatna *et al*., 2005a).

Larva: A mature larva is 2.50 ±2.75mm long, soft, pearly white, legless, fleshy and very thick bodies (Fig. 1B). The head and mouth parts are usually intact structures as food fragments. The larval instar could be identified by measuring the width of the head capsules of larvae dissected from kernels. In corn, the average widths of tunnels are 0.33 mm, 0.55 mm, 0.80 mm and 1.49 mm for first, second, third and fourth instars, respectively (Jumroenma, 1992). The mean developmental periods for stadia 1-4 at 27°C and 69±3 % R.H. are 3.6, 4.7, 4.8 and 5.0 days, respectively.

Pupa: The pupa is uniformly white in color when it is first formed, then gradually darkens and assumes adult structure before fully mature (Fig. 1C). A pupa

is 3.75-4.25 mm long and 1.75 mm wide. Both pupa length and width are not constant because its body size depends on the kind of food rearing.

Adult: Adults reach a length of 3.0 - 3.5 mm long. Body color varies from dull red-brown to almost black. Some adults have four reddish orange circular markings on elytra more clearly defined, being light red to yellow (Anonymous, 2010) (Fig.1D). Snout is cylindrical with dorsal margin straight; antennae inserted near base of snout, just in front of eyes, funicle with 6 segments and club with basal segment shiny, pygidium largely exposed. The thorax is densely pitted with irregularly shaped punctures, except for the smooth narrow strip extending down the midline of the dorsal side. Thorax is longer than wide constricted near apex, sides feebly curved, gradually divergent to base; disk densely and coarsely punctured, body length depends upon food rearing (Table 1).



(A) (B)

(C) (D)

Figure 1 Life stages of maize weevil, *Sitophilus zeamais* (Motschulsky).
(A) Egg (Floyd and Newson, 1959) (C) Pupa
(B) Larva (D) Adult

Table 1 The effect of food type on the body length of *Sitophilus zeamais* (Motschulsky) adult.

Type of food	Adult body length (mm)	Length from beginning of pronotum to end of elytra (mm)
	Mean (range)	
Corn	4.38 (3.9-4.9)	3.35 (3.0-3.6)
Wheat	3.70 (3.0-4.6)	2.80 (2.2-3.3)
Milled rice	3.60 (2.9-4.3)	2.80 (24-3.0)
Rough rice	3.40 (2.7-3.2)	2.70 (2.5-3.1)
Shelled rice	3.17 (2.3-3.9)	2.42 (1.8-2.9)

Source: Maceliski and Korunic (1973)

Development

Total development period ranges at approximately 30 to 45 days. The actual length of life cycle depends on the quality of grain being infested such as in different varieties of maize (Sukprakarn *et al.,* 1996). Most eggs were deposited in the endosperm, but 28% were in or around the germ. Maximum daily rate of fecundity (6.7 eggs per female in 24 hours), duration of development, and number of progeny produced were optimal at 30°C and 75% R.H. The duration of immature development of rice weevils is longer when multiple eggs are deposited in a kernel and when more than one weevil larva occurs in a wheat kernel, the larger one will eat the smaller one. The lower limit for development from eggs to adult weevils was 15.6°C and the upper limit was 32.5°C at 75% R.H. The adult maize weevil will also feed on the grain during its lifespan, which is approximately 5 to 8 months, before dying (Throne, 1994).

The incubation period of the egg is about 6 days. Upon hatching, the larva begins to feed inside the grain, which takes approximately 20 to 30 days. At this

point, the larvae become pupae, and they begin the transformation into the adult weevil form. This process takes approximately 6 days. During these 6 days, the pupae do not eat or move. The adult also feed within the grain but, unlike the larvae, they move from one grain to another (Pury, 1968).

Distribution

Sitophilus zeamais (Motschulsky) is strong and can fly very far, it can propagate rapidly and is found throughout the warmer, tropical parts of the world where maize and other cereals are growing and more humid regions of the world (Longstaff, 1981; Dobie *et al*., 1984). It has been recorded from Europe (Spain, Italy, Turkey); Africa (Angola, Arabia, South Africa, East Africa, Ethiopia, Ghana, Madagascar, Madeira, Mauritius, Morocco, Mozambique, Nigeria); Asia (India, Tibet, Malaysia, Molucca Isles, Borneo, Japan, Thailand, Myanmar); Australia (Australia-New South Wales, Queensland, West Australia, New Guinea, New Zealand, Pacific Islands); USA (Texas, Florida); Central America (Costa Rica, Mexico, West Indies); South America (Argentina, Brazil, Guyana, Honduras, Chile, Equador, Guatemala, Nicaragua, Venezuela) (Anonymous, 2009; Dennis, 1983). The maize weevil and other stored insect pests were moved and distributed around the world because of the global transportation of stored products. Moreover, the differences in stored condition and regional climate are supportive to maize weevil adaptation and survival (Chaisaeng, 2007).

Food preference

Maize weevil is commonly associated with corn and rice in tropical storage and to a lesser extent in other raw or processed cereals, including wheat, oats, barley, sorghum, rye and buckwheat. It is the major pest of stored rice, but is of less importance in wheat. They cannot breed in finely processed grain but readily breed in manufactured flour products such as macaroni and noodles, and also in milled cereals that have become caked from excess moisture. The range of moisture contents within which it will breed has been found to be much wider than that of *S. oryzae* and it has

been found to attack fruit, such as apples, in storage. *Sitophilus zeamais* (Motschulsky) commonly infests standing crops prior to harvest, particularly maize, where the moisture contents exceed 20% (Maceljski and Korunic, 1973; Longstaff, 1981; Anonymous, 2009). Sukprakarn *et al*. (1984) conducted a survey of maize weevil in seed stores of farmer, middleman, a cooperative, export warehouse and silo during December 1983 to January 1984. They found that paddy, milled rice, maize, sorghum, wheat and barley were only infested by maize weevil. Food preference of maize weevil was inconstant, but the most favorable food was maize grain (Nawanich, 1996; Nualvatana *et al*., 2005b).

Natural enemies

Natural enemies of maize weevil is generally hymenopterous wasps: *Anisopteromalus calandrae* (Howard), *Theocolax elegans* Westwood, *Holepyris sylvanhnidis* (Bretes),*Cerocephala donodiri* Graham, *Meraparus requistitus* (Tuker), *Dibrachys cavus*(Walker) and *Lariophagus distinguendus* (Forster) (Kengkarnpanich, 2003).

Control of maize weevil

To reduce detriment in postharvest systems from maize weevil infestation, chemical pesticides are most commonly used to control insect pests. Fumigation plays a principle role in insect pest control in stored product. At present, phosphine and methyl bromide are the two common fumigants used for grain protection. Insect resistance to phosphine is a global issue now and control miscarriages have been reported in field situations in some countries (Taylor, 1989; Collins *et al*., 2002). However, there are several reasons to search for alternatives for the control of storage pests: consumer preference for food without insecticide residues, worker safety concerns, resistant insect population and deregistration of current synthetic insecticides (Hou and Field, 2003).

Plant secondary metabolites are a diverse group of molecules that are concerned in the adaptation of plants to their environment but are not part of the primary biochemical pathways of cell growth and reproduction. Plant products have been found to contain a hundred of different allelochemicals, for example; some proteins, alkaloids, terpenes and cyanogenic glucosides (Ryan, 1990; Ryan and Pearce, 1998; Bishop *et al*., 2000; Sales *et al*., 2000). These compounds are very promising because of several distinct advantages. Secondary plant chemicals have existed in nature as plant components for millions of years without any ill or opponent effects on the ecosystem. Some plants have more than one chemical as active principles responsible for their biological attribute. These may be either for one particular biological effect or may have diverse ecological effects.

Legume seeds comprise a wide range of allelochemicals with toxic and deterrent effects against stored product insects (Harborne *et al*., 1971; Bell, 1978). An admixture of yellow split-peas (*Pisum sativum* L.) with wheat resulted in reduction of survival and reproduction rate of *Sitophilus oryzae* (L.) (Coombs *et al*., 1977; Holloway, 1986). Recently, at concentrations as low as 0.01% pea protein were shown to cause adult mortality and reduced reproduction of several stored-product insect pests (Bodnaryk *et al*., 1999; Delobel *et al*., 1999).

Fields *et al*. (2001) studied repellency effect of pea (*Pisum sativum*) protein, ground pea, pea fiber or pea starch against some stored-product insects. Their result showed that there was a negative correlation between the pea protein concentrations and the number of adults found in treated grain for *S. oryzae*, but not for *T. castaneum.* Similar result was reported for *C. ferrugineus* by Mohan and Fields (2002). Pea protein reduced the number of offspring of all three species (Hou and Fields, 2003). Pea fiber repelled *C. ferrugineus* (Mohan and Fields, 2002) but not *S. oryzae* and *T. castaneum* (Fields *et al*., 2001). Pea starch did not repel any of the insects (Mohan and Fields, 2002). Ground pea increased emigration of *C. ferrugineus and T. cataneum* from the wheat (Mohan and Fields, 2002). In a two choice bioassay, the pea-protein-treated grain had significantly fewer insects (*C. ferrugineus, S. oryzae, S. zeamais, T. castaneum, and T. confusum*) than untreated grain.

Consumption of pea *(P. sativum)* protein treated with wheat flour by *T. castaneum, S. oryzae* and *R. dominica* was significantly reduced when compared with wheat flour alone. Consumption of protein-treated wheat flour was affected when the insects were exposed for 3 days. Antifeedants present in the pea protein fraction are apparently responsible for the reduced feeding response in these species (Pretheep-Kumar and Mohan, 2004).

Protein-enriched pea (*P. sativum* var. Bonneville) flour at 1% concentration treated with milled rice against the rice weevil significantly repelled *S. oryzae* (Pretheep-Kumar *et al.,* 2004b). In addition, paddy grains treated with pea flour at the same concentration repelled *T. castameum* followed by *S. oryzae* and *R. dominica* (Pretheep-Kumar *et al.,* 2004a). Mortality of *S. oryzae* was found to increase and fecundity was markedly suppressed, in rice treated with 1% pea flour extract (Pretheep-Kumar *et al.,* 2004b).

Pretheep-Kumar *et al*. (2007) studied long-term efficacy of the protein-enriched flour of pea (*P. sativum* L. var. Bonneville) in its toxicity and progeny reduction. It was evaluated by combining it with wheat flour and testing the admixture against the red flour beetle, *T. castaneum*. The toxicity and progeny-reducing effects of the wheat flour treated with protein-enriched pea flour were stable for a period of 5 months (Pretheep-Kumar *et al*., 2004b) when stored at 28°C with 75% R.H. This result was confirmed by Fields (2006) who found that insecticidal activity of pea fractions decreased after treated wheat kernels were held at 30°C, 70% R.H. for 8 months. However, the toxicity of protein pea flour was not reduced after 9 months when stored at -15°C or at room temperature as flour or mixed with grain (Hou and Field, 2003).

Hou *et al*. (2004b) compared two known repellents of stored-product insects, DEET and neem with the protein-enriched pea flour, defatted protein-enriched pea flour, and pea protein extract for their efficacy at reducing penetration and invasion by *S. oryzae*, *T. castaneum*, *C. ferrugineus*, and *Oryzaephilus surinamensis* (L.). Protein-

enriched pea flour, DEET and neem reduced the penetration of *S. oryzae*, but defatted protein-enriched pea flour and pea protein extract did not. The number of *S. oryzae*, *T. castaneum*, *C. ferrugineus*, and *O. surinamensis* entering pierced paper envelopes containing wheat and were treated with DEET was reduced by 99%, 86%, 97% and 91%, respectively. Neem was less effective than DEET in reducing penetration and invasion of insects. Protein-enriched pea flour did not prevent insects entering pierced envelopes.

The combinations of protein-rich pea flour with parasitoids were tested against stored product beetles by Hou *et al.* (2004a). They found that protein-rich pea flour was not toxic to, and did not reduce the offspring of, *Anisopteromalus calandrae* (Howard), a parasitoid of *S. oryzae*, nor did it reduce offspring of *Cephalonomia waterstoni* (Gahan), a parasitoid of *C. ferrugineus*. Thus combinations of protein-rich pea flour and parasitoids could reduce populations of *S. oryzae*.

Hou *et al.,* 2004c studied combination of protein-rich pea flour and pea extract with insecticides and enzyme inhibitors for control of stored-product beetles. The result show protein rich pea flour mixed with other products (diatomaceous earth, neem, *Bacillus thuringiensis* (Berliner), malathion and pyrethrum) in wheat reduced the concentration of protein-rich pea flour. Neem and protein-rich pea flour acted synergistically against *T. castaneum*. Malathion and protein-rich pea flour acted synergistically against *S. oryzae*. Protein-rich pea flour combined with diatomaceous earth or pyrethrum acted additively against *S. oryzae*. All other combinations acted antagonistically. An extract from protein-rich pea flour reduced feeding of *S. oryzae*, and three enzyme inhibitors, piperonyl butoxide, profenofos, and diethyl maleate, were tested for their possible synergistic effects on feeding deterrence and mortality. Piperonyl butoxide and pea extract had additive effects, and diethyl maleate had no effect on the feeding and mortality of insects. Profenofos alone killed all insects in 3 days. The flour consumption of *S. oryzae* was positively correlated with LT_{50} in flour disks treated with pea extract.

Fields (2006) studied three pea fractions (protein, fiber and starch) of yellow field pea (*P. sativum*) mixed with wheat kernels or wheat flour against insect pests. The results showed that protein-rich pea flour was more toxic than fiber, which was more toxic than starch. For the protein-rich pea flour mixed with wheat kernels, the most sensitive insects were *S. oryzae*, *S. zeamais* and *S. granarius*, followed by *C. ferrugineus* which was more sensitive than *T. castaneum* and *R. dominica*. For the protein-rich pea flour mixed with wheat flour, *C. pusillus* was most sensitive, followed by *C. turcicus* and *T. confusum*, with *T. castaneum* being the most resistant. Although protein-rich pea flour did not kill adults to a great extent when mixed with flour, it reduced offspring production significantly. Again *C. pusillus* was the most sensitive, followed by *T. confusum*, with *T. castaneum* offspring being the most resistant.

Mohan *et al*. (2007) studied penetration of polyethylene sheets coated with protein-enriched pea flour solution by two stored-product insects. They found that adults of *S. oryzae* and *R. dominica* penetrated polyethylene sheets (0.0635mm thick) coated with protein-enriched pea flour solution at 0, 1 and 5% levels, but did not penetrate the sheets coated with a 10% concentration.

Several compounds in protein-rich pea flour such as polypeptides, soyasaponins and lysolecithins were found toxic to *S. oryzae* (Taylor *et al*., 2004a, 2004b, 2004c). Three polypeptides have been isolated from peas that are toxic to stored-product insects (Delobel *et al*., 1999). These polypeptides are related to pea albumins of the PA1b type (Higgins *et al*., 1986). One of the PA1b-like polypeptides belongs to the cystine-knot family of toxins (Jouvensal *et al*., 2003). This polypeptide binds with high affinity to the microsomal fraction of susceptible, but not resistant, *Sitophilus* spp. (Gressent *et al*., 2003).

Enzyme inhibitor

Enzyme 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 could 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

α-Amylase inhibitor is a substance which binds to α-amylase and makes it loses activity. These inhibitors can inhibit mammalian α-amylase, endogenous α-amylase which is produced in seed during germination and acts as the plant defense system against pests and pathogens.

Plant α-amylase inhibitor was first reported in wheat (Gibbs and Alli, 1998) and were later found in beans (*P. vulgaris*), acorns, mangoes, rye (Gibbs and Alli,1998), millet (Shivaraj and pattabiraman, 1980), maize (Blanco-Labra and Iturbe-chinas, 1981), peanut (Irshad and Sharma, 1981), barley (Mundy *et al*., 1984) and sorghum (Kutty and Pattabiraman, 1986). These inhibitors have different structures and can be grouped into two classes: nonproteinaceous α-amylase inhibitor and proteinaceous α-amylase inhibitor.

Non-proteinaceous α-amylase inhibitors

Nonproteinaceous α-amylase inhibitor contains diverse types of organic compounds, such as acarbose, isoacarbose, acarviosine-glucose, hibiscus acid and cyclodextrins. Acarviosine-glucose and cyclodextrin from roselle tea (*Hibiscus sabdariffa*) inhibit the porcine pancreatic α-amylase and the human pancreatic α-amylase (Hansawasdi *et. al*., 2000). The cyclic structure of organic compounds binds to catalytic site or carbohydrate binding site near the catalytic site of α-amylase and makes it loses activity. X-ray crystallography study suggested three α-cyclodextrin bound to the porcine pancreatic α-amylase. α-Cyclodextrin I and II bound near the catalytic site, while α-Cyclodextrin III bound at an accessory site (Larson *et. al*., 1994). The inhibition effect on the porcine pancreatic α-amylase using acarbose and α-cyclodextrin as an inhibitor indicated that acarbose is a strong inhibitor, while α-cyclodextrin is a weak inhibitor and the type of inhibition are mixed noncompetitive inhibition (Koukiekoulo *et al*., 2001).

Proteinaceous α-amylase inhibitors

Plant α-amylase inhibitors are abundant in cereals and leguminosae. These inhibitors exhibit different specificities against α-amylase from different sources. Some α-amylase inhibitors act only against mammalian α-amylase or insect α-amylase. Other inhibitors have high affinity for both mammalian and insect α-amylases. They can be grouped by similarity in sequence and three dimensional structures into six classes: knottin-type, kunitz-like, cereal-type, γ-thionin-like, thaumatin-like and lectin-like (Richardson, 1990).

Leguminosae seed contains a family of the plant defense proteins including α-amylase inhibitor which inhibits digestive enzyme in the midgut of insect larvae and prevents the growth of larvae that infest in seed. Thus these inhibitors are attractive candidates for controlling of seed weevils as the insects are highly dependent on starch as energy source. There were many examples of leguminosae seed containing the proteinaceous amylase inhibitors which obstruct digestion through

their action on insect gut digestive amylases and proteinases which play a key role in the digestion of plant starch and proteins (Grossi de sa *et al.,* 1997; Franco *et al*., 2002). The plant α-amylase inhibitors inhibit the α-amylase in a digestive tract of *Tenebrio molitor* (Frels and Rupnow, 1984), *C. chinensis*, *C. maculatus* (Ishimoto and Kitamura, 1989) *Zabrotes subfaciatus* (Suzuki *et al*., 1993) and *Bruchus pisorum* (Ieluk *et al*., 2000) resulting in suppression of growth and development of insect larvae.

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*) and mung bean (*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 α-amylase inhibitors from different varieties of the common bean does not inhibit plant, fungal or bacterial α-amylases, but blocks the activity of salivary and pancreatic amylases of humans and mammals and the α-amylases of insects (Powers and Whitaker, 1977). Plant α-amylase inhibitors are abundant in cereals and leguminosae. These inhibitors exhibit different specificities against α-amylase from different sources. Some α-amylase inhibitors are act only against mammalian α-amylase or insect α-amylase. Other inhibitors have high affinity for both mammalian and insect α-amylases (Richardson, 1990).

Power and Culbertson (1982) reported that amylase from yellow meal worm (*Tenebrio molitor*) larvae, Mediterranean flour moth (*Anagasta kuhniella*) larvae, red flour beetle (*T. castaneam*) adult as well as adults and larvae of confuse flour beetle (*T. confusum*) were inhibited by bean inhibitor.

Gatehouse *et al*. (1985) prepared protein α-amylases inhibitors from wheat and their effects were tested against storage insect pests both *in vitro* against the insect α-amylases and *in vivo* in insect feeding trials. Inhibitor fraction A was found to inhibit porcine pancreatic α-amylases but not insect α-amylases, whereas fractions B, C and D did not inhibit porcine pancreatic α- amylase but were strong inhibitors of digestive α-amylases from larvae of *T. confusum*, a storage pest of wheat products, and *C. maculatus*, a storage pest of legume seeds.

Ishimoto and Kitamura (1989) studied inhibitors of α-amylases present in the common bean, *Phaseolus vulgaris*. They found that bean seeds contain at least two different α-amylases inhibitors called αAI-1 and αAI-2. It inhibited several mammalian α-amylases and the larval midgut amylases of the azuki bean weevil (*C. chinensis*) and of the cowpea weevil. This result was confirmed by Shade *et al*. (1994).

α-Amylase inhibitors have also been identified in other legumes of genus *Vigna*, such as *Vigna sublobala*, cowpea (*V. unguiculata*) (Piergiovanni and Della, 1994) and mung bean (*Vigna radiata*) (Haq *et al*., 2005). Kokiladevi *et al*. (2005) compared inhibitory activity of α-amylase inhibitors in the seed of *Vigna* genotypes and found that *V. umbellata*, *V. sublobata* and *V. glabracens* belonged to a group with higher levels of inhibitory activity. *Vigna trilobata* showed a moderate level of inhibition while *V. radiata* and *V. unguiculata* exhibited a lower inhibitory activity.

Seeds with α-amylase inhibitor (αAI) in several cultivars of the common beans play a protective role against bruchid pests. The αAI strongly inhibited the larval midgut α-amylase activities of *C. chinensis* and *C. maculatus* and non pest species of the common beans. Bean αAI-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 mung bean, *V. sublobata* (Sahu, 1996).

Engkagul *et al.* (2004) studied *in vitro* and *in vivo* effect of mung bean α-amylase inhibitor on *C. maculatus* and suggested that α-amylase inhibitors from gramma radiated mutant mung bean had 4-5 times more inhibitory activity against *C. maculatus* and barley malt α-amylase than those from the standard variety. The *in vivo* studied indicated that the crude extract from protein part did not produce effects on the average numbers of egg laid on seeds. However, significant reductions in the average number of emerging adults were observed in mutant seeds as compared with that in the standard variety.

The effect of α-amylase inhibitor on α-amylase activity *in vitro* was also studied. One hundred percent inhibition of the protein part to α-amylase activity of *C. maculatus* was found in all variety/line mung bean seeds. Similar study was also conducted against α -amylase extracted from barley. The effects were less than those on α-amylase of the weevil (Bannakan *et al*., 2007).

Wisessing *et al.* (2008) investigated the underlying mechanism using both proteomics and biochemical characterization. The α-amylase inhibitor from the gramma irradiated mutant mung bean seed, M5-16, was purified in two step procedures involving ammonium sulfate precipitation and gel filtration chromatography using sephadex G-100. This α-amylase inhibitor exits as a monomer and has a molecular weight of 27 kDa. The α-amylase inhibitor from the mung bean seed can inhibit *C. maculatus* α-amylase but can not inhibit human salivary α-amylases. 2D gel electrophoresis suggested that gamma-induced mutant line decrease expression of α-amylase inhibitor and other protein comparing with wild type.

MAETERIALS AND METHODS

1. Mass rearing insect

Sitophilus zeamais (Motschulsky) was obtained from Postharvest Technology Research and Development Laboratory, Postharvest and Product Processing Research and Development Office, Department of Agriculture, Bangkok. One hundred of maize weevil adults, 1-to 2-weeks-old, were transferred to 200 g milled rice in 400 ml glass bottles covered with blotting paper. The milled rice (Thai Pathumthani fragrant rice) was used as food and ovipositing media for the weevils. The rice was frozen at -20°C for at least one week before use to ensure that it was free of any living insects. The parent weevils were transferred to new ovipositing bottles every three days and were allowed to lay eggs for one month. The weevil cultures were maintained at 27±2°C, 75±5% R.H. and 12:12 h (light: dark) photoperiod for at least three generations before being used in experiments (Fig.2).

Figure 2 Bottles containing milled rice infested with maize weevils.

Callosobruchus maculatus was obtained from Postharvest Technology Research and Development Laboratory, Postharvest and Product Processing Research and Development Office, Department of Agriculture, Bangkok. This weevil was used for α-amylase inhibitor assay.

2. Preparation of whole bean flour

Three types of legume, whose seeds were used to test against maize weevils, were mung bean (KPS2), *Vigna radiata* (L.), navy bean, *Phaseolus vulgaris* (Pangda1) and red kidney bean, *Phaseolus vulgaris* L. (Mokjam). Mung bean was obtained from Chai Nat Field Crops Research Center Thailand, navy bean and red kidney bean were purchased from Royal Project Foundation of Thailand. Each type of bean was ground to powder with Fitz- mill Comminutor with size 60 mesh (250μ) (Fitzpatrick Elhurst, Illinois 60125, USA) followed by Alpine pinmill with size 100 mesh (150μ) (Augsburg, Germany). This whole bean flour was used for deterrent tests.

3. Extraction of protein rich bean flour

One hundred grams of bean flour was extracted with 200 ml of 20 mM phosphate buffer (PBS), pH 7, and stirred with magnetic stirrer at 4°C for 3 hours. After centrifugation (Hermle refrigerate centrifuge model Z383K, Hermle Labortechnik GmbH, Germany) at 10,000xg for 30 minutes at 4°C, the protein in clear supernatant (S_1) was precipitated with 80% saturation ammonium sulfate and centrifuged again at 10,000xg for 20 minutes at 4°C to yield the protein pellet. The protein pellet was then dissolved in minimum volume of PBS, followed by dialysis against PBS. The dialysate was air dried (Freeze dryer; FD3 Heto, Denmark) and the quantity of protein was estimated by the method of Lowry *et al.* (1951). The protein was kept at -80°C until use in making protein-enriched bean flour, which was subsequently used for deterrent and penetration tests.

4. Deterrent test

The cup bioassay technique as described by Pretheep-Kumar *et al*. (2004b) was used to evaluate the deterrent effect of whole bean flour and protein-enriched bean flour against *S. zeamais* (Fig. 3). One hundred grams of rice was treated with each type of whole bean flour at 0%, 0.1%, 1%, 10% and 20% w/w (whole bean flour/rice grain) concentrations. The treated rice was then placed in a 350 ml perforated cup with round holes, each with a diameter of 2 mm, adequately large to allow the insects to pass through. Twenty adults of the maize weevil, age 1 to 2 weeks, were placed on the rice at the center of the perforated container. The cups were kept in a clear plastic box (17 cm width × 25 cm length × 9 cm height). The deterrent of the whole bean flour was measured in terms of the number of weevils moving out of the perforated cups, away from the treated rice. The deterred weevils were trapped in a tray below the cups. The numbers of weevils leaving the treated and untreated rice were determined at 24, 48 and 72 hours after exposure and the experiment was repeated three times.

Deterrent of protein-enriched bean flour was also tested. One hundred grams of rice was soaked in protein-enriched bean flour dissolved in phosphate buffer (pH7) at 0%, 0.01%, 0.1%, 1% and 5% w/w concentrations (protein-enriched bean flour/rice grain). After soaking for 1 hour, the treated rice was air-dried for approximately 3 hours. Thereafter, the deterrent of treated rice was evaluated by the same procedures as described above.

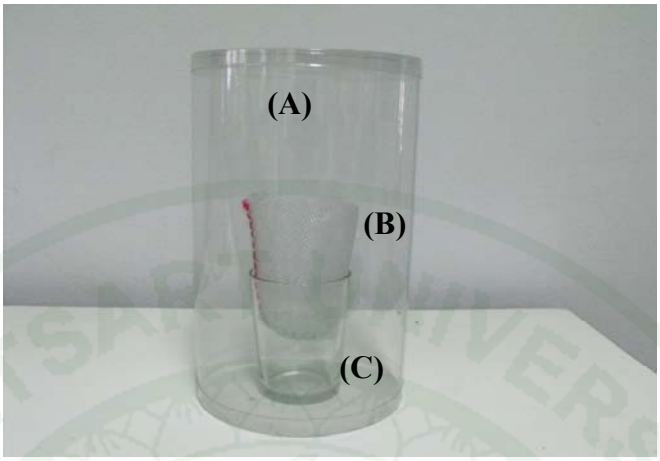


Figure 3 The cup bioassay technique for deterrent test.
(A) clear plastic box
(B) perforate cup
(C) lower cup

5. Penetration test

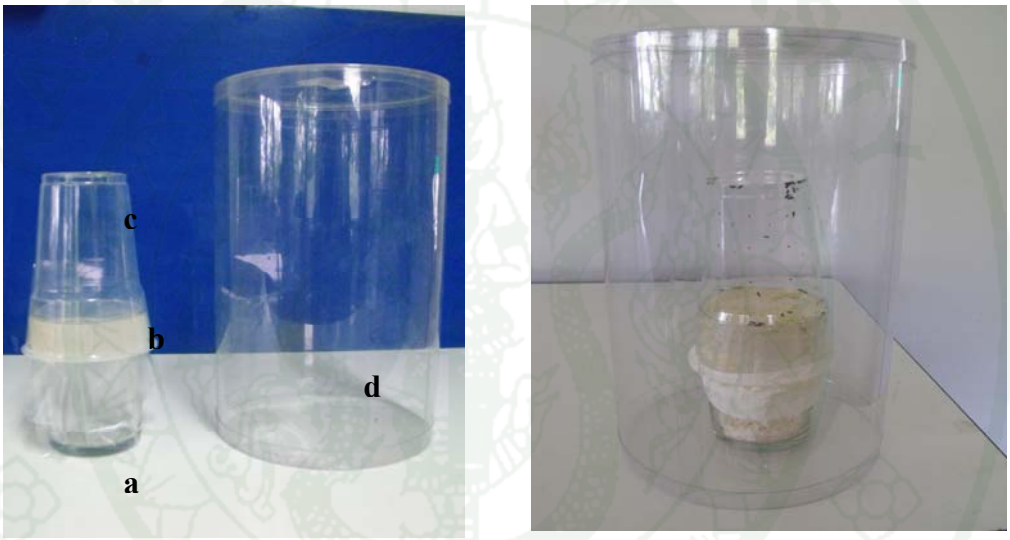
5.1 Test material

Polyethylene sheet, 0.06mm thick, was provided by Advance Packaging Corporation Limited. The sheet was used for packaging food grains. Protein-enriched bean flour was dissolved in basic water (pH 8.5-9) at concentrations of 0% and 5% w/w (protein-enriched bean flour/rice grain). The basic water was prepared by dissolving sodium hydroxide (98%) in distilled water. The solution was thoroughly stirred and was applied to the polyethylene sheet by dipping and spraying method (Bloszyk *et al.*, 1990). The treated sheet was air-dried for 24 hours at room temperature before use.

5.2 Test procedure

The 'cup test' apparatus, described by Gerhardt and Lindgren (1954), was used to test the penetration of the insects. The apparatus comprised of two plastic

cups with plastic lids. Ten grams of rice grains was placed at the bottom of the lower cup and 50 adult weevils were placed in the top cup. A treated plastic sheet was held between the two cups and tightened by clips. The weevils in the top cup and above the film would either chew through the film to reach food, or eventually die of starvation. After 4 weeks, the film was observed for the penetration by the weevils, as indicated by the presence of circular holes of 1-2mm in diameter (Fig. 4). The number of holes on polyethylene sheet coating with protein bean solution was compared with untreated control. Three replications for each treatment were made.



(A) (B)

Figure 4 The cups used in the penetration test.

(A) cup test and clear plastic box

a. lower cup
b. polyethylene sheet
c. upper cup
d. clear plastic box

(B) cup test coated with protein bean flour solution in clear plastic box

6. Characterization of *S. zeamais* and *C. maculatus* α-amylase

6.1 Preparation of α-amylase extract

α-Amylase was extracted from whole body of adult maize weevil. The weevils were frozen at -20°C for 30 minutes. Subsequently, two grams of the frozen weevils were homogenized at 4°C in 8 ml of 20 mM phosphate buffer pH 7.0 and then centrifuged at 10,000 xg for 20 minutes at 4°C. If precipitate was still present, it would be removed again by centrifugation. The protein, in clear supernatant, was used as crude amylase. The enzyme extracts were then divided into small portions (1 ml) and kept at -80°C for subsequent determination of enzyme-specific activity of α-amylase. The protein concentration of the extracts was determined using the method described by Lowry *et al.* (1951). α-Amylase was also extracted from cowpea weevil, *C. maculatus*, by the same procedure described above.

6.2 α-Amylase activity assay

α-Amylase activity was measured using the method of Bernfeld *et al*. (1955). A mixture of 100 µl α-amylase and 250 µl of 1% starch solution, in 200 mM phosphate buffer pH 6 containing 20 mM $CaCl_2$, was incubated at 60°C for 10 min. The reaction was terminated by adding 250 µl of 3,5-dinitrosalicylic acid reagent (1% 3, 5-dinitrosalicylic acid, 0.4 M sodium hydroxide and 1.06 M potassium sodium tartate), followed by boiling in water bath (Memmert, Germany) for 5 min. The reaction mixture was diluted with 2 ml of distilled water and the absorbance was measured at 540 nm. Maltose (0.1-1.0 µmol) was used for preparation of the calibration curve. The α-amylase-specific activity is defined as µmol of maltose produced min^{-1} mg $protein^{-1}$ under the specific reaction condition. One unit of amylase inhibitor activity was defined as the amount of inhibitor giving inhibition of one α-amylase activity unit under the given assay conditions.

7. Determination of optimum pH for α-amylase of maize weevil

The optimum pH for α-amylase activity was determined by varying pH values. The pH was adjusted by using the following buffers: glycine-hydrochloric buffer for the pH 2.0, citrate-phosphate buffer for the pH 3.0-5.0, phosphate buffer for the pH 6.0-8.0 and carbonate-bicarbonate for the pH 9.0-11.0 and potassium chloride-sodium hydroxide for pH 12.0. The reaction mixture consisted of 50 µl of crude enzyme extract and 200 µl of 1% starch solution in the buffer containing 20 mM $CaCl_2$ at different pH levels. This mixture was then incubated at room temperature for 10 min. The reaction was terminated by adding 250 µl of 3, 5-dinitrosalicylic acid reagent, followed by heating in boiling water bath for 5 min. The reaction mixture was diluted with 2 ml of distilled water and the absorbance was measured at 540 nm. The absorbance at each pH was used to calculate the α-amylase activity by comparing with the maltose standard curve. Optimum pH was determined by the pH that gave the highest α-amylase activity.

8. Determination of optimum temperature for α-amylase of maize weevil

The optimum temperature for α-amylase activity was determined by varying the temperatures between 30-80°C. The activity of α-amylase was determined by incubating 50 µl of amylase and 200 µl of 1% starch solution in 200 mM phosphate buffer pH 6 containing 20 mM $CaCl_2$ at different temperatures for 10 min. The reaction was terminated by adding 250 µl of 3, 5-dinitrosalicylic acid reagent, followed by heating in boiling water bath for 5 min. The reaction mixture was diluted with 2 ml of distilled water and the absorbance was measured at 540 nm. The absorbance of each temperature was used to calculate the α-amylase activity by comparing with the maltose standard curve. Optimum temperature was determined by the temperature that gave the highest α-amylase activity.

9. α-Amylase inhibitor assay

9.1 Preparation of crude protein from beans

Thirty grams of each bean powder, i.e. mung bean, navy bean and red kidney bean was extracted with 100 ml of 20 mM phosphate buffer (pH 7) by stirring with magnetic stirrer at 4°C for 3 hours. The soluble fraction was filtered through muslin cloth and then centrifuged at 10,000xg for 30 minutes. The supernatant was kept at -20 °C for use in ensuing experiments.

9.2 Inhibitory effect of crude protein of the three bean types

α-Amylase inhibitor activity was measured with a modification of the Bernfeld method (Bernfeld *et al*., 1955). The assays were performed at optimum conditions of each enzyme i.e. pH 6 at 60°C for crude *S. zeamais* α-amylase, pH 6 at 50°C for crude *C. maculatus* α-amylase (Wisessing, 2008), pH 7 at 37°C for human salivary amylase and pH 4 at 50°C for barley malt amylase (Bannakan, 2007).

To determine the inhibitory effect of crude protein from each bean type, 50 µl of crude enzyme of *S. zeamais* and 100 µl of crude protein from bean were pre-incubated at room temperature for 30 minutes. The reaction was initiated by adding 200 µl of 1 % starch solution containing 20 mM $CaCl_2$ and 200 mM buffer at optimum pH 6. The incubation was carried out at the optimum temperature of 60°C for10 minutes. The reaction was terminated by adding 250 µl of 3, 5-dinitrosalicylic acid reagent, followed by boiling in water bath for 5 minutes. The reaction mixture was cooled down, diluted with 2 ml of water, and the absorbance was measured at 540 nm. The blank was the reaction mixture without the enzyme.

Inhibitory effects of crude protein from each bean type on crude α-amylase of *C. maculatus*, human salivary α-amylase and barley malt α-amylase were determined by the same method described above. Maltose (0.1-1.0 µmol) was used for preparation of the calibration curve. The α-amylase specific activity is defined as

μmol of maltose produced min^{-1} mg $protein^{-1}$ at the specific reaction condition. One hundred percent of α-amylase inhibitor activity is defined as the amount of inhibitor giving a complete inhibition of 10 mg/ml α-amylase under the specified assay conditions.

Statistical analysis

1. Deterrent test

Effects of type of legume seeds, type of bean product, concentration and time interval on number of insects moving out of the container were analyzed by Kruskal-Wallis one-way analysis of variance (Conover, 1980; Siegel and Castellan, 1988). The differences among median number of insects moving out of the container were further analyzed by multiple comparison tests after Kruskal-Wallis one-way analysis of variance.

2. Penetration test

Effects of type of legume seeds on the number of holes on polyethylene sheet were analyzed by using Kruskal-Wallis one-way analysis of variance. The differences among median number of holes were further analyzed by multiple comparison tests after Kruskal-Wallis one-way analysis of variance.

3. α-Amylase inhibitor assay

Inhibitory effect of three bean types was calculated by one hundred percent of α-amylase inhibitor activity, which was defined as the amount of inhibitor giving a complete inhibition of 10 mg/ml α-amylase under the specified assay conditions.

RESULTS AND DISCUSSIONS

1. Deterrent test

1.1 Deterrent effect of whole bean flour

An average number of *S. zeamais* that was deterred from rice grains treated with whole flour of all three beans, after 24-hour were not significantly different among concentrations (Table 2). On the contrary, Pretheep-Kumar *et al*. (2004b) found that 1% whole pea flour caused 15% repellency against *Rhyzopertha dominica* at 24 hours.

The deterrent effect of whole bean flour was detected at 48-and 72-hour. An average number of *S. zeamais* that was deterred from rice grains treated with whole flour of navy bean, after 48-hour, was significantly different among concentrations (Table 3). The deterrent effect of rice grains treated with 1%, 10% and 20% whole flour of navy bean against *S. zeamais*, after 48 hours, was significantly higher than that of the lower concentrations.

Similar results were observed for all kinds of bean after 72-hour (Table 4). The deterrent effects of 20% whole flour of red kidney bean against *S. zeamais*, after 72 hours, was also significantly higher than that of the lower concentrations. Similar outcome was observed for mung bean. There were no significant differences among deterrent of rice grains treated with various concentrations of whole flour of navy bean.

The deterrent effect of rice grains treated with 1, 10 and 20% whole flour of navy bean against *S. zeamais*, after 72 hours, was significantly higher than that of the lower concentrations. The deterrent effect of rice treated with 20% whole flour was significantly different among bean types (Table 5). The deterrent effect of whole flour at lower concentrations was not significantly different among bean types.

The results indicated that whole flour of red kidney bean and of mung bean at 20 % could deter *S. zeamais*.

The deterrent of whole bean flour in this study could be reflected by reduced number of maize weevils on treated rice grains. Similarly, Mohan and Fields (2002) reported that ground pea increased the movement of *C. ferrugineus* and *S. oryzae* out of the grain at the hightest concentration. Based on this result, the deterrent effect of whole bean flour tended to depend on concentration and bean type. The deterrent effect of whole bean flour, in this study, may be due to the flour particle, 150μ, which causes irritation of insect spiracle.

In addition, the deterrent effect of whole bean flour could be due to some compounds in seeds such as starch, fiber or protein. For example, Bodnaryk *et al*. (1999) reported that pea starch was not repellent but the pea protein possessed repellent activity against *T. castaneum*, *S. oryzae* and *R. dominica*. Field *et al*. (2001) studied repellency effect of pea, *P. sativum*, fractions against stored product insects. They found that for the three pea fractions, the rank order of toxicity as well as repellency was protein>fiber>starch. They suggested that the deterrent might have resulted form chemosensory effects of pea protein fractions, either olfactory or gustatory.

In practice, bean flour could be used in combination with other control procedures. To increase the deterrent acyivity, bean flour might be mixed with other natural products such as diatomaceous earth, neem (Hou *et al.,* 2004c). Hou *et al*. (2004a) combined protein-rich pea flour with parasitoids. They found that protein-rich pea flour was not toxic to, and did not reduce the offspring of *Anisopteromalus calandrae* (Howard), a parasitoid of *S. oryzae*, nor did it reduce offspring of *Cephalonomia waterstoni* (Gahan), a parasitoid of *C. ferrugineus*. The combination of protein-rich pea flour and parasitoids reduced populations of *S. oryzae*.

Table 2 Number of *S. zeamais* (mean±S.E.) moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean after 24-hour (n=3): Kruskal-Wallis one-way analysis of variance.

	Number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.1	1	10	20	χ^2	*P*-value
Red kidney bean	0.0±0.0	0.7±0.3	3.7±2.1	2.7±1.7	2.7±2.1	6.09	0.19
Mung bean	0.0±0.0	1.3±0.3	2.3±1.2	3.0±2.5	1.7±0.3	5.27	0.25
Navy bean	0.0±0.0	0.3±0.3	5.0±1.7	6.3±3.4	6.7±4.1	8.46	0.07

[1](whole bean flour/rice grain)

*Significant difference at $P = 0.05$

Table 3 Number of *S. zeamais* (mean±S.E.) moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean after 48-hour (n=3): Kruskal-Wallis one-way analysis of variance.

	Number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.1	1	10	20	χ^2	*P*-value
Red kidney bean	0.0±0.0	1.3±0.9	5.0±2.0	6.3±2.9	6.3±3.0	8.97	0.06
Mung bean	0.0±0.0	1.3±0.3	4.0±1.5	3.0±2.5	5.3±2.3	7.49	0.11
Navy bean	0.0±0.0a	0.0±0.0a	7.7±3.1b	9.3±4.2b	9.0±3.8b	10.90	0.02*

[1](whole bean flour/rice grain)

Means followed by the same letter in the same row are not significantly different at *P*= 0.05.

Table 4 Number of *S. zeamais* (mean±S.E.) moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean after 72-hour (n=3): Kruskal-Wallis one-way analysis of variance.

	Number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.1	1	10	20	χ^2	*P*-value
Red kidney bean	0.0±0.0a	3.0±1.2a	6.7±2.2a	3.7±2.2a	10.3±0.9b	10.98	0.02*
Mung bean	0.0±0.0a	2.0±1.0a	5.7±1.7a	4.0±2.1a	8.3±1.2b	11.01	0.02*
Navy bean	0.0±0.0a	0.3±0.3a	10.0±2.0b	11.0±3.2b	15.0±0.6b	11.89	0.01*

[1](whole bean flour/rice grain)

Means followed by the same letter in the same row are not significantly different at $P = 0.05$.

Table 5 Number of *S. zeamais* moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean at the same concentration after 72-hour (n=3): Kruskal-Wallis one-way analysis of variance.

Concentration (% w/w)[1]	Number of *S. zeamais*			χ^2	*P*-value
	Red kidney bean	Navy bean	Mung bean		
0	0.0±0.0	0.0±0.0	0.0±0.0	NaN	NA
0.1	3.0±1.2	0.3±0.3	2.0±1.0	4.43	0.11
1	6.7±2.2	10.0±2.0	5.7±1.7	3.62	0.16
10	3.7±2.2	11.0±3.2	4.0±2.1	3.35	0.19
20	10.3±0.9b	15.0±0.6c	8.3±1.2a	6.06	0.05*

[1](whole bean flour/rice grain)

Means followed by the same letter in the same row are not significantly different at $P = 0.05$.

NaN = not a number

NA = no available

1.2 Deterrent effect of protein-enriched bean flour

The deterrent effect of rice grains treated with protein-enriched flour solution of red kidney bean and mung bean, after 24-hour, was significantly different among concentrations (Table 6). However, the deterrent effect of rice grains treated with protein-enriched flour solution of navy bean, at 24 hours, was not affected by concentrations (Table 6).

After 24-hour, the deterrent effect of rice grains treated with 1 and 5 % protein-enriched flour solution of red kidney bean was significantly higher than that of the lower concentrations (Table 6). Similar result was noticed for rice grains treated with 5 % protein-enriched flour solution of mung bean (Table 6).

After 48-and 72-hour, the deterrent effect of rice treated with protein-enriched flour solution of each bean type was significantly different among concentrations (Table 7-8).

After 48-hour, the deterrent effect of rice grains treated with 1 and 5 % protein-enriched flour solution of red kidney bean and navy bean were significantly higher than that of the lower concentrations (Table 7). The deterrent effect of rice grains treated with 5% of mung bean protein-enriched flour solution was significantly higher than that of the lower concentrations (Table 7).

After 72-hour, the deterrent effect of rice grains treated with 1 and 5 % protein-enriched flour solution of each bean type was significantly higher than that of the lower concentrations (Table 8).

Similar to whole bean flour, the deterrent effect of protein-enriched flour solution of each bean type against *S. zeamais* tended to depend on concentration and bean type. Likewise, Fields *et al*. (2001) found that there was a significant positive correlation between pea flour extract concentration and the number of adult *S. oryzae* moving out of treated milled rice. Moreover, paddy grains treated with the protein-

rich fraction of Bonneville pea flour at 1% w/w basis exposed for a period of 72 hours significantly repelled *S. oryzae*, *T. castaneum* and *R.* dominica (Mohan and Fields, 2000).

Based on the results, the deterrent effect of protein bean flour was less effective against *S. zeamais* than those previously reported. For example, Bodnaryk *et al*. (1999) found that pea protein had repellent activity against *S. oryzae*. This might be due to the protein isolation process, for which Bodnaryk *et al*. (1999) used chloroform for defatting, aqueous methanol for extraction, and silica chromatography. The key natural products of pea extracts have been separated and purified in the laboratory by thin layer, flash, ion exchange and high performance liquid chromatography (HPLC). According to Bodnaryk *et al*. (1999), protein-rich pea flour was more toxic than the pea flour itself. In contrast, bean protein in this experiment was extracted by standard procedure, with 200 ml of 20 mM phosphate buffer (PBS), pH7. The protein, in clear supernatant, was precipitated with 80% saturation ammonium sulfate. This protein precipitation process may not yield proteins with deterrent activity. Thus the difference in deterrent effect of proteins may be due to the difference in the method of protein extraction.

The less effective deterrent effect of protein from bean flour might be related to the size of bean flour used for protein extraction. The protein flour for protein extraction in this experiment was 150µ. On the contrary, pea flour of smaller grain size, 53µ, was used for extracting pea protein-enriched fraction by Pretheep-Kumar *el al*. (2004b).

Table 6 Number of *S. zeamais* (mean±S.E.) moving out of rice grains treated with protein of red kidney bean, mung bean and navy bean after 24-hour (n=3): Kruskal- Wallis one-way analysis of variance.

	Number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Type	0	0.01	0.1	1	5	χ^2	*P*-value
Red kidney bean	0.0±0.0a	0.0±0.0a	0.3±0.3a	1.0±0.6b	1.7±0.3b	9.33	0.05*
Mung bean	0.0±0.0a	0.0±0.0a	0.3±0.3a	0.3±0.3a	1.7±0.3b	9.66	0.04*
Navy bean	0.0±0.0	0.3±0.3	0.3±0.3	1.0±0.6	1.7±0.6	6.99	0.13

[1](protein-enriched bean flour/rice grain)

Means followed by the same letter in the same row are not significant difference at $P = 0.05$.

Table 7 Number of *S. zeamais* (mean±S.E.) moving out of rice grains treated with protein solution of red kidney bean, mung bean and navy bean after 48-hour (n=3): Kruskal-Wallis one-way analysis of variance.

	Number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.01	0.1	1	5	χ^2	*P*-value
Red kidney bean	0.0±0.0a	0.0±0.0a	0.3±0.3a	1.3±0.3b	2.0±0.0b	12.28	0.01*
Mung bean	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.7±0.3b	1.7±0.3b	11.68	0.01*
Navy bean	0.0±0.0a	0.3±0.3a	0.3±0.3a	1.3±0.3b	2.3±0.3b	11.08	0.02*

[1](protein-enriched bean flour/rice grain)

Means followed by the same letter in the same row are not significant difference at $P = 0.05$.

Table 8 Number of *S. zeamais* (mean±S.E.) moving out of rice grains treated with protein of red kidney bean, mung bean and navy bean after 72-hour (n=3): Kruskal-Wallis one-way analysis of variance.

	Number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.01	0.1	1	5	χ^2	*P*-value
Red kidney bean	0.0±0.0a	0.0±0.0a	0.3±0.3a	1.3±0.3b	2.0±0.0b	12.28	0.01*
Mung bean	0.0±0.0a	0.3±0.3a	0.0±0.0a	1.0±0.0b	3.0±0.5b	12.44	0.01*
Navy bean	0.0±0.0a	0.0±0.0a	0.3±0.3a	1.7±0.3b	3.0±0.5b	12.48	0.01*

[1](protein-enriched bean flour/rice grain)

Means followed by the same letter in the same row are not significant difference at $P = 0.05$.

2. Penetration test

The number of holes that *S. zeamais* penetrated polyethylene sheets treated with 5% protein flour solution of red kidney bean was significantly higher than that of the untreated control (Table 9). The number of holes on polyethylene sheets coated with 5% protein flour solution of mung bean and navy bean was not significantly different from the control. This study shows that polyethylene sheets coated with protein flour solution of the three beans cannot prevent insect penetration. On the other hand, Hou *et al*. (2004b) revealed that protein-enriched pea flour alone significantly reduced the number of *S. oryzae* penetrating treated napkin paper, compared to pea protein fractions. They also found that DEET treated paper bags were not penetrated by stored-product insects. Neem was repellent enough to reduce insect immigration into package. Mohan *et al*. (2007) found that adults of *S. oryzae and R. dominica* penetrated polyethylene sheets (0.0635mm thickness) coated with protein-enriched pea flour solution at 0, 1 and 5% levels, but did not penetrate the sheets coated with a 10% concentration.

The difference in prevention of insect penetration by bean or pea protein may due to the protein extraction and isolation processes. These processes may destroy the bioactive ingredient that prevents insect penetration. This was supported by Hou *et al*. (2004b) who suggested that repellent compounds which was removed by chloroform, or the defatting procedure destroyed the repellent compounds in the protein enriched pea flour.

Based on the result, the bean protein may attract maize weevil into packages so it would not provide protection to packaging. This was confirmed by Hou *et al*. (2004b). Otherwise, protein may have a plasticizing action and is not compatible with polyethylene sheet, which are currently used in manyfood packages (Hou *et al*., 2004b). Hou *et al*. (2004b) found that DEET has a plasticizing action and is not compatible with wax paper and plastic sheet.

Packages made of polyethylene sheet can now be impregnated with anti-feedant compounds to prevent insect penetration (Graham *et al*., 2002). These results

indicate that polyethylene sheet coated with protein bean flour solution did not have the potential to prevent penetration of *S. zeamais*. However, Fields *et al*. (2001) suggested that pea protein is well suited as a natural stored-grain protectant. In addition, Pretheep-kumar *et al*. (2004b) found that protein-enriched pea flour did not affect the appearance, flavor, taste, texture and overall acceptability of rice.

Table 9 Number of *S. zeamais* (mean±S.E.) that penetrated polyethylene sheet coated with protein solution of red kidney bean, mung bean and navy bean (N=3): Kruskal-Wallis one-way analysis of variance.

	Number of hole			
	Concentration (% w/w)[1]			
Bean Type	0	5	χ^2	*P*-value
Red kidney bean	0.3±0.3a	2.7±0.6b	4.09	0.04*
Mung bean	0.7±0.3	2.3±0.6	2.72	0.09
Navy bean	0.3±0.3	1.7±0.3	3.33	0.06

[1](whole bean flour/rice grain)

Means followed by the same letter in the same row are not significant difference at $P = 0.05$.

3. Optimum temperature of α-amylase from maize weevil

The α-amylase activity was determined at 30°C to 80°C. The optimum temperature was at 60°C. The enzyme activity sharply declined at higher temperatures (Fig 5). The relatively high optimal temperature for α-amylase of *S. zeamais* may result from adaptation to high temperature of the surrounding environment as in the silo.

This condition differed from those found in other insect amylases, such as α-amylases from *Z. subfasciatus*, *T. castaneum* and *T. molitor*, which showed higher activities at 37 °C (Sivakumar *et al.*, 2006).

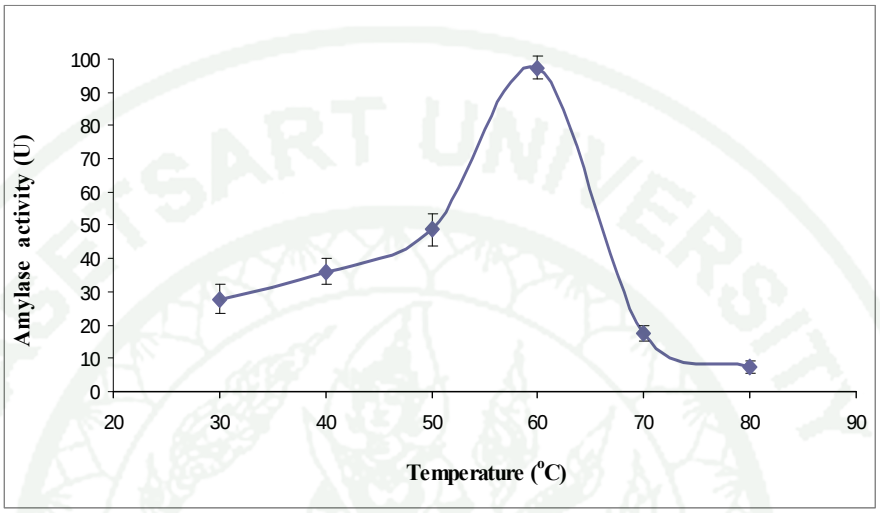


Figure 5 Effect of the temperature on *S. zeamai*s α-amylase activity.

4. Optimum pH of α-amylase from maize weevil

The effect of pH, 2 to12, on the α-amylase activity is shown in Figure 6. The optimum pH for *S. zeamais* α-amylase activity was at pH 6. This finding was similar to several earlier reports on pH optima for amylase from *V. angularis* (Mar *et al*., 2003), *Z. subfasciatus* (Pelegrini *et al*., 2006) and *C. chinensis* (Podoler and Applebaum, 1971).

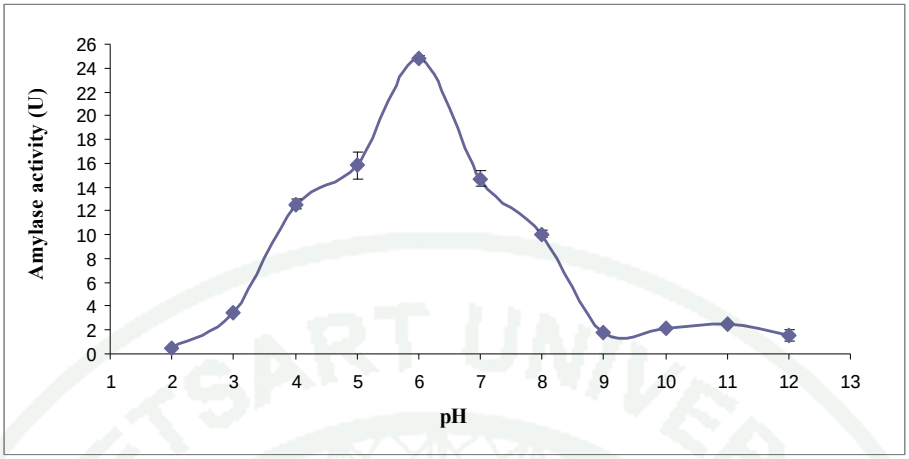


Figure 6 Effect of the pH on *S. zeamais* α-amylase activity.

5. Effects of crude protein on activities of *S. zeamais* α-amylase, *C. maculatus* α-amylase, human salivary α-amylase and barley malt α-amylase.

The inhibitory effect of crude protein from mung bean, red kidney bean and navy bean was tested against α-amylase of *S. zeamais* α-amylase, *C. maculatus*, human salivary and of barley malt. The optimum condition was pH 6, at 60 °C for *S. zeamais* α-amylase, pH 6, at 50 °C for *C. maculatus* α-amylase, pH 7 (Wisessing, 2008), at 37 °C for human salivary α-amylase and pH 4, at 50°C for barley malt α-amylase (Bannakan, 2007).

α-Amylase activities (10 mg/ml) of *S. zeamais*, *C. maculatus*, human salivary and barley malt preincubated with crude protein from mung bean, red kidney bean and navy bean (Fig 7). α-Amylase of *S. zeamais* was inhibited by crude protein from all bean types. The inhibitory effect of inhibitor from mung bean was greater than that of navy bean and red kidney bean. Likewise, α-amylase of *C. maculatus* was inhibited by crude protein from three bean types. The inhibitory effect of crude protein from mung bean was greater than that of red kidney bean and navy bean.

This result was supported by Pueyo (1995) who reported that insect α-amylase was inhibited by proteinaceous inhibitors from red kidney bean *(P. vulgaris)*. This was in agreement with the results of the experiment by Bannakan *et al*. (2007) who

reported that α-amylase inhibitor of seed meal from four mung bean varieties/lines inhibited *C. maculatus* amylases 100 percent.

Barley malt α-amylase was inhibited by mung bean α-amylase inhibitor as well as the navy bean α-amylase inhibitor and followed by red kidney bean α-amylase inhibitor. This result was accorded with Bannakan *et al*. (2007) who found that the inhibitors from both standard and mutant lines giving 100 percent inhibition for α-amylase of barley malt.

Human salivary α-amylase was unlikely to be inhibited by α-amylase inhibitors from all three beans.

α-Amylase inhibitors from all three beans were classified as αAI-2 type because they inhibited α-amylase from *S. zeamais*, *C. maculatus* and barley malt but not human salivary. Similar result was previously reported in *Z. subfasciatus* by Grossi de Sa *et al*. (1997). α-Amylase inhibitors from three beans may not directly affect deterrent activity against *S. zeamais*. α-Amylase inhibitors in treated rice may affect *S. zeamais* by inhibiting digestion necessary for growth and development. As a result, the maize weevils were deterred from treated rice and urged to find more suitable food. These inhibitors have potential to be used as insecticidal protein for controlling insect pests without any harm to comsumers and environment.

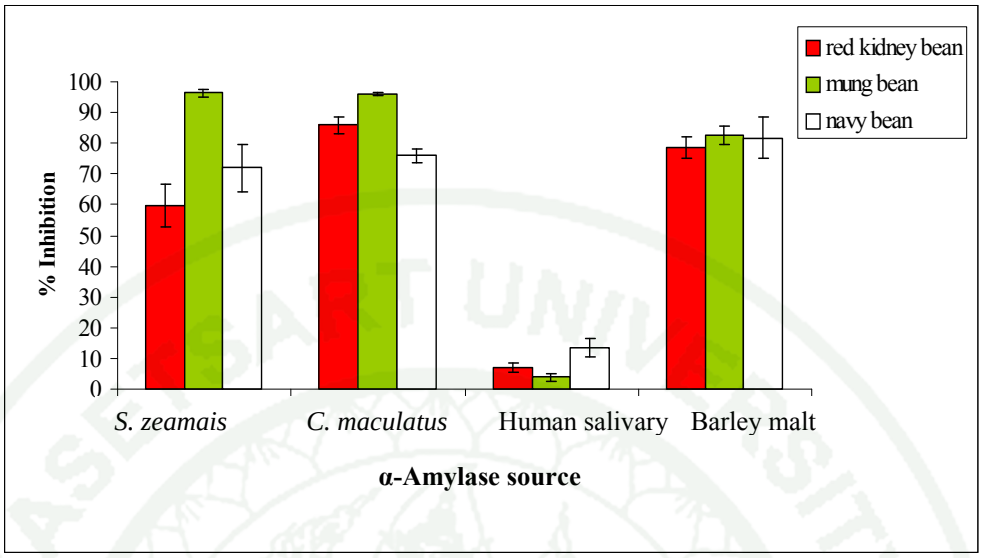


Figure 7 Percent inhibition of α-amylase inhibitor from red kidney bean, mung bean, and navy bean against α-amylase of *S. zeamais*, *C. maculatus*, human salivary and barley malt

CONCLUSIONS

The whole flour of red kidney bean, mung bean and navy bean could be used as deterrent against *S. zeamais*. The deterrent effect of rice treated with 20% whole flour was significantly different among bean types. The deterrent effect of whole bean flour in this study could be reflected by reduced number of maize weevils on treated rice grains. The deterrent effect of whole bean flour may be due to the flour particle, 150μ that causes irritation of spiracle. Based on the results, the deterrent effect of whole bean flour tended to depend on concentration and bean type.

Protein flour solution of red kidney bean, mumg bean and navy bean treated with rice grains has little deterrent effect against *S. zeamais.* However, these bean protein solutions increased insect moving out of the rice grains when compared with the control. The deterrent effect of protein bean flour also tended to depend on concentration and bean type. Protein flour solution does not deterring *S. zeamais* due to protein isolation process and size of bean flour. The ineffectiveness of protein flour solution as deterrent may be due to large size of bean flour used in protein isolation process.

Polyethylene sheet coated with protein flour solution of red kidney bean, mung bean and navy bean cannot prevent *S. zeamais* penetration. Protein bean flour solution may attract maize weevil into packages so it would not provide protection to packaging.

α-Amylase inhibitors from all three beans were classified as αAI-2 type because they inhibited α-amylase from *S. zeamais*, *C. maculatus* and barley malt but not human salivary. α-Amylase inhibitors play an important role in indirectly deterred *S. zeamais* from the treated rice.

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APPENDICES

Appendix A

Deterrent test

1. Deterrent test

1.1 Deterrent effect of whole bean flour

Appendix Table A1 The median number of *S. zeamais* moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean after 24-hour: Kruskal-Wallis one-way analysis of variance table.

	Median number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.1	1	10	20	χ^2	*P*-value
Red kidney bean	0	1	3	5	5	6.09	0.19
Mung bean	0	1	5	1	6	5.27	0.25
Navy bean	0	0	8	12	10	8.46	0.07

[1](whole bean flour/rice grain)

* Significantly different at *P*=0.05

Appendix Table A 2 The median number of *S. zeamais* moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean after 48-hour: Kruskal-Wallis one-way analysis of variance table.

	Median number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.1	1	10	20	χ^2	*P*-value
Red kidney bean	0	1	2	2	1	8.97	0.06
Mung bean	0	1	3	1	2	7.49	0.11
Navy bean	0	0	5	7	3	10.90	0.02*

[1](whole bean flour/rice grain)

* Significantly different at *P*=0.05

Appendix Table A 3 Comparison of differences in observed values in the number of *S. zeamais* moving out of rice grains treated with whole flour of navy bean in comparison to untreated control after 48-hour.

Concentration (% w/w)[1]	Difference in observed values			
	Concentration (% w/w)[1]			
	0.1	1	10	20)
0	0.00 (NS)	7.00* (Sig.)	7.67* (Sig.)	7.83* (Sig.)

[1](whole bean flour/rice grain)

Critical difference = 4.68

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 4 The median number of *S. zeamais* moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean after 72-hour: Kruskal-Wallis one-way analysis of variance table.

	Median number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.1	1	10	20	χ^2	*P*-value
Red kidney bean	0	3	5	2	10	10.98	0.02*
Mung bean	0	1	4	3	9	11.01	0.02*
Navy bean	0	0	12	12	15	11.89	0.01*

[1](whole bean flour/rice grain)

* Significantly different at *P*=0.05

Appendix Table A 5 Comparison of differences betweens concentration in observed values of the number of *S. zeamais* moving out of rice grains treated with whole flour of red kidney bean after 72 hours exposure.

Concentration (% w/w)[1]	Difference in observed values Concentration (% w/w)[1]			
	0.1	1	10	20
0	5.00 (NS)	8.50 (NS)	5.17 (NS)	11.33* (Sig.)
0.1	-	3.50 (NS)	0.17 (NS)	6.33 (NS)
1	-	-	3.33 (NS)	2.83 (NS)
10	-	-	-	6.17 (NS)

[1](whole bean flour/rice grain)

Critical difference = 10.25

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 6 Comparison of differences between concentrations in observed values of the number of *S. zeamais* moving out of rice grains treated with whole flour of mung bean after 72-hour.

Concentration (% w/w)[1]	Difference in observed values			
	Concentration (% w/w)[1]			
	0.1	1	10	20
0	4.33 (NS)	8.50 (NS)	6.00 (NS)	11.17* (Sig.)
0.1	-	4.17 (NS)	1.67 (NS)	6.83 (NS)
1	-	-	2.50 (NS)	2.67 (NS)
10	-	-	-	5.17 (NS)

[1](whole bean flour/rice grain)

Critical difference = 10.25

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 7 Comparison of differences between concentrations in observed values of the number of *S. zeamais* moving out of rice grains treated with whole flour of navy bean after 72-hour.

Concentration (% w/w)[1]	Difference in observed values			
	Concentration (% w/w)[1]			
	0.1	1	10	20
0	1.00 (NS)	6.33 (NS)	7.50 (NS)	10.16 (NS)
0.1	-	5.33 (NS)	6.50 (NS)	9.17 (NS)
1	-	-	1.17 (NS)	3.83 (NS)
10	-	-	-	2.67 (NS)

[1](whole bean flour/rice grain)

Critical difference = 10.25

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 8 Comparison of differences in observed values in the number of *S. zeamais* moving out of rice grains treated with whole flour of navy bean in comparison to untreated control after 72-hour.

Concentration (% w/w)[1]	Difference in observed values			
	Concentration (% w/w)[1]			
	0.1	1	10	20
0	1.00 (NS)	6.33* (Sig.)	7.50* (Sig.)	10.16* (Sig.)

[1](whole bean flour/rice grain)

Critical difference = 4.68

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 9 Comparison median number of *S. zeamais* moving out of rice grains treated with whole flour of red kidney bean, mung bean and navy bean at the same concentration after 72 hours exposure.

Concentration (% w/w)[1]	Median number of *S. zeamais*			χ^2	*P*-value
	Red kidney bean	Navy bean	Mung bean		
0	0	0	0	NaN	NA
0.1	3	0	1	4.43	0.11
1	5	12	4	3.62	0.16
10	2	12	3	3.35	0.19
20	10	15	9	6.06	0.05*

[1](whole bean flour/rice grain)

* Significantly difference at *P*=0.05

NaN = not a number

NA = no available

1.2 Deterrent effect of protein enriched bean flour

Appendix Table A 10 The median number of *S. zeamais* moving out of rice grains treated with protein of red kidney bean, mung bean and navy bean after 24-hour: Kruskal- Wallis one-way analysis of variance table.

	Median number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.01	0.1	1	5	χ^2	*P*-value
Red kidney bean	0	0	0	1	2	9.33	0.05*
Mung bean	0	0	0	0	2	9.66	0.04*
Navy bean	0	0	0	0	1	6.99	0.13

[1](whole bean flour/rice grain)

* Significantly difference at *P*=0.05

Appendix Table A 11 Comparison of differences in observed values in the number of *S. zeamais* moving out of rice grains treated with protein from red kidney bean and mung bean, in comparison to untreatedcontrol after 24-hour

		Difference in observed values			
		Concentration (% w/w)[1]			
Bean types	Control	0.01	0.1	1	5
Red kidney bean	0	0.00 (NS)	2.00 (NS)	5.00* (NS)	8.00* (Sig.)
Mung bean	0	0.00 (NS)	2.17 (NS)	2.17 (NS)	8.17* (Sig.)

[1](whole bean flour/rice grain)

Critical difference = 4.68

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 12 The median number of *S. zeamais* moving out of rice grains treated with protein solution of red kidney bean, mung bean and navy bean after 48-hour: Kruskal-Wallis one-way analysis of variance table.

	Median number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.01	0.1	1	5	χ^2	*P*-value
Red kidney bean	0	0	0	1	2	12.28	0.01*
Mung bean	0	0	0	1	2	11.68	0.01*
Navy bean	0	0	0	1	2	11.08	0.02*

[1](whole bean flour/rice grain)

* Significantly difference at P=0.05

Appendix Table A 13 Comparison of differences in observed values in the number of *S. zeamais* moving out of rice grains treated with protein from red kidney bean, mung bean, and navy bean in comparison to untreated control after 48-hour

		Difference in observed values			
		Concentration (% w/w)[1]			
Bean types	Control	0.01	0.1	1	5
Red kidney bean	0	0.00 (NS)	1.83 (NS)	6.67* (NS)	9.00* (Sig.)
Mung bean	0	0.00 (NS)	0.00 (NS)	4.33 (NS)	8.17* (Sig.)
Navy bean	0	1.83 (NS)	1.83 (NS)	6.67* (Sig.)	9.67* (Sig.)

[1](whole bean flour/rice grain)
Critical difference = 4.68

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference

Appendix Table A 14 The median number of *S. zeamais* moving out of rice grains treated with protein of red kidney bean, mung bean and navy bean after 72-hour: Kruskal-Wallis one-way analysis of variance table.

	Median number of *S. zeamais*						
	Concentration (% w/w)[1]						
Bean Types	0	0.01	0.1	1	5	χ^2	*P*-value
Red kidney bean	0	0	0	1	2	12.28	0.01*
Mung bean	0	0	0	1	3	12.44	0.01*
Navy bean	0	0	0	2	3	12.48	0.01*

[1](whole bean flour/rice grain)

* Significantly difference at *P*=0.05

Appendix Table A 15 Comparison of differences in observed values in the number of *S. zeamais* moving out of rice grains treated with protein of red kidney bean, mung bean and navy bean in comparison to untreated control after 72-hour.

		Difference in observed values			
		Concentration (% w/w)[1]			
Bean types	Control	0.01	0.1	1	5
Red kidney bean	0	0.00	1.83	6.67*	9.00*
		(NS)	(NS)	(Sig.)	(Sig.)
Mung bean	0	2.00	0.00	6.00*	9.50*
		(NS)	(NS)	(Sig.)	(Sig.)
Navy bean	0	0.00	1.67	6.67*	9.17*
		(NS)	(NS)	(Sig.)	(Sig.)

[1](whole bean flour/rice grain)

Critical difference = 4.68

* Significant difference (difference in observed values > critical difference at *P*=0.05)

NS, not significant difference.

2. Penetration test

Appendix Table A 16 The median number of *S. zeamais* that penetrated polyethylene sheet coated with protein solution of red kidney bean, mung bean and navy bean: Kruskal-Wallis one-way analysis of variance table.

	Median number of hole			
	Concentration (% w/w)[1]			
Bean Types	0	5	χ^2	*P*-value
Red kidney bean	0	2	4.09	0.04*
Mung bean	1	3	2.72	0.09
Navy bean	0	2	3.33	0.06

[1](whole bean flour/rice grain)

* Significantly difference at *P*=0.05

Appendix B

α-Amylase inhibitor

Protein Determination

Lowry's method (Lowry *et al*., 1951)

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: Dissolving sodium carbonate 10 g in 960 ml distilled water with 3 N NaOH 35 ml

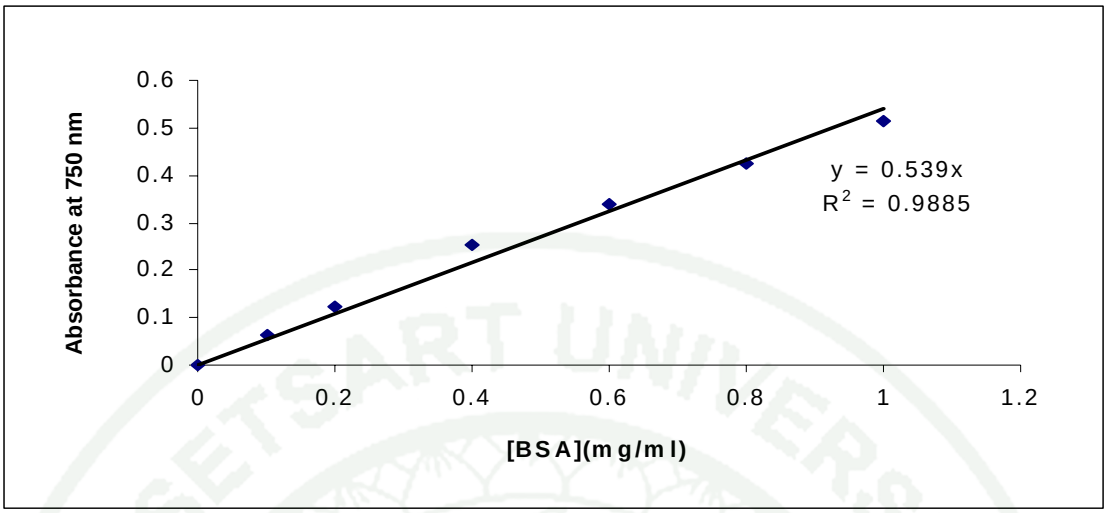
B: Dissolveing copper sulfate 1 g in 100 ml distilled water

C: Dissolving potassium sodium tartrate 2 g in 100 ml distilled water

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

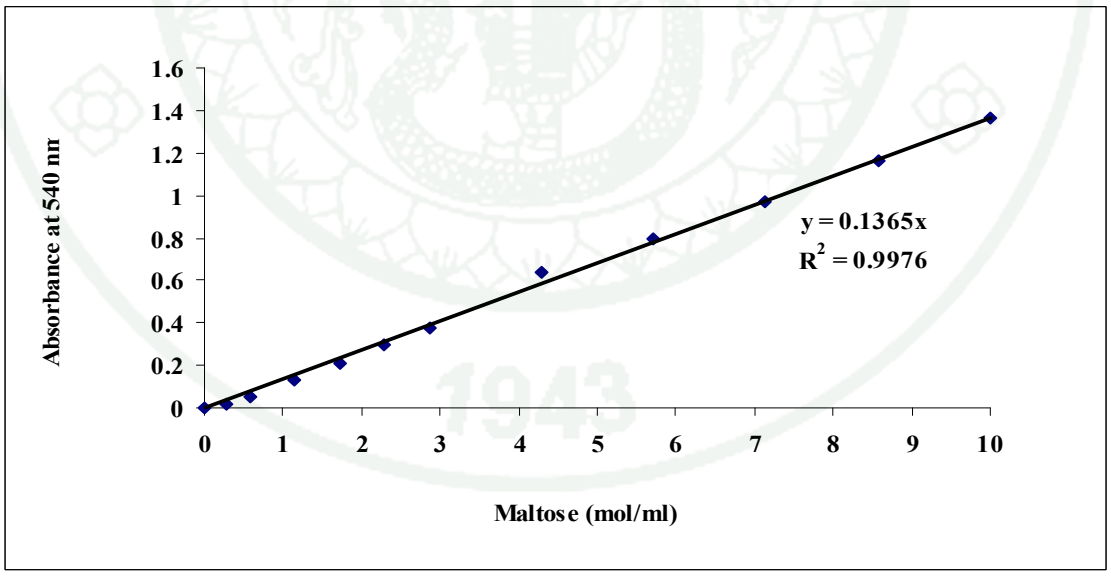
DNS Color Reagent

1. Dissolving 3, 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



Appendix Figure B 1 Standard curve of Bovine Serum Albumin (BSA) for protein determination.

α-Amylase activity



Appendix Figure B 2 Standard curves for the determination of maltose.

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