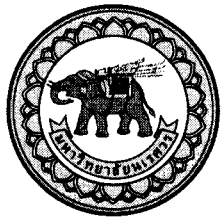

รายงานวิจัยฉบับสมบูรณ์

โครงการ การโคลนและการศึกษาคุณสมบัติทางชีวเคมีของเอนไซม์แอลฟ่าอะไมเลสจากมอดข้าวสาร

cDNA cloning and biochemical characterization of alpha-amylase from rice weevil (Sitophilus oryaze L.)

โดย รองศาสตราจารย์ ดร.สุขกิจ ยะโสธรศรีกุล และคณะ

เดือนกันยายน 2555

สัญญาเลขที่ R2554B082

รายงานวิจัยฉบับสมบูรณ์

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คณะผู้วิจัย สังกัด

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คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร

สนับสนุนโดยงบประมาณแผ่นดินปี 2554 มหาวิทยาลัยนเรศวร

สรุปรายงานการวิจัย

โครงการวิจัยเรื่อง การโคลนและการศึกษาคุณสมบัติทางชีวเคมีของเอนไซม์แอลฟ่าอะไมเลสจากมอดข้าวสาร
(รหัสโครงการ R2554B084)

CHAPTER I

INTRODUCTION

Rationale of the study

Rice is one of the most important agricultural products of Thailand and also very important for Thai's life (Srinives, Charinpanitkul and Tanthapanichakoon, 2005). It is the most important staple food crop of Thai's population. In addition, it is played a pivotal role in economy of Thailand throughout its history (Chongkid, 2004; Pitathawatchai, 2005; Choeun, Godo and Hayami, 2006). Thais have cultivated rice for a long time and have improved it continuously. Rice cultivation has many caused that reduce productivity such as rice diseases, rice insect pests, animal pests. Furthermore, stored-grains product have interfered with insect pests for example Angoumonis grain moth (Boshra, 2007), Rice moth (Coelho, Marangoni and Macedo, 2007), Lesser grain borer (Bashir, et al., 2003) and Rice Weevil (*Sitophilus oryzae*) (Grenier, Mbaiguinam and Delobel, 1997; Jutharat Attajarusit, 2003; Ministry of Agriculture and Cooperatives, 2005).

Rice weevil (*Sitophilus oryzae*) is one of the main disasters which cause rice damage in many countries. It is considered a primary stored-grain insects in warm climate areas and cause significant losses to stored grains, especially cereals (Batta, 2004). Moreover, this specie is a primary pest that can easily infest within the hollow husk of the grain kernel (Athanassiou, 2006).

The rice insect pest can produce alpha-amylase enzyme to hydrolyze alpha (1, 4) glycosidic bonds of amylose and amylopectin in rice grains for their growth and development. Alpha-amylase plays an important role in carbohydrate metabolism. Organisms with a starch-rich diet depend on the effectiveness of it amylases for survival (Titarenko, et al., 2000; Grossi DE SA, M. F. and Chrispeels, M. J., 1997; MacGrego, et al., 1993; Strobl, et al., 1997).

The current trend about stored-product pest management is to use chemical control (insecticides), which are the most efficient and effective methods. However, several problems have emerged with the use of conventional chemical control methods such as the increasing cost of inorganic chemical, hazards to the environmental including the resistance of many species of stored-product pests (Vardeman, et al., 2006). A recent review describes that plant inhibitors active towards insect a-amylases and includes aspects of transgenic plants against insect predators in order to control endogenous alpha-amylase activity or defend against pathogens and pests (Svensson, et al., 2004).

Plant seeds are rich sources of a large number of different proteinaceous inhibitors acting on alpha-amylase or other polysaccharide processing enzymes. Therefore, alpha-amylase inhibitors are effective against stored-product pests that rely on starch as a major food source (Pytelkova, et al., 2009). The alpha-amylase inhibitors are found in nature, it can recognize to different alpha-amylases. In addition, some alpha-amylase inhibitors have high affinity to both mammalian and insect alpha-amylases (Svensson, et al., 2004).

Understanding biochemical properties of alpha-amylase enzyme will probably help us control the damage of stored rice grains by rice weevil in the future. Moreover, cloning and analyse nucleotide sequence of alpha-amylase from rice weevil (*Sitophilus oryzae*) has not been investigated. Therefore, the objective of this research is cloning and analysing nucleotide sequences of alpha-amylase genes from rice weevil to get the information for further study of its susceptibility to alpha-amylase inhibitor in rice.

Objectives of this study

1. To clone and analyse nucleotide sequence of alpha-amylase genes from rice weevil (*Sitophilus oryzae*).

2. To investigate biochemical properties of alpha-amylases from rice weevil (*Sitophilus oryzae*) against rice alpha-amylase inhibitor.

Scope of this study

1. The insect used in this experiment was adult stage of rice weevil (*Sitophilus oryzae*).

2. The RNA was extracted from rice weevil (*Sitophilus oryzae*).

3. The total RNA was used as a template for generating complementary DNA using Reverse transcriptase-polymerase chain reaction (RT-PCR) method.

4. The cDNA was cloned by TA cloning and nucleotide sequences were analyzed by DNA sequencing.

5. The full-length of alpha-amylase gene was generated using 5'/3' RACE PCR amplification.

6. The protein expression and biochemical properties were investigated.

Hypotheses

The rice weevil is one of the rice damage causes by alpha-amylase enzyme that hydrolyses alpha (1, 4) glycosidic bonds of amylose and amylopectin in rice grains. If nucleotide sequences, enzyme activity and biochemical properties of alpha-amylase enzyme from rice weevil are known, relevance measures can be developed to protect rice damage from this insect pest.

Anticipated Outcomes

1. To provide Nucleotide sequences of rice weevil (*Sitophilus oryzae*) and information for studying alpha-amylase of other insect.

2. To study biochemical properties of rice weevil alpha-amylase.

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Rice (*Oryza sativa* L.)

Rice (*Oryza sativa*) is the most important food crops and staple food of the world's living populations, especially in Asia, Middle East, Latin America and the West Indies (Chun-hong, et al., 2010). Cultivated rice is the Gramineae or grass family and the clan Oryzeae. There are two distinct types of rice, Oryza sativa (Asian rice) and Oryza glaberrima (African rice) as shown in Figure 1 (Sweeney and Mccouch, 2007; Samuel, 1991).

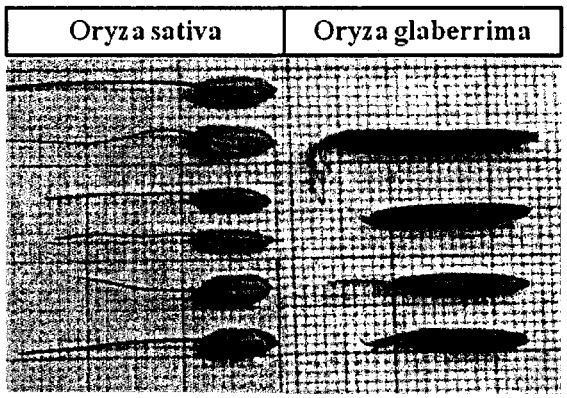


Figure 1 *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice)

Source: http://www.hongthongrice.com/knowledge_detail-28.html

Components of rice seed

Rice is composed of protein, starch, lipids, minerals, and other constituents. The most components of rice seed are starch by found that milled rice has about 84 to over 90% starch. Starch consists of two types of molecules, amylose and amylopectin (Figure 2). Amylose is necessary a linear α-1,4-linked glucan, whereas amylopectin has various branch points that form by α-1,6 linkages joining linear chains (Yao, Zhang and Ding, 2002; Samuel, 1991).

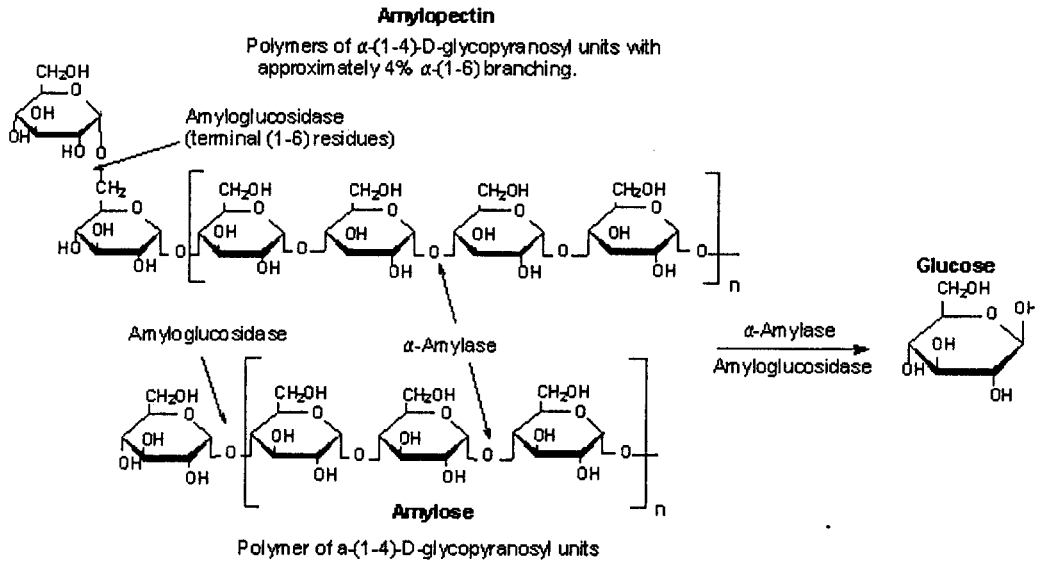


Figure 2 Structure of starch consist of amylopectin and amylase.

Source: http://www.sigmaaldrich.com/life-science/metabolomics/enzymatic-kits.html

Rice pest

The problems of rice cultivation in tropical area are rice diseases, rice animal pests, rice weeds and rice insect pests. They happen every year and effect to rice yield loss (Ministry of Agriculture and Cooperatives, 2005). Moreover, the pathogens such as fungi, bacteria and virus cause rice damage (Franco, et al., 2002).

1. Rice diseases

Rice diseases consist of fungal, bacterial, viral, mycoplasma and nematode diseases. The example fungal diseases are Blast, Narrow brown spot, Brown spot and Sheath blight. The example bacterial diseases are Bacterial blight and Bacterial leaf streak. The example viral diseases are Yellow orange leaf disease, tugro and Ragged stunt. The example mycoplasma disease is Orange leaf disease. The example nematode diseases are Rice root nematode, *Hirschmanniella oryzae*, Rice stem nematode, *Ditylenchus angustus*, and White tip nematode, *Aphelenchoides besseyi* (Figure 3) (Zhan-yu, Jing-feng and Rong-xiang, 2008; Samuel, 1991).

Rice diseases management has several methods such as host resistance, cultural management and chemical control (Mongkol Chan, 1993).

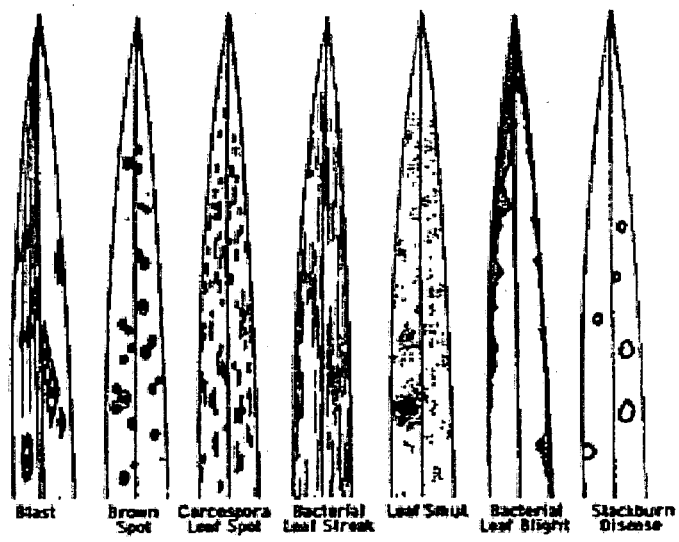


Figure 3 Typical leaf lesions of seven rice diseases

Source: Samuel, 1991

2. Rice animal pests

Rice animal pests that found in Thailand are spoted munia, ricefield crab, roof rat, ship rat, ricefield rat, great bandicoot, lesser bandicoot and golden apple snail. The golden apple snail (*Pomacea canaliculata Lamarck*) (Figure 4) is a major rice pest in a number of Asian countries, including Taiwan, Japan, the Philippines, Indonesia and Thailand. If it is not controlled it can cause devastative damage to rice crops, thus incurring huge loss to farmers. The adult snails cut the young paddy seedlings or tillers that are less than 21 days old. Attacks mainly take place during the night. The signs of the attack are the cut rice tillers floating in the water. Attacks tend to be worse in areas where the water is more than 1 cm deep, or in stagnant water.

Controlling the snails is difficult and costly. The snails are prolific and females may lay 2000 - 3000 eggs in one year. The natural predator is unknown and the use of pesticides may kill other types of beneficial snails and aquatic organisms. Current measures to control the snails are quarantine action, collecting, destroying eggs and adults, as well as introducing ducks to feed on the snails. Sometimes the snails are gathered in the field, crushed and fed to penned ducks (Zubir Bidin, 2002).

Figure 4 Golden apple snail (*Pomacea canaliculata Lamarck*)

Source: Food and agriculture organization of the United Nations

3. Rice weeds

Rice weeds are a co-major problem in rice production in Thailand. The results of weed have effected to yield loss, thus incurring huge loss to farmers (Vongsaroj, 1993). Weeds that found in rice field, there are varied species such as *Echinochloa* spp., *Ageratum conyzoides* L., *Paspalum distichum* L., and *Cyperus difformis* L. Some weeds compete more with cereals because of their similar growth behavior, rooting profile and nutrient requirements (Manandhar, Shrestha and Lekhak, 2007).

Considerable progress in weed control has been achieved with various measures such as ensuring the purity of rice seed, proper selection of cultivar and seeding rate, proper planting method, good land preparation and water management, hand weeding and chemical weed control and crop rotation used together in a system of integrated weed management (Samuel, 1991).

4. Rice insect pests

Many insects are found in rice field and stored-product. Only a few insects are considered as rice pests. Insect pests damage rice by feeding on the leaves, stems, roots, and grain (University of Arkansas, 2010). The rice insect pests can be classified into 2 groups. The first group is insect pests that attack all stages of plant growth and the second group is insect pests of stored-product. The examples of the insect pest that attack all stages of plant growth are rice stem borers, leafhoppers and plant hoppers and grain sucking insects and root feeders. The examples of the insect pests of stored-product are Angoumois grain moth, Rice moth, Lesser grain borer and Rice Weevil.

4.1 The insect pests that attack all stages of plant growth

4.1.1 Rice stem borers

The rice stem borers are important pests of rice-growing throughout Asia. Rice stem borers infest plants from the seedling stage to maturity. The larvae can inflict three forms of damage consisting of yellowing leaf sheaths, exit holes on the stem and broken stems. Stem borers are difficult to control with insecticides because they hide within the stems where they are protected (Sudhir, 2003).

4.1.2 Leafhoppers and Plant hoppers

Leafhoppers are one of the largest families of plant-feeding insects. The nymphs and adults of the leafhopper pierce and suck the sap of tender plant shoots. The female adults lay eggs into the tender stems which results in leaf edge yellowing, leaf tine curling, vein reddening, plant shoot growing slowly and stagnating and withering. The leafhopper usually causes significant yield loss and deterioration in plant quality. They can move very quickly when disturbed. Therefore, it is very difficult to control (Jin, M. and Baoyu, H, 2007).

Plant hoppers in the order Hemiptera remove xylem and phloem sap from the leaves and stems of rice. Excessive feeding causes plants to wilt. It acts as vectors in transmitting rice viruses that cause rice diseases.

4.1.3 Grain sucking insects and Root feeders

Grain sucking insects are several species in the Heteroptera suborder attack developing rice grains. Both nymphs and adults feed on the grain by inserting their sucking mouthparts between the lemma and the palea. They prefer rice at the milk stage but will also feed on soft and hard dough rice grains. Removal of the liquid milky white endosperm results in small and unfilled grains. When the bugs feed on soft or hard dough endosperm, they inject enzymes to predigest the carbohydrate. In the process, they contaminate the grain with microorganisms that cause grain discoloration or "pecky" rice. Damage from feeding at this stage reduces grain quality rather than weight. Pecky rice grains are prone to break during milling (Heinrichs and Barrio, 2004).

Root feeders are normally found in well-drained fields and are not a problem in irrigated environments. The example root feeders are termites (order Isoptera) and the rice water weevil; *Lissorhoptrus oryzophilus* (order Coleoptera). Termites occur in patches and often kill the plants, especially when rainfall is lacking. The water weevil is a major insect pest of irrigated rice in the USA. The adult water weevil feeds on the leaves and causes little damage while the larvae feed on the roots and severely reduce the root system. Plants with reduced root systems grow poorly and have low yields (Heinrichs and Barrio, 2004).

4.2 Stored-product insects

4.2.1 Angoumois grain moth, *Sitotroga cerealella* Oliv, Lepidoptera: Gelechiidae

The Angoumois grain moth, *Sitotroga cerealella* (Olivier), is a cosmopolitan pest of stored corn, wheat, rice, barley, sorghum, and other cereals, and it also attacks cereals in the field before harvest. This insect develops within grain kernels, causing considerable direct damage, as well as making the grain a more suitable medium for reproduction of secondary insect pests (Boshra, 2007).

The Angoumois grain moth deposits its eggs singly or in groups on or near grain, and newly hatched larvae burrow into the kernels or enter through cracks in the pericarp (Figure 5). Larval-pupal development is completed within the kernel and pupation occurs in a silk-lined chamber in the burrow. Before pupation, the larva cuts a channel to the outside, leaving only a weakly fastened flap of pericarp through which the adult moth will emerge (Arbogast and Mullen, 1987).

The life cycle of this insect varies with temperature, relative humidity and diet. Previous study reported that total development time of this insect from egg to adult was completed in 25 days when reared in sorghum at 30°C and 70% RH. Total development time was 28 days when the insects were reared on corn kernels mixed with some flour at 30°C and 80% RH and 36 days when the insects were reared in corn at ambient temperature and relative humidity. The larva is dormant for 4 to 5 months during the winter in colder climates. There are generally 4 to 5 generations per year, although in heated warehouses there may be as many as 10 to 12 generations (Perez-mendoza, Weaver and Throne, 2004; Mason, 2003).

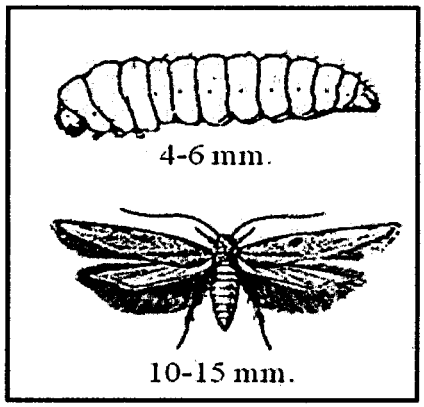


Figure 5 Angoumois grain moth, *Sitotroga cerealella* Oliv

Source: Linda, 2003

4.2.2 Rice moth, *Corcyra cephalonica* Stal, Lepidoptera: Pyralidae

Rice moth, *Corcyra cephalonica* Stal is a worldwide-distributed insect adapted to warm humid environments. The larvae are general feeders and considered one of the key pests of rice, cocoa, biscuits, pearl millet, sorghum and seeds. Larvae also contaminate foods by secreting silken threads that web together food particles, dusts and frass "debris or excrement produced by insects".

The adult moth has pale buff-brown forewings with 12-15 mm. wingspan, hindwings almost transparent and uniform in colour (Figure 6). The wing tips are rounded and tightly folded to the body when at rest. There are no distinctive markings on the wings, although veins may be slightly darkened. The larvae are dull white with long fine hair and dark brown head. Cocoons are white in colour. Adults do not feed and live for 1 to 2 weeks. Through this period and within short-range, rice moth males are attracted to the female abdominal-tip pheromone that causes the male moths to search for a mate and attempt copulation. Mated females lay from 100 to 200 eggs near food sources. Eggs hatch in 4-10 days. Larvae spin silken threads as they feed and web grains, debris and other particles into galleries in which they live and feed. When they are fully-grown, they form dense white cocoons to pupate. Pupae are usually found in food or they may be found between pallets and sacks. Adults emerge from pupae within 4 to 8 weeks and repeat their life cycle. At optimal temperatures of

86 - 90.5°F and relative humidity of 70%, the life cycle lasts 28-35 days. The low minimum temperature and low relative humidity (RH) of the rice moth are 60°F and 20%, respectively. However, there are up to six generations per year in tropical countries and one generation in temperate climate. Control of these insects generally requires the use of chemical insecticides that are toxic to humans and domestic animals and harmful to the environment (Coelho, Marangoni and Macedo, 2007).

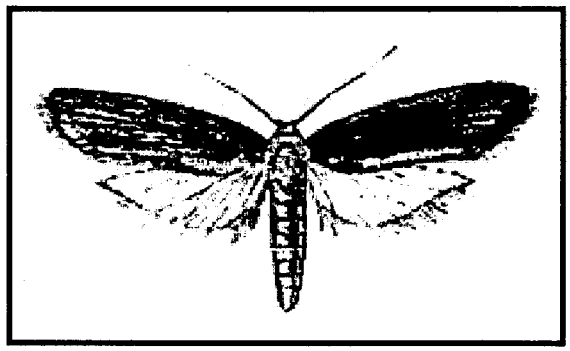


Figure 6 Rice moth, *Corcyra cephalonica* Stal, Lepidoptera

Source: www.ricethailand.go.th

4.2.3 Lesser grain borer, Rhyzopertha dominica F., Coleoptera: Bostrichidae

Lesser grain borer (*Rhyzopertha dominica* (F.)) is a strong flyer and is commonly found flying outside and inside grain-handling facilities during warm months. It is a destructive pest of stored grains throughout the world. Both larvae and adults are able to attack whole grain (Bashir, et al., 2003; Wright and Morton, 1995).

Appearance of Lesser grain borer is a beetle of 2-3 mm length, red-brown to black-brown, slim, cylindrical in body. The hood-shaped, rounded neck shield extends beyond the head; the spots on the shield gradually become smaller towards the rear. The three last segments of the antennae form a loose club. The larvae are white, similar to grubs and have brown head capsules. The white pupal stage is passed inside the grain kernel.

Adults are long-lived and lay an average of one to seven eggs per day over several months by female deposits 300-500 eggs in grain and similar crops. Eggs are laid externally on the wheat kernels. After hatching, larvae bore into and feed inside the kernels. Larvae pupate inside the kernels and adults remain inside for several days after eclosion (Perez-Mendoza, et al., 1999; Vardeman, et al., 2007).

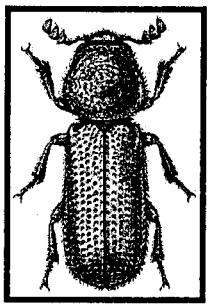


Figure 7 Lesser grain borer, *Rhyzopertha dominica* F.

Source: www.grainscanada.gc.ca

4.2.4 Rice Weevil, *Sitophilus oryzae* L., Coleoptera: Curculionidae

Rice weevils (*Sitophilus oryzae*) are considered primary stored-grain insects in warm climate areas. They cause significant loss to stored-grains. This species infest sound kernels of various cereals such as wheat, barley, rice, and maize at conditions favourable to their development (25–35°C and low RH) (Grenier, Mbaiguinam and Delobel, 1997; Batta, 2004; Athanssiou, et al., 2006; Athanassiou, et al., 2008). Both the adults and larvae feed on whole grains. The adult rice weevil can fly and is attracted to lights. When disturbed, adults pull in their legs, fall to the ground, and feign death. The larval rice weevil must complete its development inside a seed kernel or a man-made equivalent, like macaroni products. Larval rice weevils have been known to develop in hard caked flour. The adult female bores a cavity into a seed and then deposits a single egg in the cavity, sealing in the egg with secretions from her ovipositor.

Appearance of rice weevil (Figure 8) is small 2.5 to 4 mm and stout in appearance. It is very similar in appearance to the granary weevil (Peng, et al., 2003). However, the rice weevil is reddish-brown to black in color with four light yellow or reddish spots on the corners of the elytra (the hard protective forewings). The snout is 1 mm long, almost 1/3 of the total length. The head with snout is as long as the prothorax or the elytra. The prothorax (the body region behind the head) is strongly pitted and the elytra have rows of pits within longitudinal grooves. The larva is legless and stays inside the hollowed grain kernel. It is fat with a cream colored body and dark head capsule.

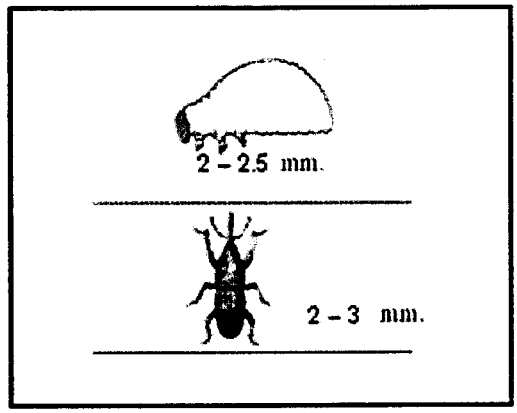


Figure 8 Rice Weevil, *Sitophilus oryzae* L.

Source: Department of Ministry of Agriculture and Cooperatives Rice

The life cycle of rice weevil may live for 7 to 8 months (Figure 9). The adult female lays an average of 4 eggs per day (producing 250-400 eggs). A single generation can be completed in around 30 to 34 days. The eggs hatch in about 3 to 6 days. The larvae feed inside the grain kernel for an average of 18 days. The pupal stage lasts an average of 6 days (5 to16 range). The new adult will remain in the seed for 3 to 4 days.

The previous study found that the insect in genus *Sitophilus* (Coleoptera, Curculionidae) can produce alpha-amylase enzyme to hydrolyze alpha (1, 4) glycosidic bonds of amylose and amylopectin in rice grains. Alpha-amylases play an important role in carbohydrate metabolism (Titarenko and Chrispeels, 2000; Grossi DE SA and Chrispeels, 1997; MacGrego, 1993; Strobl, et al., 1997).

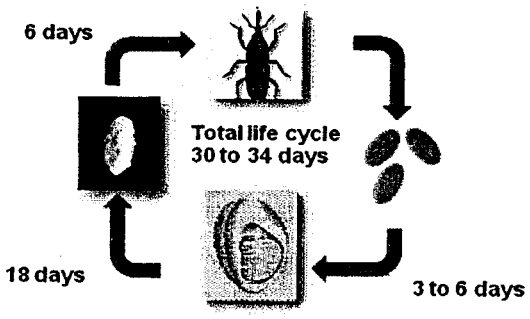


Figure 9 Life cycle of Rice Weevil, *Sitophilus oryzae* L.

Amylase

Amylases (E.C: 3.2.1.0) are enzyme which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units. They can be derived from several sources such as plants, animals, insect (Zverlov, Holl and Schwarz, 2003) and micro-organisms for example *Bacillus* species and *Aspergillus* species (Gouda and Elbahloul, 2008; Pandey, et al., 2000). Then they can specifically cleave the O-glycosidic bonds in starch, a storage polysaccharide present in seeds, tubers etc. of various plants. Starch consists of two components, a linear glucose polymer, amylose which contains α-1,4 linkages and a branched polymer, amylopectin in which linear chains of α-1,4 residues are inter linked by α-1,6 linkages. Starch depolymerization by amylases is the basis for several industrial processes such as the preparation of glucose syrupse, bread making and brewing. Amylases are instrumental in starch digestion in animals resulting in the formation of sugar, which are subsequently used in various metabolic activities (Nirmala and Muralikrishna, 2003).

Amylases compose of alpha, beta and gamma-amylases and can be classified into two categories, endoamylases and exoamylases. Endoamylases are alpha-amylase to catalyse hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases are beta-amylase and glucoamylase to act from the non-reducing end successively resulting in short end product (Reddy, Nimmagadda and Sambasiva, 2003; Pandey, et al., 2000).

1. Alpha-amylase

Alpha-amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) constitute a family of endoamylases that catalyze the hydrolysis of α-D-(1,4)-glucan linkages in starch components, glycogen and various other related carbohydrates. They play a vital role in carbohydrate metabolism of animals, plants and microorganisms (Strobl, et al. 1998). Alpha-amylases belong to family 13 in the classification of glycoside hydrolases. Family 13 is the most diverse of all glycoside hydrolase families (Nahoum, et al., 2000). It is a group of enzymes that occur in a wide variety of organisms such as animal, fungi, plant, bacteria and insect (Grossi De Sa and Chrispeels, 1997; Oliveira-Neto, et al., 2003; Kekos and Macris, 1983). The structures of these α-amylases commonly consist of three domain, a structurally conserved $(\beta/\alpha)_8$-barrel domain first observed in triose phosphate isomerase (Domain A), an additional domain inserted within Domain A (Domain B) and the C-terminal domain (Domain C). All known α-amylases contain calcium ions that contribute to stabilization of the structures (Nonaka, et al., 2003).

1.1 Alpha-Amylase in animals

For alpha-amylase of animals, it is found in saliva, pancreatic juice and other tissues (Brayer, Luo and Withers, 1995). This enzyme is a digestive enzyme classified as a saccharidase (an enzyme that cleaves polysaccharides) and is calcium metalloenzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, alpha amylase enzyme of animal breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin.

The structure of human pancreatic α-amylase has been using X-ray diffraction techniques. This enzyme is a 56 kDa protein consisting of 496 amino acids in a single polypeptide chain and is found to be composed of three structural domains (Figure 10). The largest is Domain A (residues 1-99, 169-404), which forms a central eight-stranded parallel β-barrel, to one end of which are located the active site residues Asp 197, Glu 233 and Asp 300. Also found in this vicinity is a bound chloride ion that forms ligand interactions to Arg 195, Asn 298 and Arg 337. Domain B is the smallest (residues 100-168) and serves to form a calcium binding site against the wall of the β-barrel of Domain A. Domain C (residues 405-496) is made up of anti-parallel β-structure and is only loosely associated with Domains A and B (Brayer, Luo and Withers, 1995).

The structure of saliva α-amylase consists of three domains: domain A (residues 1-99, 170-404), domain B (residues 100-169) and domain C (residues 405-496). The domain A adopts a $(\beta/\alpha)_8$ barrel structure bearing three catalytic residues Asp197, Glu233 and Asp300. The domain B occurs as an excursion from domain A and contains one calcium-binding site. Domain C forms an all β-structure and seems to be an independent domain with as yet unknown function (Ramasubbu, et al., 2005).

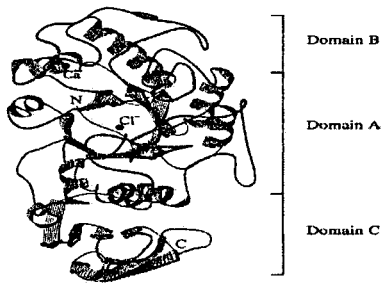


Figure 10 Stereo drawing of a schematic representation of the polypeptide chain fold of human pancreatic α-amylase. Also indicated are the relative positionings of the three structural domains present in this protein (Domain A, Domain B and Domain C). Along with locations of the calcium and chloride binding sites.

Source: Brayer, Luo and Withers, 1995

1.2 Alpha-Amylases in fungi

The alpha-amylases are found in fungi and are typically three domain structures consisting of the catalytic core domain linked to a C-terminal eight-stranded β-sheet domain A third domain, termed domain B, is found inserted between the third β-sheet and the helix of the $(\beta/\alpha)_8$-barrel and may play a role in both enzyme stability and substrate binding. A single structural calcium ion is found bridging the A and B domains. For example α-amylase in fungal is *Aspergillus oryzae* (TAKA-amylase) and has been determined by X-ray crystallography. The TAKA-amylase structure consists of three domains. The catalytic core A domain is comprised of a $(\beta/\alpha)_8$-barrel; the catalytic center of the enzyme being located on the C-terminal side of the barrel is the case in all $(\beta/\alpha)_8$-barrel enzymes. A short decoration occurs between the third barrel strand and helix. This forms domain B (residues 122-176), whose main structural feature is a short three-stranded antiparallel β-sheet. The interface between the A and B domains is maintained by a structural, conserved, "high-affinity" calcium site, whose ligands are donated by both the A and B domains. The final domain in the TAKA-amylase structure is an eight-stranded β-sandwich domain (residues 384-478). The substrate binding groove is formed by the fissure along the A and B subunit interface (Figure 11) (Brzozowski and Davies, 1997).

Figure 11 Schematic diagram of the TAKA-amylase structure drawn with the MOLSCRIPT program (Kraulis, 1991). The hexasaccharide ligand is shown in ball-and-stick mode and the single calcium ion as a shaded sphere, and the A-C domains are labeled.

Source: Brzozowski and Davies, 1997

1.3 Alpha-Amylase in plants

Alpha-Amylase (EC 3.2.1.1) of the plant plays a key role in the metabolism by hydrolyzing starch in the germinating seed and in other tissues. This is finished primarily through the 1,4-α endoglycolytic cleavage of amylose and amylopectin, the principal components of starch granules in plant cells (Huang, Stebbins, Rodriguez, 1992).

There is the study of the barley alpha-amylase. The α-amylase in germinating barley seed contains two main isozymes, AMY1 and AMY2, both of which are involved in starch degradation to provide energy for the development of the plant embryo, of the barley alpha-amylase, insight into the catalytic mechanism is gained from the X-ray crystal structure of its molecular complex with acarbose. For example, the overall structure of AMY2, including the two acarbose-binding sites, is

shown in Figure 12. In brief, the structure folds into three domains. The largest of the three domains (domain A) consists mainly of a parallel $(\beta/\alpha)_8$-barrel supersecondary structure. A small irregular loop domain (domain B) protrudes from domain A connecting strand β_3 and helix α_3. In AMY2, domain B binds three calcium ions, which are most probably critical for proper folding and conformational stability. The carboxy-terminal domain (domain C) is organized as a five-stranded anti-parallel β-sheet (Kadziola, et al., 1998; Vallee, et al., 1998).

Figure 12 Ribbon diagram of the AMY2 structure in complex with acarbose fragments shown as ball-and-sticks. White spheres correspond to the calcium ions. This Figure was produced with MOLSCRIPT and Raster3D

Source: Kadziola, et al., 1998

1.4 Alpha-amylase in Bacteria

The alpha-amylase is found in bacteria and has been studied extensively from various aspects, including structure, function, secretion and industrial application. Alpha-amylase has two aspartic residues and one glutamic acid residue that are conserved among species and are presumed to be the catalytic residues (Kagawa, et al., 2003).

The previous study determined the crystal structures of Wild-type *Bacillus* sp. Strain KSM-K38 (AmyK38) as shown in Figure 13. The main chain folding of AmyK38 is almost homologous to that of *Bacillus licheniformis* α-amylase and it consists of 473 amino acid residues. The AmyK38 molecule consists of three domains (A, B, and C) containing a $(\beta/\alpha)_8$-barrel motif (Domain A) commonly observed in various glycosidases (Nonaka, et al., 2003).

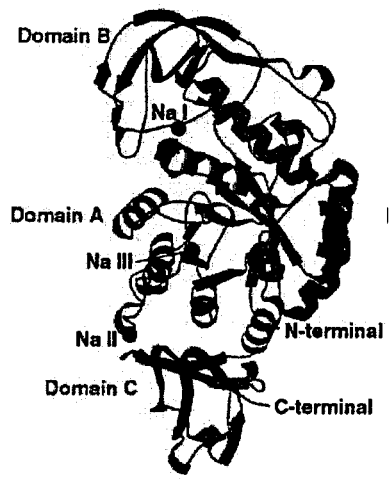


Figure 13 Stereo view of the ribbon model of the overall structure of AmyK38. Three domains (A, B, and C) are shown in red, blue, and green, respectively. Three sodium ions, Na I, Na II, and Na III, are shown as yellow spheres. These figures were drawn by MOLSCRIPT and Raster3D.

Source: Nonaka, et al., 2003

1.5 Alpha-Amylase in insect

Alpha-amylase from insect plays a central role in carbohydrate metabolism with a starch-rich diet depend on the effectiveness of their amylases for serious. This is certainly the case for insects that are serious agricultural pests because they consume starch-rich plant organs such as seed and roots (Titarenko and Chrispeels, 2000). Many insects, especially those that feed on grain products during larval and adult life depend on their amylases for survival.

The previous study of the three-dimensional structure of the α-amylase from *Tenebrio molitor* (TMA) (Figure 14) found that the three-dimensional structure consists of a single polypeptide chain of 471 amino acid residues with a molecular mass of 51 kDa, one calcium ion, one chloride ion and 261 water molecules. TMA is an acidic protein with a calculated pI of 4.3 and the pH optimum for the cleavage of starch was determined to be 5.8. It has been composed of 3 domains that are domain A, domain B and domain C. Domain A is composed of two segment and forms an eight stranded, parallel β-barrel, which is embraced by a concentric circle of eight helical segments, thus displaying the fold of a $(\beta/\alpha)_8$-barrel. Domain B, inserted into domain A between the third β-sheet and third α-helix and forms a cavity against the β-barrel of domain A in which the calcium ion is bound. The cation is of fundamental importance for the structural integrity of molecule. Domain C is located exactly opposite to domain B on the other side of domain A. No contact exists between domain B and C. The C domain comprises the C-terminal and a Greek key motif and its functional role is yet to be established (Tripathi, et al., 2007; Strobl, et al., 1998).

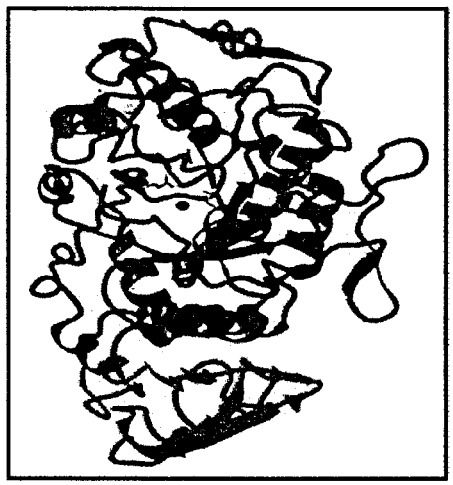


Figure 14 Ribbon plot of TMA. Domain A is shown in blue, domain B in green, and domain C in red. The chloride anion and the calcium cation are represented by a purple and a yellow sphere, respectively, and the active site residues Asp 185, Glu 222, and Asp 287 are depicted in pink.

Source: Strobl, et al., 1998

2. Beta-amylase

Beta-amylase (α-1, 4-glucan maltohydrolase, E.C.3.2.1.2) is an exohydrolases that attacks the non-reducing ends of starch molecules, producing β-maltose and β-limit dextrin as product (Douglas, Stanley and Laurens, 1982; Ziegler, 1999).

3. Gamma-amylase (Glucoamylase)

Gamma-amylase (EC.3.2.1.3) is a group of exoamylase that catalyses the hydrolylsis of α-1, 4-linkages and α-1,6-linkages in starch components. It has been found in several microorganisms like bacteria and fungi.

Application of amylase enzyme

The property of amylase enzyme has been applied in many other fields such as clinical, medicinal and analytical chemistries, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries (Kumar, Singh and Rao, 2005; Pandey, et al., 2000).

Alpha-amylase inhibitor

The number of proteinaceous alpha-amylases inhibitors isolated and identified so far is extremely large. They occur in microorganisms, higher plants and animals (Payan, 2004). The enzyme inhibitors impede digestion through their action on insect gut digestive alpha-amylases and proteinases, which play a key role in the digestion of plant starch and proteins. The natural defences of crop plants may be improved through the use of transgenic technology (Franco, et al., 2002).

Proteinaceous alpha-amylase inhibitors can have different polypeptide scaffolds and can be grouped by their tertiary structures into six classes: lectin-like, knottin-like, cereal-type, Kunitz-like, y-purothionin-like and thaumatin-like. Plant seeds are rich sources of a large number of different proteinaceous inhibitors acting on α-amylases or other polysaccharide processing enzymes. They abundant in cereals and leguminosae, have been extensively studied. The present mini-review addresses the diverse group of alpha-amylase inhibitors with focus on recent insights in structure and function. Some alpha-amylase inhibitors show strict target enzyme specificity and recognize only one out of several closely related isozymes or enzymes from different species (Sensson, et al., 2004).

CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Insect

Rice weevil (*Sitophilus oryzae)*

2. Equipment

2.1 Horizontal gel electrophoresis (Sunrise Garment Co., LTD., USA)

2.2 Heating block (Labnet International, Inc., USA)

2.3 Px2 Thermal cycle (Applied Biosystem Co., LTD., UAS)

2.4 Refrigerated Centrifuge (Tomy kogyo Co., LTD., Japan)

2.5 Gel documentation system (Biolmaging Systems Co., LTD., England)

2.6 Hot air oven (MMM Medcenter Einrich-tungen GmbH Co.,LTD., Germany)

2.7 Autoclave (Sanyo electric Co., LTD., Japan)

2.8 Microcentrifuge (Labnet International, Inc., USA)

2.9 Microwave oven (Sharp, Japan)

2.10 Water bath (Julabo Labortechnik GMBH Co., LTD., Germany)

2.11 pH meter (Mettler-Toledo International Inc., Switzerland)

2.12 Incubator (ShelLab Co., LTD., USA)

2.13 UV/Vis Spectrophotometer (Beckman Coulter Co., LTD., USA)

2.14 High intensity ultrasonic processor (Becthai Bangkok Equipment & Chemical Co., LTD., USA)

2.15 Micro pipette

2.16 Pipette tips

2.17 Beakers

2.18 Magnetic Bar

2.19 Plastic culture dish

2.20 Spreader

2.21 Loop

2.22 Tube, PCR tube

2.23 Cutter

2.24 Duran bottle

2.25 Erlenmeyer flasks

2.26 Cylinders

3. Chemicals

3.1 RNA isolation

3.1.1 Trizol reagent (Invitrogen, USA)

3.1.2 Chloroform (BDH, England)

3.1.3 Isopropanol (TEDIA, USA)

3.1.4 Ethanol (Merck, Germany)

3.2 Removal of genomic DNA from RNA preparation

3.2.1 DNase I

3.2.2 10X DNase I reaction buffer with $MgCl_2$

3.2.3 DEPC-treated Water

3.3 Complementary DNA (cDNA) synthesis kit (Fermentas, USA)

3.3.1 RevertAid^{TM} M-MuLV Reverse Transcriptase (200 U/µl)

3.3.2 RiboLock^{TM} RNase Inhibitor (20 U/µl)

3.3.3 5X Reaction Buffer

3.3.4 Oligo(dT) Primer

3.3.5 Random Hexamer Primer

3.3.6 Forward GAPDH Primer

3.3.7 Reverse GAPDH Primer

3.3.8 Control GAPDH RNA

3.3.9 DEPC-treated Water

3.4 Polymerase chain reaction (PCR) (Invitrogen, USA)

3.4.1 10X PCR buffer

3.4.2 50 mM $MgCl_2$

3.4.3 Taq DNA polymerase 5 U/µl

3.4.4 10 mM dNTP mix

3.5 Terminal Transferase (BIO-RAD, USA)

3.5.1 10X Terminal transferase reaction buffer

3.5.2 Terminal transferase (20 U/µl)

3.6 Agarose gel electrophoresis

3.6.1 Tris base (Usb, USA)

3.6.2 Boric acid (Usb, USA)

3.6.3 Ethylenediaminetetraacetic acid (EDTA) (BIO-RAD, USA)

3.6.4 Agarose (Usb, USA)

3.6.5 Ethidium bromide (BIO-RAD, USA)

3.7 illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthecare, UK)

3.7.1 Capture buffer type 2

3.7.2 Wash buffer type 1

3.7.3 Elution buffer type 4

3.7.4 Elution buffer type 6

3.8 TA cloning kit (Fermentas, UAS)

3.8.1 Vector pTZ57/T, 55 ng/µl

3.8.2 5X Ligation Buffer

3.8.3 T4 DNA Ligase, 5 U/µl

3.8.4 Control PCR Fragment, 42 ng/µl

3.8.5 Vector pTZ57R (without insert), 0.1 µg/µl

3.8.6 Control DNA (pTZ57R with insert), 0.1 µg/µl

3.8.7 Water, nuclease-free

3.9 LB broth and LB agar

3.9.1 Agar powder (Himedia Laboratories Pvt. Ltd., India)

3.9.2 Difco™ LB broth, Lennox (Becton, Dickinson and Company, USA)

3.10 eparation of competent cell

3.10.1 Calcium chloride (Ajax Finechem Pty Ltd., New Zealand)

3.10.2 Glycerol (MERCK, Germany)

3.11 striction enzyme (Invitrogen, USA)

3.11.1 *Xba* I

3.11.2 *Hind* III

3.11.3 *Xho* I

3.11.4 *Nde* I

3.11.5 10X REact®2

3.11.6 10X REact®6

3.12 emical for protein analysis

3.12.1 Glycine (BIO-RAD, USA)

3.12.2 Tween 20 (SIGMA, USA)

3.12.3 Triton X-100 (BIO-RAD, USA)

3.12.4 Acetic Acid (Merck, Germany)

3.12.5 Acrylamide/Bis Solution 19:1 (BIO-RAD, USA)

3.12.6 Ammonium persulfate (BIO-RAD, USA)

3.12.7 2-Mercaptoethanol (BIO-RAD, USA)

3.12.8 Methanol (LAB-SCAN, Ireland)

3.12.9 Sodium dodecyl sulphate (SDS) (Amersham, USA)

3.12.10 Coomassie Billiant Blue G-250 (BIO-RAD, USA)

3.12.11 Ponceau S Solution (SIGMA, USA)

3.13 DNA ladder

3.13.1 100 bp Plus DNA ladder (Fermentas, USA)

3.13.2 200 bp DNA ladder (Bio basic inc., Canada)

3.13.3 λDNA/*Hind*III (Fermentas, USA)

3.14 rotein marker (BIO-RAD, USA)

3.14.1 Precision Plus Protein™ All Blue Standards

3.14.2 Precision Plus Protein™ Unstained Standards

Methods

1. Sample preparation

Rice weevil (*Sitophillus oryzae)* was maintained in rice at room temperature for 28 days. About 200 mg of *Sitophilus oryzae* as adult stage were selected and transferred to 1.5 ml steriled microgentrifuge tube. The samples were stored at -80°C for further use.

2. RNA extraction

Total RNA was extracted from adult stage of *Sitophilus oryzae* using Trizol reagent (Invitrogen, USA) according to the instruction's manual. Briefly, 200 mg of *Sitophilus oryzae* were ground in liquid nitrogen. The sample was incubated with 700 µl of Trizol reagent at room temperature for 5 min. Mixing solution was rapidly mixed with 200 µl of chloroform and incubated at room temperature for 3 min. After incubation, sample tube was centrifuged at 10,000xg for 15 min at 4°C. The RNA was collected from the aqueous phase and precipitated with 600 µl of isopropanol, and incubated at room temperature for 10 min. Following centrifugation at 10,000xg for 15 min at 4°C, the pellet was washed with 700 µl of 75% ethanol. Then the RNA pellet was centrifuged at 5,000xg for 5 min at 4°C and air dried. Finally, the RNA pellet was dissolved with 12 µl of RNase free water.

3. Complementary DNA (cDNA) synthesis

For RT-PCR applications, RNA template must be free of DNA contamination. Prior to cDNA synthesis, 1µg of RNA template was incubated with solution component of 1 µl 10X DNase I buffer, 1 µl DNase I and adjusted with DEPC-treated water to 9 µl at 37°C for 30 min. Then, 1 µl of 25 mM EDTA was added to sample solution and incubated at 65°C for 10 min. Pure RNA was directly used in first-strand cDNA synthesis reaction using RevertAid^{TM} First strand cDNA synthesis kit (Fermentas, USA) according to manufacture's protocol. Firstly, reagent components of 1 µl Oligo (dt_{18}) primer and 1 µg total RNA was incubated at 65°C for 5 min. After that, the sample was incubated with mixture of 4 µl 5X reaction buffer, 1 µl of 20 units Ribolock Ribonuclease Inhibitor, 2 µl 10 mM dNTP mix and 1 µl 200 units RevertAid M-MuLv Reverse transcriptase at 42°C for 60 min. Finally, the reaction mixture was then incubated at 70°C for 5 min for stopping the reaction. The

reverse transcription reaction product was directly used in polymerase chain reaction (PCR) amplification.

4. Polymerase chain reaction (PCR) amplification

The primers for PCR amplification were designed from sequence information of nine known insect alpha-amylases, *Anthonomus grandis* (Amylag2), *Anthonomus grandis* (Amylag1), *Tribolium castaneum*, *Zabrotes subfasciatus*, *Callosobruchus chinensis*, *Diabrotica virgifera virgifera*, *Blaps mucronata* (Amy2), *Blaps mucronata* (Amy1) and *Phaedon cochleariae* (Figure15).

PCR amplification was performed using 10-100 ng of the first-strand cDNA reaction as a template. The reaction mixture composed of 0.5 µl first-strand cDNA, 0.5 µl of 10 pmol primer as a putative alpha-amylase insects and β-actin (internal control), 2.5 µl of 10X PCR buffer, 0.5 µl of 10mM dNTPs, 0.75 µl of 50 mM $MgCl_2$, 0.25 µl of 5 U/µl Taq DNA Polymerase and distilled water to make the volume up to 25 µl. Samples were incubated for 2 min at 94 °C and followed by 40 cycles of 30 sec at 94°C, 30 sec at 49°C, 1 min at 72°C with a final 10 min extension step at 72°C. The resulting DNA fragments were separated with 1% agarose gel electrophoresis.

Table 1 Oligonucleotide primers used for DNA amplification of partial alpha-amylase gene and β-actin

Primer name	***Direction***	***Sequence(5'→ 3')***
β-actin primer	Forward	GTTCCCATCCATCGTAGGTCG
β-actin primer	Reverse	GCAGAGCGTAACCTTCGTAGAT
Alpha-amylase primer	Forward	TGGTGGGA**R**A**V**RTACCA
Alpha-amylase primer	Reverse	GGATG**V**GC**Y**A**D**C ATR**A**A

Alpha-amylase primers are degenerate primer. D: A+T+G, R: A+G, V: A+C+G and Y: C+T

Figure 15 The comparison of alpha-amylase nucleotide sequence from nine known insects. The boxed regions represented sequences for primer design.

5. Gel electrophoresis

PCR products were analyzed by electrophoresis on 1% agarose gel in 0.5X TBE buffer. PCR samples were mixed with 6X loading dye. Electrophoresis was performed at constant 75 volt for 90 min using 0.5X TBE buffer. After electrophoresis, the gel was stained with 10 μg/ml of ethidium bromide solution for 15 min and destained with distilled water. The band in the gel was visualized by Gel documentation system (BioImaging Systems Co., LTD., England).

6. Purification of PCR product from agarose gels

PCR products in agarose gel were extracted using the FXTM PCR DNA and Gel Band Purification kit (GE, UK). PCR products were cut out of the gel and transferred into 1.5 ml steriled microcentrifuge tubes. The sample was dissolved with 400 μl capture buffer type 2 and incubated at 60° C until the agarose was completely dissolved. Then, the sample mixture was centrifuged briefly to collect the liquid at the bottom of the tube. 600 μl of sample mixture was transferred to the column and incubated at room temperature for 1 min. After centrifugation at 16,000xg for 30 sec, sample was washed with 600 μl of wash buffer type 1 and centrifuged again at 16,000xg for 30 sec. The flow-through was discarded and centrifuged again to dry the column matrix. Finally, the purified DNA was eluted with 40 μl of sterile water. The resulting purified DNA was determined by measuring the absorbance at 260 nm.

7. cDNA Cloning and DNA sequencing

7.1 Preparation of competent cells

Competent *E. coli* DH5α cells were prepared using the $CaCl_2$. A single colony of *E. coli* was transferred to 3 ml of LB broth and incubated at 37°C for 16-18 hr in shaking incubator. The next day, 1 ml of the growing cells was transferred into 100 ml LB broth and incubated at 37°C for 3 hr or mid log phase (OD_{600}~0.4-0.5). The cells were transferred to a cold centrifuge tube and centrifuged at 6,000xg at 4°C for 10 min. The culture medium was decanted from the cell pellet, and pellet was suspended in 50 ml of cold 50 mM $CaCl_2$ and stored on ice for 20 min. The cells were centrifuged again at 6,000xg at 4°C for 10 min and suspended in 10 ml of cold 50 mM $CaCl_2$ (15% glycerol). The sample was held on ice for 2-3 hr. The aliquots of 50 μl sample were transferred to 1.5 ml steriled tubes and stored at -80° C until used.

7.2 Ligation reaction

Purified DNA was ligated to pTZ57R/T plasmid vector according to the plasmid supplier's recommendation. The reaction mixture was contained of 1.5 µl of pTZ57R/T plasmid vector, 2 µl of purified DNA, 3 µl of 5X T4 DNA ligase buffer, 0.5 µl of T4 DNA ligase and 3 µl of steriled water. The reaction mixture was incubated at 4°C overnight.

7.3 Transformation and selection

Competent cells were thawed on ice. The product of a ligation reaction was pipetted to the thawed competent cells and incubated for 30 min on ice. The cells were heat shocked at 42° C for 90 sec, then immediately transferred to ice bath for 2 min and 200 µl of LB medium were added to the cells. The cultures were incubated at 37° C for 1.5 hr. The cells were spreaded on LB medium agar contained 50 mg/ml ampicillin, 40 µl of 100 mM IPTG and 40 µl of 20 mg/ml X-Gal. The agar plate was incubated at 37°C overnight, recombinant clones were identified as white colonies. The cells contained intact vector DNA gave blue colonies. The recombinant clones were confirmed by restriction enzyme digestion.

7.4 Isolation and purification of plasmid DNA from *E.coli*

The plasmid DNA was isolated from an overnight bacterial culture using nucleic acid extraction kit (Vivantis, USA) following the supplier's protocols. A white colony was grown in LB broth with 50 µg/ml of ampicillin at 37°C overnight. The cells were collected by centrifugation at 6,000xg for 2 min. The cell pellets were resuspended in 250 µl of S1 buffer by vortex or pipette up and down, followed by adding 250 µl of S2 buffer. The mixture was gently mixed by inverting the tube 5 times. Then 400 µl of buffer NB was added and gently mix by inverting the tube for 8 min. The DNA solution was separated by centrifugation at 14,000xg for 10 min at room temperature. The supernatant was transferred to column, followed by centrifugation at 10,000xg for 1 min. The plasmid solution was packed into column and it was washed with 600 µl of wash buffer. Plasmid was eluted from column by adding 80 µl of steriled water to the column, leaving it to stand for 1 min and centrifuging at 10,000xg for 1 min. The purified plasmid DNA was confirmed by restriction enzyme digestion and automated DNA sequencing.

7.5 Restriction analysis

The plasmid DNA was digested using restriction enzyme according to the manufacture's recommendation. The reaction mixture contained 1 μg of purified plasmid DNA, 3 μl of 10X buffer, 10 U/μl of restriction enzyme (*Xba*I and *Hin*dIII) and adjusted volume to 15 μl by steriled water. After incubation at 37°C for 2 hr, the reaction was stopped by adding agarose gel loading dye. Finally, the products of restriction enzyme digestion were analyzed by agarose gel electrophoresis.

7.6 DNA sequencing and Comparison of database

The recombinant clone was analyzed by DNA sequencing (1st Base, Malaysia). The resulted nucleotide sequences were blasted in the GenBank database. This software is available on the internet: http://blast.ncbi.nlm.nih.gov/Blast.cgi. The nucleotide sequence was translated into the protein sequence using the 'ORF Finder' at the NCBI site (http://www.ncbi.nlm.nih.gov/projects/gorf/). The signal peptide cleavage site was determined using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The theoretical pI and Mw (molecular weight) were estimated using the 'Compute pI/Mw tool' option on the ExPasy server (http://www.expasy.ch/tools/pitool.html). Sequence alignments were obtain using website (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

8. 5'/3' Rapid Amplification of cDNA End (RACE PCR amplification)

8.1 5' RACE PCR amplification

The partial sequence was used for design 5' RACE primer (Table 2). The 5' RACE utilized a gene-specific and Oligo (dT) primer for DNA amplification. The total RNA was isolated from rice weevil (*Sitophilus oryzae*) as described in section 2.2, and used as a template for cDNA synthesis using gene-specific primer. The reaction mixture for cDNA synthesis contained 10 μl of total RNA, 0.3 μl of gene-specific primer and 1.7 μl of DEPC-treated water. The sample was mixed, briefly centrifuged and incubated at 65°C for 5 min. Then 4 μl of 5X reaction buffer, 1 μl of RiboLock™ RNase inhibitor, 2 μl of 10 mM dNTP mix and 1 μl of RevertAid™ M-MuLV Reverse transcriptase were added to the reaction mixture, followed by centrifugation and incubation at 65°C for 60 min. The reaction was stopped by heating at 70°C for 5 min. After that, the cDNA was purified using the FXTM PCR DNA and Gel Band Purification kit (GE, UK) as described in section 2.6. The dA homopolymer

was added to purified cDNA at 3' end using terminal deoxynucleotidyl transferase (TdT) enzyme. The DNA tailing reaction was composed of 5 µl of 10X TdT buffer, 5 µl of 2.5 mM $COCl_2$ solution provided, 20 µl of DNA, 0.5 µl of 10 mM dNTP, 0.5 µl of terminal transferase and 19 µl of steriled water. Then DNA was amplified using gene-specific primer and Oligo(dT) primer. The total volume of PCR reaction contained 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTP mix, 1.5 µl of 50 mM $MgCl_2$, 0.4 µl of Taq polymerase, 1 µl of Oligo-dT primer, 1 µl of gene-specific primer, 2.5 µl of DNA template and 37.6 µl of sterile water. Amplification conditions were 35 cycles of 30 sec at 94°C, 30 sec at 56°C and 1 min at 72°C. Then the PCR product was analyzed by agarose gel electrophoresis.

8.2 3' RACE PCR amplification

The partial sequence was for design 3' RACE primers (Table X). The 3' RACE was utilized a gene-specific and Oligo(dT) primer for DNA amplification. The total RNA was isolated from rice weevil (*Sitophilus oryzae*) as described in section 2.2, and the first strand cDNA was synthesized from total RNA as described in section 2.3. After that, the 50 µl total volume of PCR reaction contained 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTP mix, 1.5 µl of 50 mM $MgCl_2$, 0.4 µl of Taq polymerase, 1 µl of Oligo-dT primer, 1 µl of gene-specific primer, 2.5 µl of DNA template and 37.6 µl of steriled water. Amplification conditions were 32 cycles of 30 sec at 94°C, 30 sec at 56°C and 1 min at 72°C. Then the PCR product was analyzed by agarose gel electrophoresis.

Table 2 Oligonucleotide primers used for DNA amplification of 5' RACE and 3'RACE

Primer name	*Direction*	*Sequence(5'→ 3')*
gene-specific primer (5'RACE)	Reverse	CCAAACAAGAACTCGCACAC
gene-specific primer (3'RACE)	Forward	TTCATCTTCCAGGAAGTCAT
Oligo-dT primer		TTTTTTTTTTTTTTTTTTTTTTTT

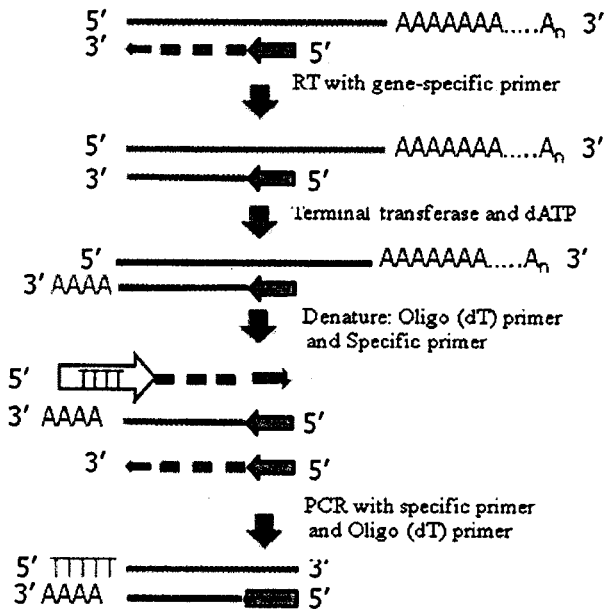


Figure 16 The method of 5' RACE PCR amplification for full-length of alpha-amylase gene.

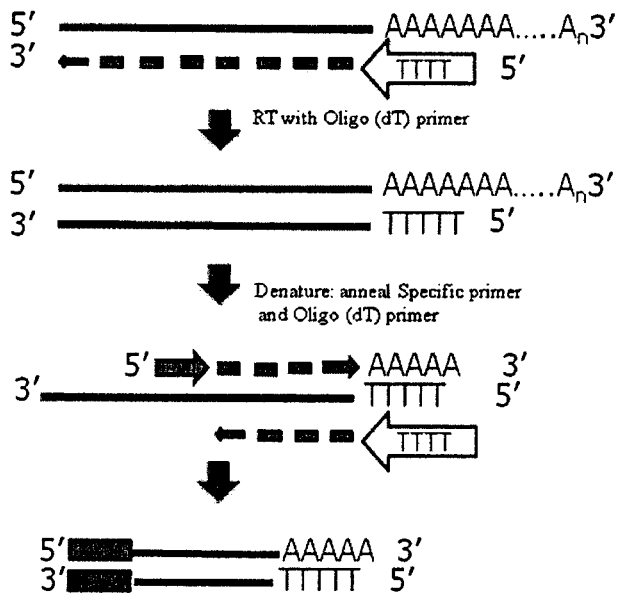


Figure 17 The method of 3' RACE PCR amplification for full-length of alpha-amylase gene.

9. Amplification of full length alpha-amylase gene

The nucleotide sequence of 5' RACE and 3' RACE were used for design primer using in full length alpha-amylase gene PCR amplification. The oligonucleotide primers were shown in table 3. The cDNA in 2.3 was as a template for amplification of full length alpha-amylase gene. The 50 µl total volume of PCR reaction contained 5 µl of 10X PCR buffer, 1 µl of 10 mM dNTP mix, 1.5 µl of 50 mM $MgCl_2$, 0.4 µl of Taq polymerase, 1 µl of Oligo-dT primer, 1 µl of gene-specific primer, 2.5 µl of DNA template and 37.6 µl of steriled water. Amplification conditions were 35 cycles of 30 sec at 94°C, 30 sec at 56°C and 1 min at 72°C. The PCR product was analyzed by agarose gel electrophoresis. After that, purification of DNA fragment was done as described in section 2.6. The DNA fragment was inserted into TA cloning vector and then determined by DNA sequencing as described in section 2.7.

10. Expression of alpha-amylase gene in *E.coli*

10.1 Construction of pET-21b(+) – alpha-amylase gene

cDNA coding for putative alpha-amylase of *Sitophilus oryzae* was expressed in *E.coli* expression system (Novagen, Madison, WI). The pET-21b(+) expression vector was digested by *Nde* 1 and *Xho* 1 restriction enzyme. The restriction mixture was composed of 2 µl of pET-21b(+) vector, 1 µl of *Nde* 1 (5 unit/ml), 1 µl of *Xho* 1 (10 unit/ml), 2 µl of 10X REact®2 and 14 ml of distilled water. The mixture was incubated at 37°C overnight. The samples were analyzed by 1% agarose gel electrophoresis and then DNA was purified from gel as described in section 2.6. The recombinant plasmid from section 2.9 was digested by *Nde* 1 and *Xho* 1 restriction enzyme. The DNA fragment was subcloned into the expression vector pET-21b(+) between *Nde* 1 and *Xho* 1 multiple cloning site. The pET-21b(+)-alpha-amylase plasmid was transformed into *E.coli* strain BL21(DE3)pLysS. The plasmid was added to 100 µl of thawed Origami (DE3) competent cells and the tube was stored on ice for 30 min. The cells were heat shocked at 42°C for 90 sec. After incubation, sample tube was transferred to ice bath for 2 min. The transformed cells were grown by adding 400 ml of LB medium and incubated at 37°C for 90 min, then plated directly on LB plate containing 50 µg/ml of ampicillin, followed by overnight incubated at 37°C. The recombinant clones were confirmed by DNA sequencing.

Table 3 Oligonucleotide primers used for DNA amplification of alpha-amylase gene expression. The restriction endonuclease recognition sites are underlined.

Primer name	*Direction*	*Sequence(5'→ 3')*
Alpha-amylase (*Nde*I)	Forward	CGCCATATGAAGGTACTAGCCCTTTTAG
Alpha-amylase (*Xho*I)	Reverse	GTCTCGAGCAATTTAGCATTGACATGGATGG CA

10.2 Expression of recombinant pET-21b(+)–alpha-amylase and variants in *E. coli* BL21(DE3)pLysS

The alpha-amylase gene was cloned into the *Ned*I-*Xho*I site of pET-21b(+) to construct pET-21b(+)-alpha-amylase. *E.coli* BL21(DE3)pLysS transformed with pET-21b(+)-alpha-amylase was grown in LB medium containing 50 mg/ml of amplicilin at the 37°C overnight. 1% bacterial culture was incubated in fresh medium and cultured at 37°C until OD at 600 nm reach 0.5-0.8. The culture was induced by adding 1 mM IPTG and then incubated for 4 hr at 28°C. Precooled bacterial cells were collected by centrifugation at 4,000 rpm for 15 min, and then kept at -80°C.

10.3 Extraction of recombinant pET-21b(+)– alpha-amylase from *E. coli* BL21(DE3)pLysS

After growth and induction as described above, the cells were resuspended in lysis buffer and lysed by sonication on ice at highest output (100 W) for 30 sec, 5 times using probe sonicator (Becthai Bangkok Equipment & Chemical, USA). Soluble and insoluble proteins were separated by centrifugation at 14,000x*g* for 15 min. Protein concentration were determined by Bradford assay (Bradford, 1976). Samples were analyzed by SDS-PAGE.

11. Protein determination

Protein concentration was measured according to the method of Bradford (Bradford, 1976) using bovine serum albumin (Bio-Rad, Germany) as a protein standard. The concentration of BSA standard was varied at 1, 0.75, 0.5, 0.25, 0.125 and 0 mg mL^{-1}. The diluted sample (5 µl) was mixed with 1X dye reagent

(250 µl) and then incubated for 15 min at room temperature. After that, the mixture was measured the absorbance at 620 nm by using microplate reader.

12. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) for alpha-amylase

The recombinant alpha-amylase protein from rice weevil was determined by SDS-PAGE. Protein samples (~10 µg) were mixed with 4 volumes of 5X SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.1% bromophenol blue, and 14.4 mM 2-mercaptoethanol), and boiled at 95°C for 5 min to denature the protein. The samples were analyzed on 4% stacking gel and 10% separating gel for 2 hr at 20 mA. Protein bands were visualized by staining in staining solution containing 0.1% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid for 1 hr, followed by several washes with destaining solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. Prestained SDS-PAGE standard (BIO-RAD, USA) ranging from 10 to 250 kDa was used as a protein marker.

13. Western blot analysis

For western blot analysis, separated proteins were transferred from polyacrylamide gel to polyvinylidene fluoride (PVDF) membrane (Amersham, USA) with a wet blotting apparatus for 2 hr at 400 mA. After blotting, the gel was stained with Coomassie Brilliant blue, while membrane was stained with Ponceau S to confirm complete transferring of protein from gel to membrane. After that, stained gel was destained with Coomassie blue destaining solution. The stained membrane was wash 3 times for 5 min with 1X Tris Buffer Saline Tween20 (TBST). The membrane was incubated with protein blocking solution containing 5% (w/v) non-fat milk in TBST at 4°C overnight. After decantation of blocking buffer, the membrane was incubated with primary antibody (anti-His Tag antibody) (Millipore, USA) for 2 hr at room temperature. The primary antibody was used at a dilution of 1:1,500 in blocking solution. The membrane was washed 3 times for 5 min with 1X TBST and then membrane was incubated with secondary antibody (goat anti-mouse antibody) (Millipore, USA) at a dilution of 1:5,000 in blocking solution for 2 hr at room temperature. Following 3 washes for 5 min with 1X TBST, the membrane was detected with chemiluminescence (GE Healthcare, UK).

14. Identification of protein by Liquid chromatography- mass spectrometry (LC-MS/MS)

The protein band of about 54kDa from SDS-PAGE was excised and subjected to in-gel digestion using an in-house method developed by Proteomics Research Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC) (Jaresitthikunchai, et al., 2009). The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10mM ammonium bicarbonate at room temperature for 1 hr and alkylated at room temperature for 1 hr in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/ 10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Extracted peptides were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

The digested protein was injected into Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 80% acetonitrile) was run in 40 min. The MS/MS data were submitted to database search using MASCOT (http://www. matrixscience.com). The data was searched against the NCBI database for protein identification.

15. Nondenaturing gel electrophoresis (Native PAGE) for alpha-amylase

Native-PAGE analysis of alpha-amylase protein was carried out by loading samples with an average of 0.2 μg per well. The running was carried out at the current of 10 mA for the stacking gel and the 15 mA for the separating gel. Both electrophoresis chamber and running buffer were pre-chilled in refrigerator before the sample has been loaded and the gel was run at 1-5°C. The stacking gel, separating gel, and the running buffer were prepared in the same way as the SDS-PAGE, except that no SDS and 2-mercaptoethanol was used. The gel was directly used in zymogram analysis.

16. Zymogram analysis

Zymogram analysis as a procedure that modified from those described by Dojnov, et al. (2008). After protein samples were electrophoresed in Native-PAGE, the gel was washed in distilled water before renaturing protein by soaking the gel in the 2% Triton-X 100 for 45 min at room temperature. The gel was transferred to substrate buffer solution (1.0% (w/v) starch, 50 mM Sodium acetate, 2.0 mM NaCl, 0.1 mM $CaCl_2$, pH 5.0) at 30°C for 30 min, follow by washing the gel in sodium acetate buffer with 20 mM NaCl and 0.1 mM $CaCl_2$ for 30 min at 30°C. After rinsing in water, amylolytic activity was stopped by addition staining solution (1.3% (w/v) I_2, 3% (w/v) KI). Alpha-amylase activity appeared as clear bands on a dark background.

17. 3, 5-Dinitrosalicylic acid (DNS) assay

Alpha-amylase activity was analyzed by 3, 5-Dinitrosalicylic acid (DNS) procedure using soluble starch as a substrate. 25 μl of smples were incubated in 250 μl of 50 mM acetate buffer pH 5.0 containing 1.0% (w/v) starch, 2.0 mM NaCl and 0.1 mM $CaCl_2$ at 30°C for 10 min. The reaction was stopped by addition 250 μl of DNS reagent and heated in boiling water for 10 min. 3, 5-Dinitrosalicylic acid is a color reagent that the reducing groups were released from starch by alpha-amylase action are measured by the reduction of 3, 5-Dinitrosalicylic acid. The boiling water was used for stopping the alpha-amylase activity and catalyzing the reaction between DNS and reducing groups of starch. After that samples were added 2.5 ml of distilled water and measured the absorbance at 550 nm. Then, absorbance was read at 550 nm after cooling on ice for 5 min. Maltose was used as a standard. Serial dilutions of maltose (Sigma, USA) in substrate solution at pH 5.0 were made to give following range of

concentration of 2, 1, 0.5, 0.25, 0.125 mg mL^{-1} (Figure 18). A blank without substrate but with alpha-amylase extract and a control containing no alpha-amylase extract but with substrate were run simultaneously with the reaction mixture. All assays were performed in triplicate.

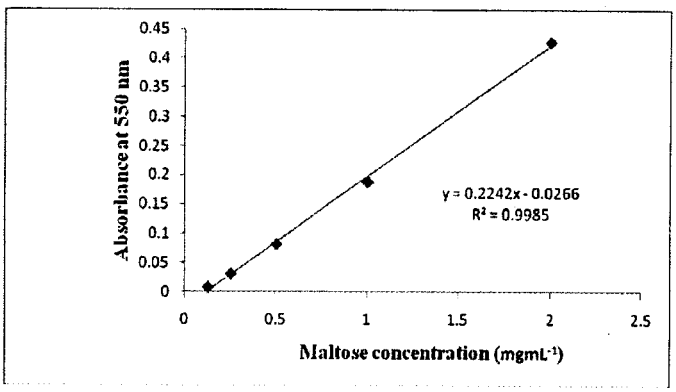


Figure 18 Standard calibration curve for the determination of maltose released in the alpha-amylase assay

18. 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate

Alpha-amylase activity was analyzed by 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate (Sigma, USA). This method is based on the principle that the hydrolysis of 2-Chloro-4-nitrophenyl- α-D-maltotrioside is catalyzed by α-Amylase, and then yields 2-Chloro-4-nirophenol that is quantitatively measured by its absorbance at 405 nm. 25 µl of samples were adding to 1 ml of the substrate solution (2-Chloro-4-nitrophenyl-α-D-maltotrioside) and incubated the mixture at 30°C for 10 min. After that, samples were added 1 ml distilled water and measured the absorbance at 405 nm.

CHAPTER IV

RESULTS AND DISCUSSION

Cloning and Analysis of nucleotide sequence of alpha-amylase genes from rice weevil (*Sitophilus oryzae*)

To clone the alpha-amylase from rice weevil, DNA sequences from nine known insect alpha-amylases were used to design oligonucleotide primers corresponding to conserved sequences. The oligonucleotide primers were used to amplify cDNA fragment obtained from adult stage of rice weevil by RT-PCR. The size PCR products were shown the 950 bp, as shown in Figure 19.

The PCR products of conserved alpha-amylase gene obtained from eluted agarose gel electrophoresis was ligated to the pTZ57R/T vector and the ligated product was transformed into *E.coli* as described in Methods. After that, a number of transformants were screened for the recombinant clone. Recombinant clones were screened with *Xba*I and *Hin*dIII restriction enzyme analysis as described in Methods. It was analyzed by agarose gel electrophoresis, as shown in Figure 20. To determine nucleotide sequences of positive clones, the nucleotide sequences of partial alpha-amylase contains 776 bp open reading frame encoding a predicted protein of 234 amino acids starting from the first ATG (see Figure 21). The obtained nucleotide sequences were compared with GenBank database of National Center for Biotechnology Information (NCBI). The result shows sequence similarity with alpha-amylase of Coleopteran such as *Anthonomus grandis* (Amylag1), *Anthonomus grandis* (Amylag2), *Tribolium castaneum*, *Phaedon cochleariae* and *Scirpophaga incertulas* which was almost 70%. The deduced nucleotide sequences were subsequently used for 5' and 3' RACE to extend the fragment in both directions.

The 5' end was obtained by 5' RACE using antisense primer, as described in Methods. The result of amplification cDNA fragment showed the appearance of 850 bp (Figure 22). After that, PCR product was eluted from agarose gel electrophoresis and used to clone into cloning vector, as described in Method. The recombinant clones were analyzed by *Xba*I and *Hin*dIII restriction enzyme analysis. Figure 23 shows the

result of restriction enzyme analysis. The derived nucleotide sequence of 5' end DNA fragment contains 856 bp open reading frame encoding a predicted protein of 263 amino acids starting from the first ATG (Figure 24). The obtained nucleotide sequences were compared with GenBank database of National Center for Biotechnology Information (NCBI). The result shows sequence similar with alpha-amylase of Coleopteran which was almost 80%.

The 3' end was obtained by 3' RACE using sense primer, as described in Methods. The result of amplification cDNA fragment showed the appearance of 900 bp (Figure 25). After that, PCR product was eluted from agarose gel electrophoresis and used to clone into cloning vector, as described in Method. The recombinant clones were analyzed by *Xba*I and *Hin*dIII restriction enzyme analysis. Figure 26 shows the result of restriction enzyme analysis. The derived nucleotide sequence of 3' end DNA fragment contains 807 bp open reading frame encoding a predicted protein of 159 amino acids starting from the first ATG (Figure 27). The obtained nucleotide sequences were compared with GenBank database of National Center for Biotechnology Information (NCBI). The result shows sequence similar with alpha-amylase of Coleopteran which was almost 80%.

The obtained nucleotides 5'/3' end were used as the information to design primers for full-length alpha-amylase gene. The result of PCR product was determined by agarose gel electrophoresis. The expected PCR product size was 1,500 bp as shown in Figure 28. The eluted full-length alpha-amylase gene was used in cloned by using TA cloning. After that, recombinant clones were screened by colony PCR. The results of colony PCR showed approximately 1,700 bp (Figure 29). The result of DNA sequencing analysis showed 1,571 bp in length. The derived nucleotide sequence from start codon contains 1,458 bp open reading frame encoding a predicted protein of 485 amino acids (Figure 30). After blasted with the GenBank database, the sequence of this clone showed almost 70% identities to Coleopteran alpha-amylases.

A comparison with other alpha-amylase amino acid sequences shows that a glutamic acid residue and two aspartic acid residues that are known to be at the active site of porcine pancreatic alpha-amylase (Asp 197, Glu 233, Asp 300) and are conserved in most other alpha-amylase, are found in *Sitophilus oryzae* alpha-amylase as Asp 202, Glu 238, Asp 303. These residues are boxed in Figure 32. In addition, the

derived amino acid sequence has a single N-glycosylation site (AsnGlySer) close to the C-terminus (Figure 33).

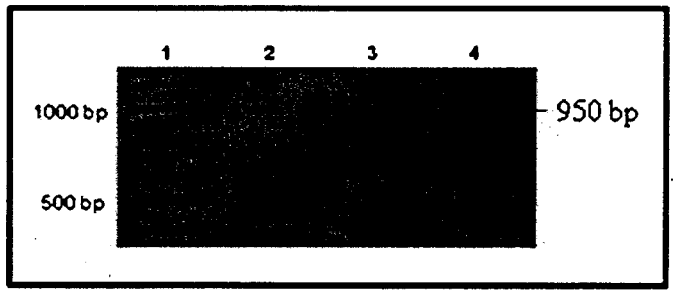


Figure 19 Agrose gel electrophoresis of PCR amplification of conserved alpha-amylase gene from rice weevil, lane 1: 100 bp Plus DNA ladder, lane 2: β-actin gene, lane 3: alpha-amylase gene and lane 4: Negative control, lane 3 and 4 using degenerate primer, resulted in the amplification of approximately 950 bp PCR product in lane 3.

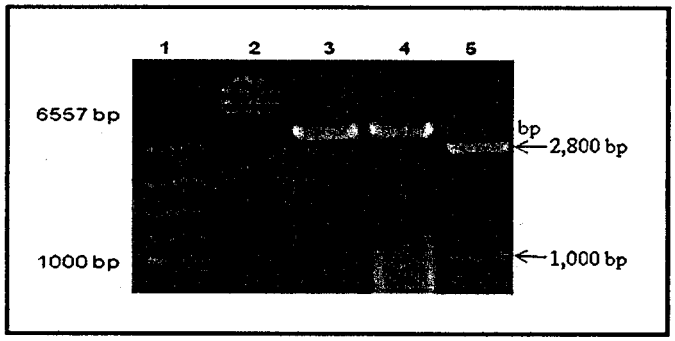


Figure 20 Agrose gel electrophoresis of recombinant plasmid digested with *Xba*I and *Hin*dIII restriction enzyme, lane 1: 100 bp Plus DNA ladder, lane 2: λ *Hin*dIII marker, lane 3: recombinant plasmid digested with *Xba*I, lane 4: recombinant plasmid digested with *Hin*dIII and lane 5: recombinant plasmid digested with *Xba*I and *Hin*dIII, resulted in the digested DNA fragments of approximately 2,800 bp and 1,000 bp.

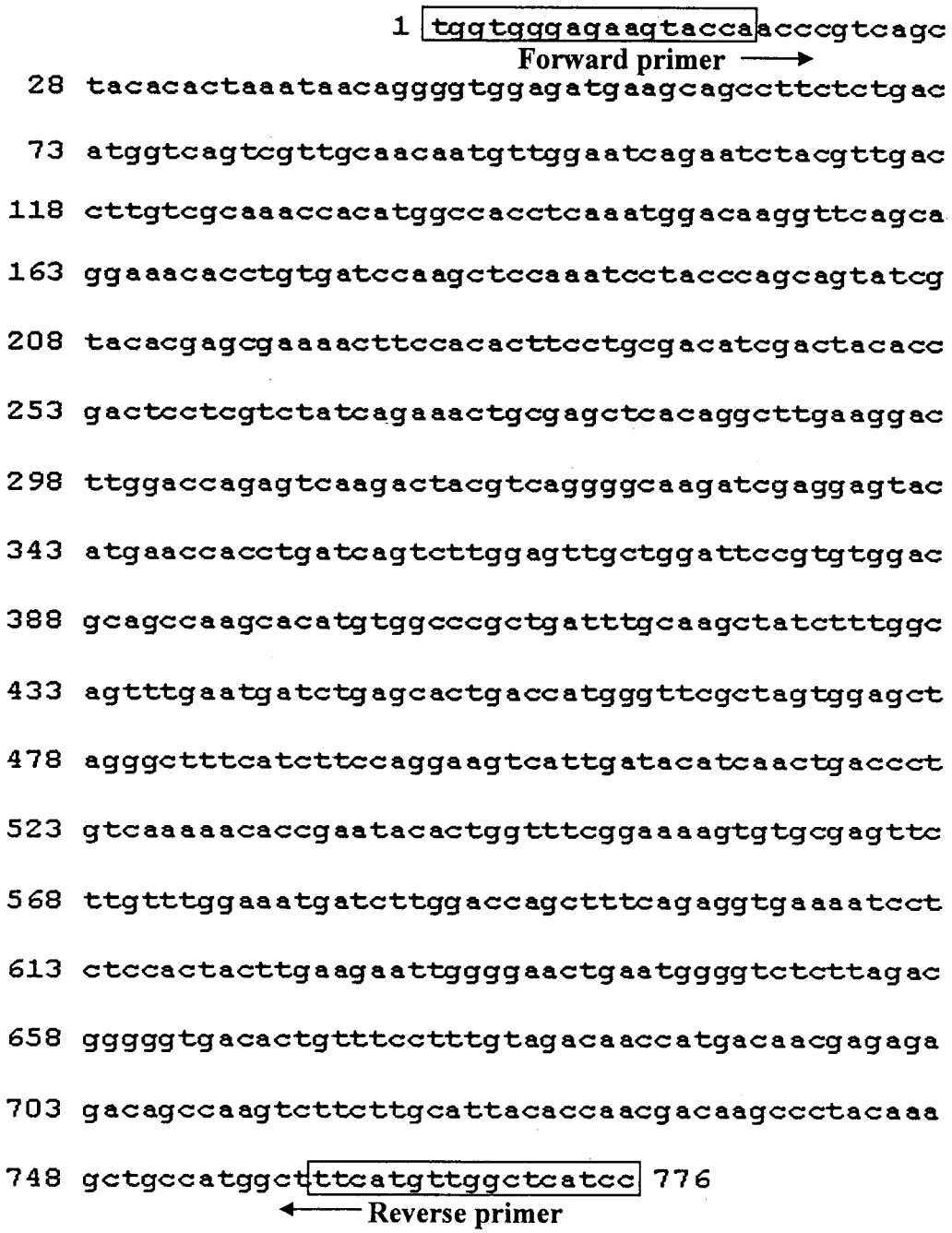


Figure 21 The partial nucleotide sequences and derived amino acid sequences of *Sitophilus oryzae*. The boxed regions represented the primer binding region.

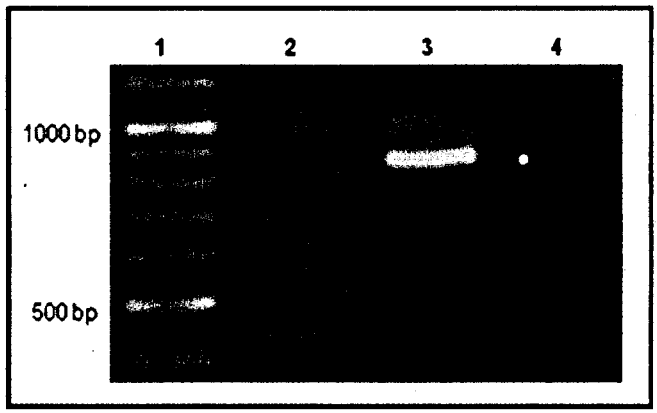


Figure 22 Agrose gel electrophoresis of PCR amplification of 5' end alpha-amylase gene, lane 1: 100 bp Plus DNA ladder, lane 2: β-actin gene, lane 3: alpha-amylase gene of 5' end and lane 4: negative control, resulted in the amplification of approximately 850 bp PCR product in lane 3.

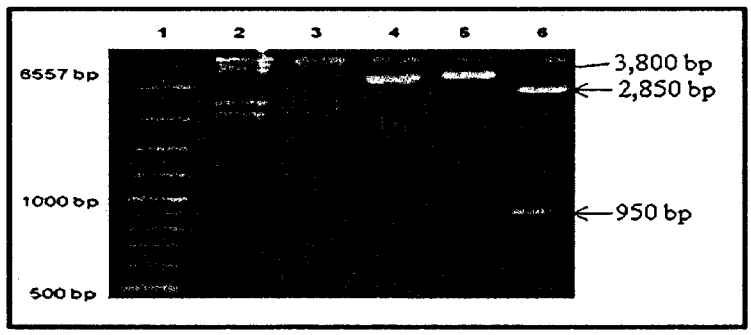


Figure 23 Agrose gel electrophoresis of recombinant plasmid digested with *Xba*I and *Hin*dIII restriction enzyme, lane 1: 100 bp Plus DNA ladder, lane 2: λ *Hin*dIII marker, lane 3: recombinant plasmid Undigested, lane 4: recombinant plasmid digested with *Xba*I, lane 5: recombinant plasmid digested with *Hin*dIII and lane 6: recombinant plasmid digested with *Xba*I and *Hin*dIII, resulted in the digested DNA fragments of approximately 2,850 bp and 950 bp.

1 GCATCTAGATCAAACAAGAACTCGCACACAATATTCAGTCTTATAGTACT

51 GATACACCAATAAACATGAAGGTACTAGCCCTTTTAGTTACTGTTTGCTT

101 TAGTGTGGCTTCTGCCCAAAAAGACCCACATTTTTTGGATGGCAGGAACA

151 CCATAGTCCATCTGTTCAAATGGAAATGGGCAGATATAGCCTCAGAATGT

201 GAAAACTTCCTGAGCGTCAAAAACTTTGCGGGTGTTCAGGTATCTCCTCC

251 CGCTGAAAGCGTTGTCGTAGAAGGCAGACCATGGTGGGAAAAATACCAAC

301 CCGTCAGCTACACACTAAATAACAGGGGTGGAGATGAAGCAGCCTTCTCT

351 GACATGGTCAGTCGTTGCAACAATGTTGGAATCAGAATCTACGTTGACCT

401 TGTCGCAAACCACATGGCCACCTCAAATGGACAAGGTTCAGCAGGAAACA

451 CCTGTGATCCAAGCTCCAAATCCTACCCAGCAGTATCGTACACGAGCGAA

501 AACTTCCACACTTCCTGCGACATCGACTACACCGACTCCTCGTCTATCAG

551 AAACTGCGAGCTCACAGGCTTGAAGGACTTGGACCAGAGTCAAGACTACG

601 TCAGGGGCAAGATCGAGGAGTACATGAACCACCTGATCAGTCTTGGAGTT

651 GCTGGATTCCGTGTGGACGCAGCCAAGCACATGTGGCCGGCTGATTTGCA

701 AGCTATCTTTGGCGGTTTGAATGATCTGAGCACTGACCATGGGTTCGCTA

751 GTGGAGCTAGGGCTTTCATCTTCCAGGAAGTCATTGATACATCGACTGAC

801 CCTGTCAAAAACACCGAATACACTGGTTTCGGAAA[GTGTGCGAGTTCTT]

← Reverse primer

851 [GTTTGG] 856

Figure 24 The nucleotide sequences and derived amino acid sequences 5' RACE, the boxed regions represented the primer.

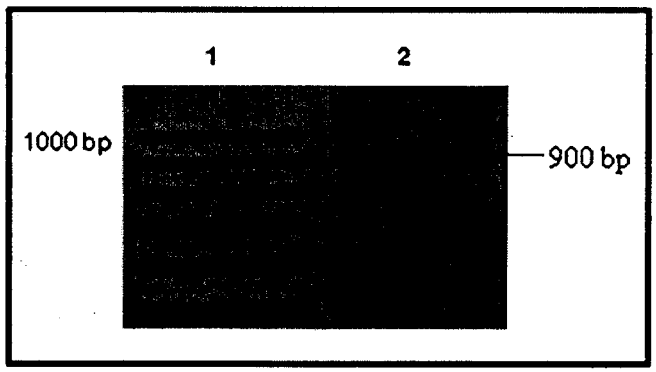


Figure 25 Agrose gel electrophoresis of PCR amplification of 3' end alpha-amylase gene, lane 1: 100 bp Plus DNA ladder, lane 2: alpha-amylase gene of 3' end, resulted in the amplification of approximately 900 bp PCR product in lane 2.

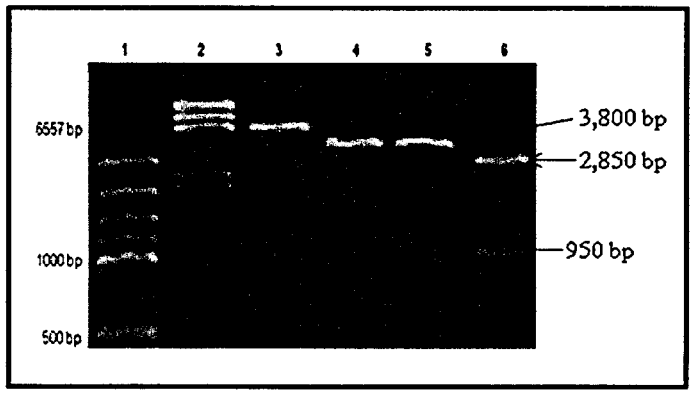


Figure 26 Agrose gel electrophoresis of recombinant plasmid digested with *Xba*I and *Hin*dIII restriction enzyme, lane 1: 100 bp Plus DNA ladder, lane 2: λ *Hin*dIII marker, lane 3: recombinant plasmid undigested, lane 4: recombinant plasmid digested with *Xba*I, lane 5: recombinant plasmid digested with *Hin*dIII and lane 6: recombinant plasmid digested with *Xba*I and *Hin*dIII, resulted in the digested fragments of approximately 2,850 bp and 950 bp.

1 TTCATCTTCCAGGAAGTCATTGACACAGGATCTGACCCTGTCTACAACAC

Forward primer ⟶

51 CGAATATACCAGCTTTGGAAAGGTTTGCGAATTCAAGTTCGGTAACTTTT

101 TGGGACCAGTATTCAGAGGAGAAAACGGACTTAAGTACCTGACCAACTGG

151 GGTACTGCCTGGGGTCTTTTGGACGGTGGAGACACAGTTTCCTTCGTAGA

201 CAACCACGACAACGAGAGAGACAGTGATGTCTACTTGCACTACAAAAACG

251 CCAAGCCATACAAGGCTGCTATCGCTTTCATGTTGGCTCACAGCTACGAC

301 ACCACTACTAGAGTACTGTCCAGTTACTACTTCGATGGACACGACCAAGC

351 TCCTCCAGCTGACGGTGACAACATTCTCTCCCCTGGCTTCAATGATGACG

401 GAAGCTGCACCAATGGCTGGGTATGTCAACATAGATGGTCTCCCATCTAC

451 AACATGGTCGAATTTAGAAACGTAGTGTCTGGTACCGAACTGACCAACTG

501 GTGGGACAATGGTGACAACCAGATTGCCTTCAGCAGAGGCGACAAAGGAT

551 TCTTCGCTGCCACAACCAGCGGTGATATCAACAGCAGTATCCCAACCGGA

601 CTGCCCGATGGTTCTTACTGTGACGTCATCCCTGGTAGTCTTTCCAGTGG

651 TTCTTGTACCGGCAAAACTTTGACAGTTAGCGGCGGAAATGTCAACGTCC

701 AGCTGAGCAGTGGTGACGAAGAAGCGGCTGTTGCCATCCATGTCAATGCT

751 AAATTGTAAACTGATGTTTGATATCCACCTTGATGACTTCCTGGAAGATG

801 AAATCGG 807

Figure 27 The nucleotide sequences and deduced amino acid sequences of 3' RACE, the boxed regions represented primer binding site.

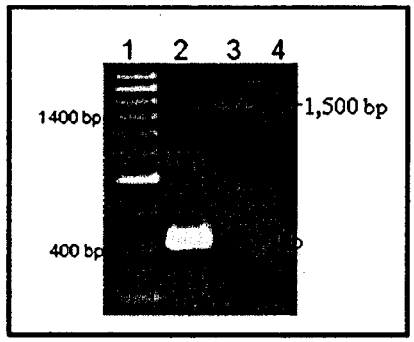


Figure 28 Agrose gel electrophoresis of PCR amplification of full-length alpha-amylase gene, lane 1: 200 bp DNA ladder, lane 2: β-actin gene, lane 3: full-length alpha-amylase gene and lane 4: negative control, resulted in the amplification of approximately 1,500 bp PCR product in lane 3.

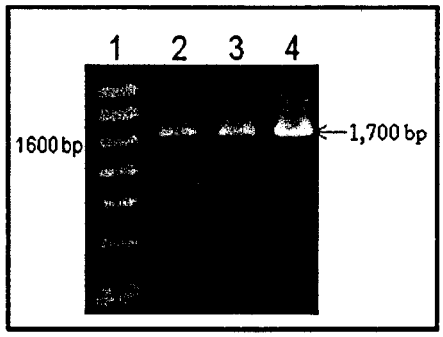


Figure 29 Agrose gel electrophoresis of PCR amplification of recombinant plasemid by colony PCR, lane 1: 200 bp DNA ladder, lane 2, 3 and 4: clone 1-3, respectively, resulted in the amplification of approximately 1,700 bp PCR product.

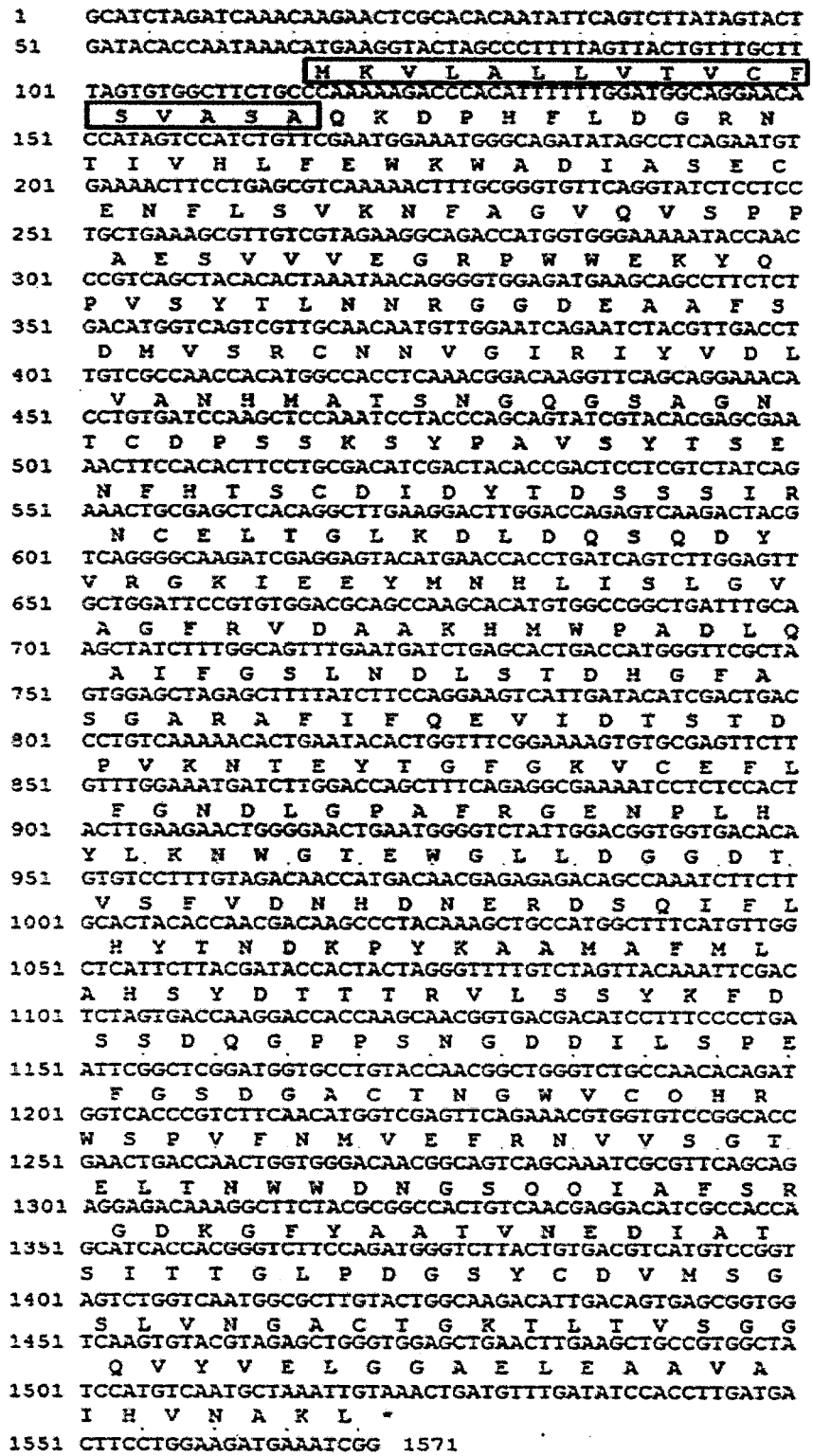


Figure 30 The nucleotide sequences and derived amino acid sequences of full-length alpha-amylase gene. The first seventeen amino acids in the red boxed represent the signal peptide.

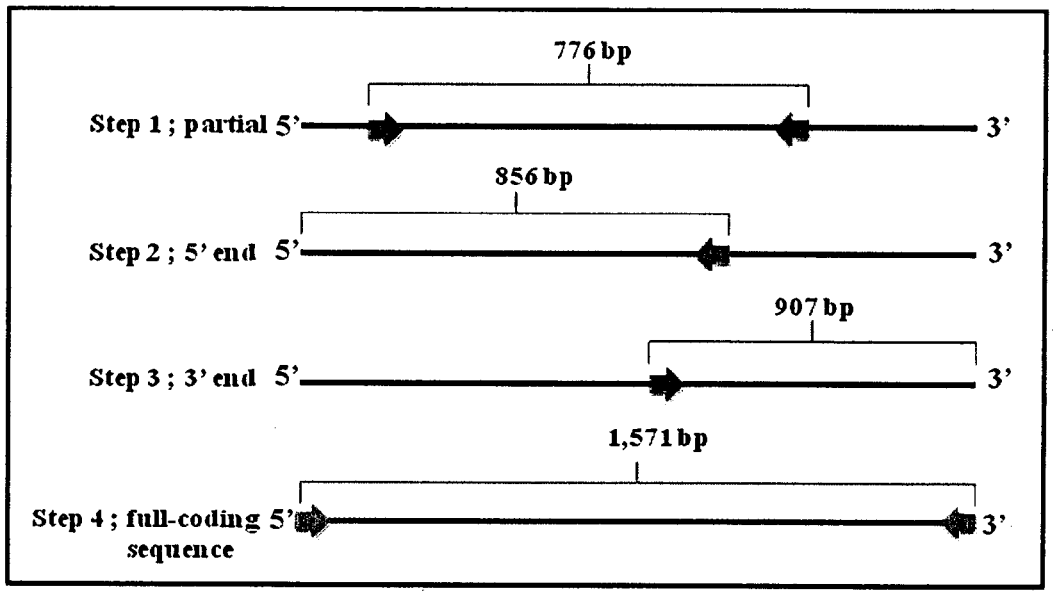


Figure 31 Schematic diagram of primer positions for alpha-amylase gene.

This diagram shows primers design for alpha-amylase gene amplification. Primers of partial nucleotide sequence of alpha-amylase gene amplification an shown in red, primers of 5'/3' end nucleotide sequence of alpha-amylase gene amplification an shown in blue and primers of complete nucleotide sequence of alpha-amylase gene amplification an shown in green.

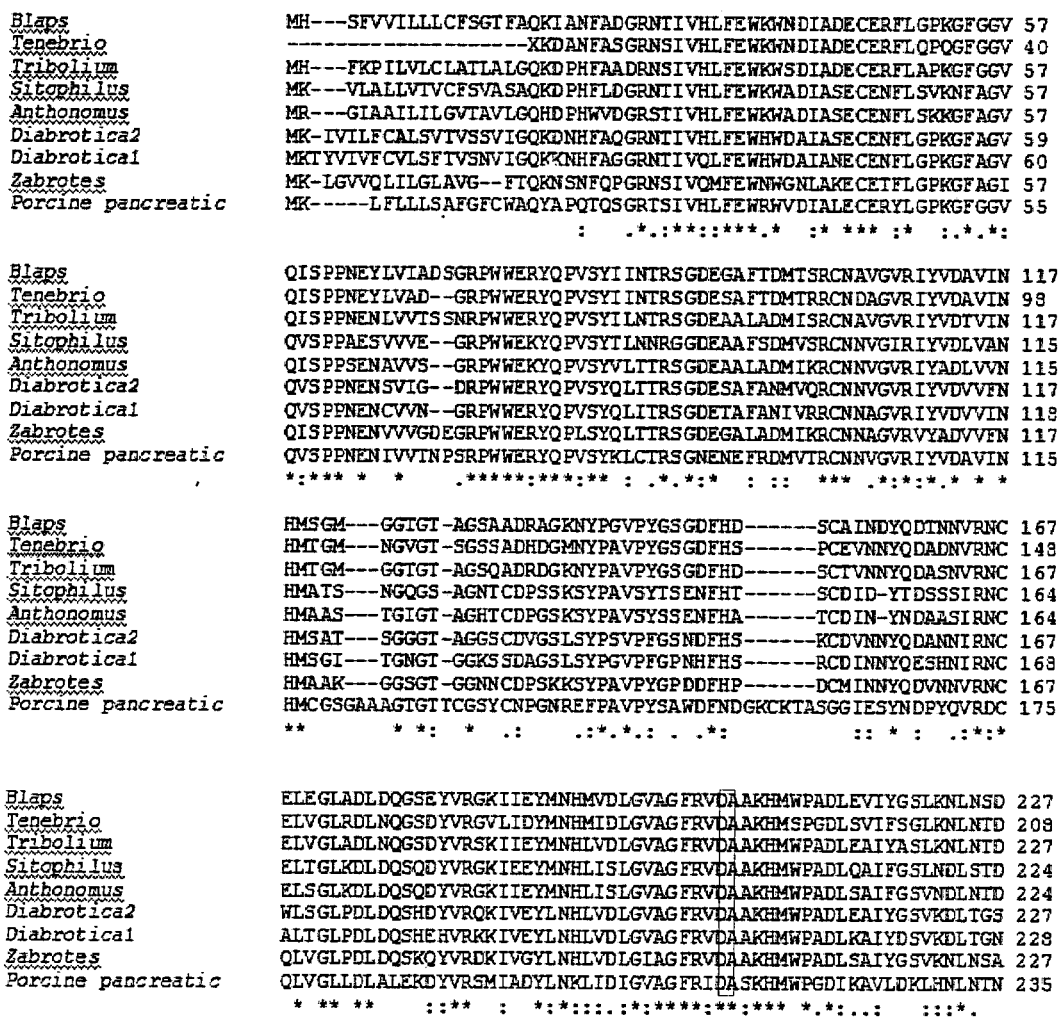


Figure 32 Amino acid sequences of alpha-amylase from *Blaps mucronata*, *Tenebrio molitor, Tribolium castaneum, Sitophilus oryzae, Anthonomus grandis, Anthonomus grandis* 2, *Anthonomus grandis* 1, *Zabrotes subfasciatus* and Porcine pancreatic. The active site residuces (Aps, Glu, Aps) are shown in boxed.

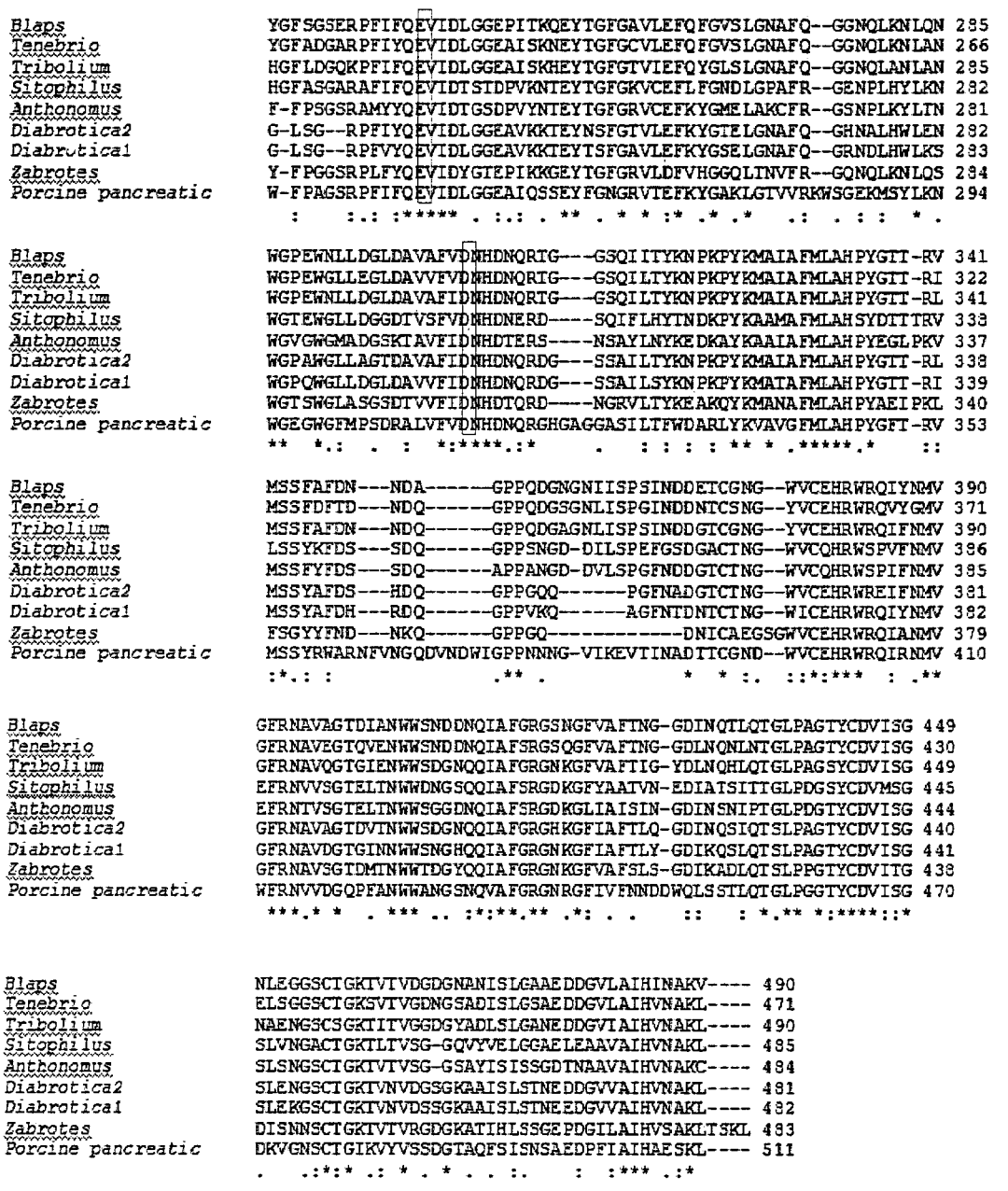


Figure 32 (Cont.)

MKVLALLVTVCFSVASAQKDPHFLDGRNTIVHLFEWKWADIASECENFLSVKNFAGVQVSPPAESVVVEGRPWWEKYQPV 80
SYTLNNRGGDEAAFSDMVSRCNNVGIRIYVDLVANHMATSNGQGSAGNTCDPSSKSYPAVSYTSENFHTSCDIDYTDSSS 160
IRNCELTGLKDLDQSQDYVRGKIEEYMNHLISLGVAGFRVDAAKHMWPADLQAIFGSLNDLSTDHGFASGARAFIFQEVI 240
DTSTDPVKNTEYTGFGKVCEFLFGNDLGPAFRGENPLHYLKNWGTEWGLLDGGDTVSFVDNHDNERDSQIFLHYTNDKPY 320
KAAMAFMLAHSYDTTTRVLSSYKFDSSDQGPPSNGDDILSPEFGSDGACTNGWVCQHRWSPVFNMVEFRNVVSGTELTNW 400
WDNGSQQIAFSRGDKGFYAATVNEDIATSITTGLPDGSYCDVMSGSLVNGACTGKTLTVSGGQVYVELGGAELEAAVAIH 480
VNAKL

Figure 33 The predicted N-glycosylation site (AsnGlySer) of amino acid sequences of *Sitophilus oryzae* close to the C-terminus is in the box.

Biochemical property investigation of alpha-amylase from rice weevil (*Sitophilus oryzae*)

1. Construction of pET-21b(+)-alpha-amylase gene

The recombinant pTZ57R/T-alpha-amylase gene plasmid was digested with *Xho*I and *Nde*I restriction enzymes. It was analyzed by agarose gel electrophoresis, as shown in Figure 34. After that, the DNA fragment of alpha-amylase gene was ligated to the pET-21b(+) expression vector as described in Methods. The recombinant plasmid clones were screened by colony PCR. The results of colony PCR showed approximately 1,500 bp PCR product (see Figure 35).

2. Expression of recombinant pET-21b(+)-alpha-amylase protein in *E.coli* BL21(DE3)pLysS

2.1 Optimization of the expression time for the recombinant pET-21b(+)-alpha-amylase protein in *E.coli* expression system

In order to optimize the protein expression system, *E.coli* BL21(DE3) pLysS harbouring the pET-21b(+)-alpha-amylase was grown at 28°C and induced with 1mM IPTG. Cells were harvested at various times (0, 1, 2, 3, 4, 5, 6, 16) before subjected to SDS-PAGE analysis. Figure 36 shows SDS-PAGE analysis of the total protein from whole-cell extracts of IPTG-induced cell cultures. In induced cells, an intense band of about 54 kDa can easily see.

2.2 Optimization of the expression temperature for the recombinant pET-21b(+)-alpha-amylase protein in *E.coli* expression system

In order to acquire the condition which yielded satisfactory amount of protein expression from the recombinant pET-21b(+)-alpha-amylase protein, comparative analysis was performed at various expression temperatures between 28°C, 37°C and 42°C and induced with 1mM IPTG. The cultures were induced for 4 hr. The expression pattern illustrated that the expression level at 28°C provided higher amount of alpha-amylase protein than 37°C (Figure 37). Therefore, the suitable condition for the recombinant pET-21b(+)-alpha-amylase expression was executed 28°C and 1mM IPTG induction for 4 hr (Figure 38).

2.3 Western blot analysis of the expression of recombinant pET-21b(+)-alpha-amylase protein in *E.coli* BL21(DE3)pLysS

After induction of recombinant plasmids pET-21b(+)-alpha-amylase in *E.coli* BL21(DE3)pLysS, the proteins were analyzed by SDS-PAGE. After that, protein samples were transferred to polyvinylidene fluoride (PVDF) membrane and detected by monoclonal antibodies against His-tagged protein. The 54 kDa reacted with the antibodies, as shown in Figure 39. These results revealed that alpha-amylase protein was expressed as a fusion protein with the C-terminal $(His)_6$.

2.4 Identification of protein by LC-MS/MS

The obtained protein was identified by LC-MS/MS. After induction of recombinant plasmids pET-21b(+)-alpha-amylase in *E.coli* BL21(DE3)pLysS host. The protein band about 54 kDa was excised from SDS-PAGE, trypsin digested and analyzed by LC-MS/MS. Mascot search of MS/MS of protein band after induction for 4 hr showed the matched peptide to the alpha-amylase of *Sitophilus oryzae* (Figure 40 and 41).

3. Analysis of alpha-amylase enzyme activity

The alpha-amylase activity in the expressed protein was determined biochemically using starch and 2-Chloro-4-nitrophenyl-α-D-maltotrioside as a substrate. The alpha-amylase activity cannot be detected in the expressed protein of *E.coli* strain BL21(DE3)pLysS. The expressed protein was also analyzed by zymograms (activity gels) using starch as a substrate. The alpha-amylase activity cannot be detected by this method (Figure 41). In addition, the expressed protein was

also analyzed by 3, 5-Dinitrosalicylic acid (DNS) assay and 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate. The alpha-amylase activity cannot be detected by these methods (Table 4 and 5). The *E.coli*-expressed alpha-amylase protein should be purified before further activity assessment or the gene should be expressed in other expression systems because of the obtained amino acid sequences have N-glycosylation site which localize at 403 amino acid residues when predicted N-glycosylation was determined from amino acid sequences using online program.

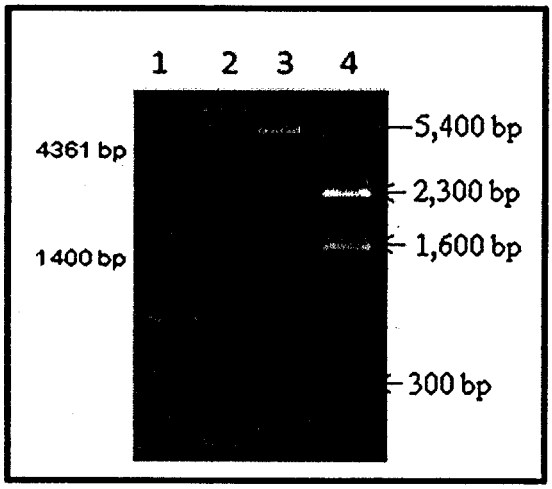


Figure 34 Agrose gel electrophoresis of recombinant pTZ57R/T–Alpha-amylase gene plasmid by restriction enzyme digestion with *Xho*I and *Nde*I, lane 1: 200bp DNA ladder, lane 2: λ *Hind*III marker, lane 3: *Xho*I and *Nde*I digested pET21b(+) (-5,400 bp) and lane 4: digested alpha-amylase recombinant plasmid with *Xho*I and *Nde*I shown three different size fragments containing with 2,300 bp, 1,600 bp and 300 bp.

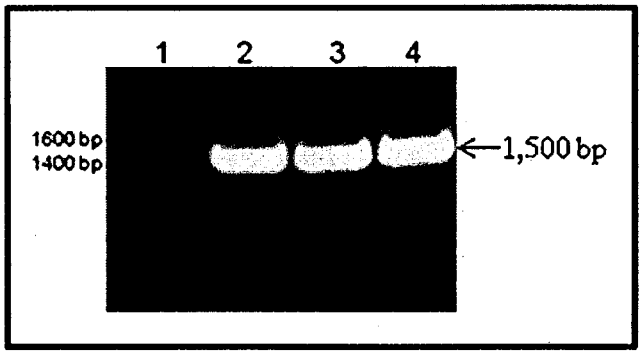


Figure 35 Agrose gel electrophoresis of colony PCR, lane 1: 200 bp DNA ladder, lane 2, 3 and 4: clone 1-3, respectively, resulted in the amplification of approximately 1,500 bp PCR product.

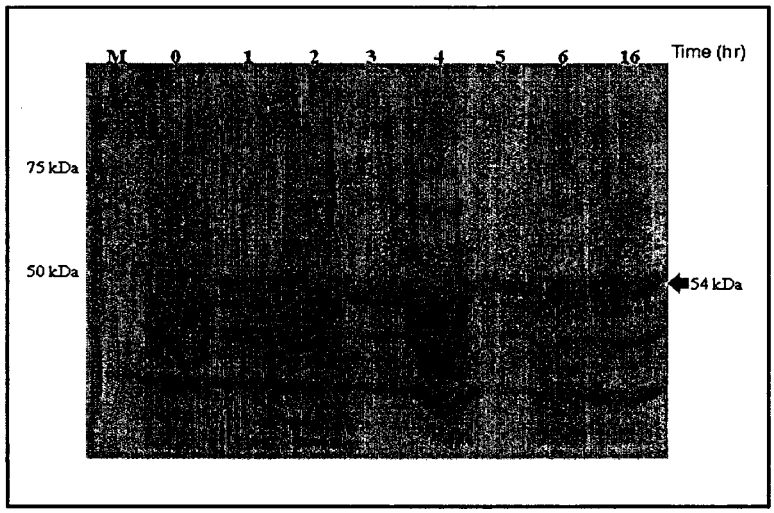


Figure 36 10% SDS-PAGE gel of recombinant pET-21b(+)-alpha-amylase was induced with 1mM IPTG at 28°C for 4 hr. lane M: Bio-Rad Precision Plus Protein™ Unstain Standards, lane 0- 16: induced pET-21b(+)-alpha-amylase cells from 0- 16 hr, respectively.

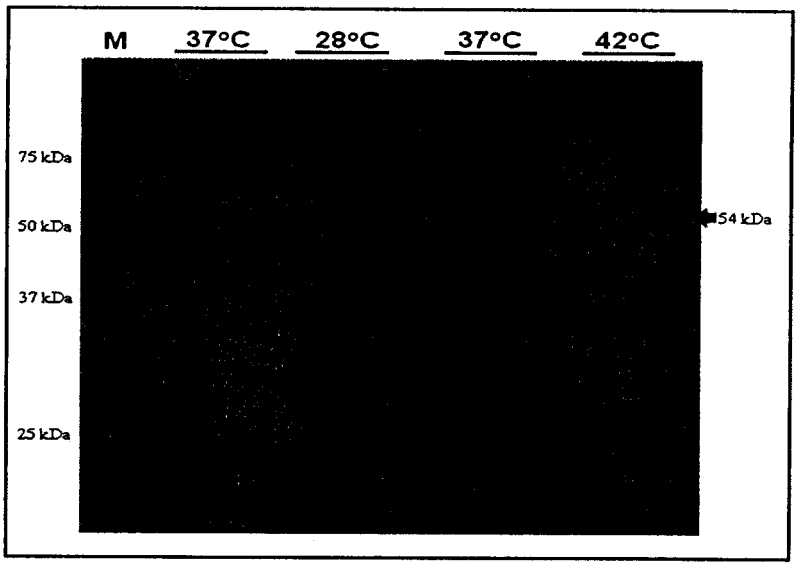


Figure 37 10% SDS-PAGE gel of recombinant pET-21b(+)-alpha-amylase was induced with 1mM IPTG at the various temperature for 4 hr. lane M: Bio-Rad Precision Protein™ Unstain Standards, where S is the Supernatant and P is the pellet.

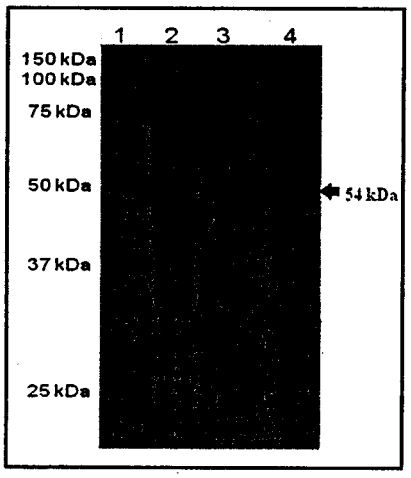


Figure 38 10% SDS-PAGE gel of recombinant pET-21b(+)-alpha-amylase was induced with 1mM IPTG at 28°C for 4 hr. lane 1: Bio-Rad Precision Plus Protein™ Unstain Standards, lane 2: induced pET-21b(+) cell, lane 3: non- induced pET-21b(+)-alpha-amylase cell and lane 4: induced pET-21b(+)- alpha-amylase cell, resulted in the intense band of approximately 54 kDa.

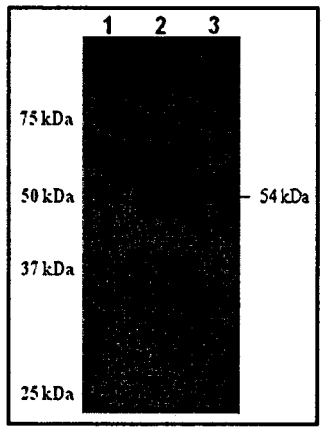


Figure 39 Western bolt analysis of recombinant pET-21b(+)-alpha-amylase that was induced with 1mM IPTG at 28°C for 4 hr. lane 1: Bio-Rad Precision Plus Protein™ Unstain Standards, lane 2: induced pET-21b(+)-alpha-amylase cells, resulted in the western bolt of approximately 54 kDa and lane 3: non-induce pET-21b(+)-alpha-amylase cells.

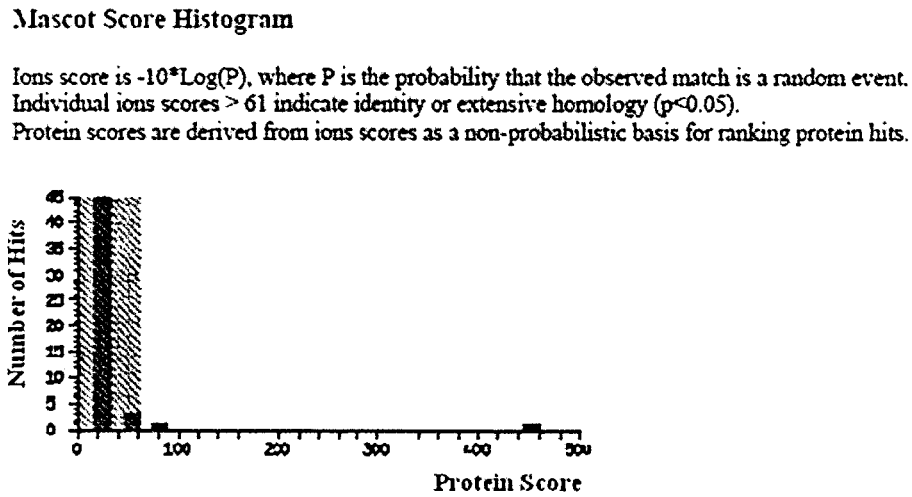
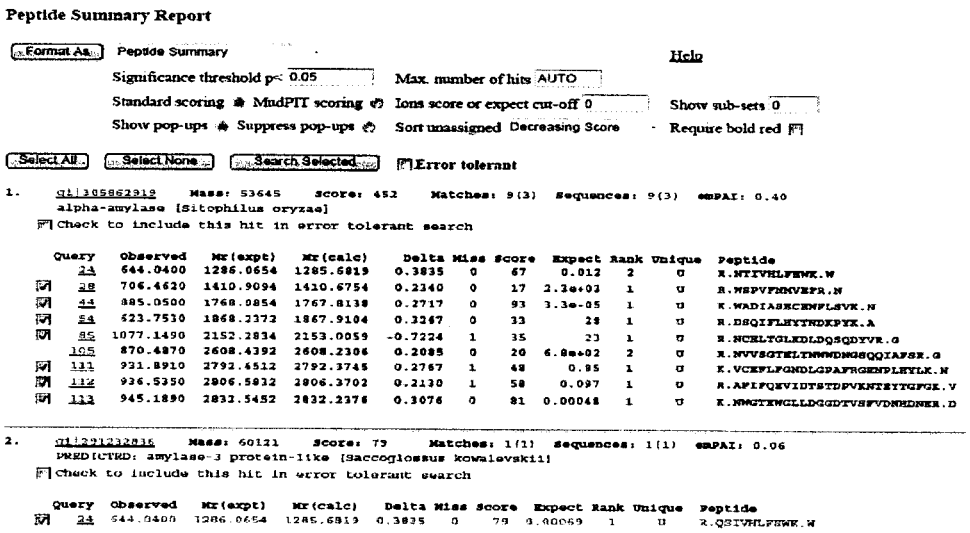


Figure 40 Mascot search of LC-MS/MS result showing that the tryptic digests of 54 kDa protein have exactly matched to alpha-amylase of *Sitophilus oryzae.*



Peptide Summary Report

Format As... Peptide Summary — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Sort unassigned Decreasing Score — Require bold red

Select All | Select None | Search Selected | Error tolerant

1. gi|305862918 Mass: 53645 Score: 452 Matches: 9(3) Sequences: 9(3) emPAI: 0.40
alpha-amylase [Sitophilus oryzae]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
24	644.0400	1286.0654	1285.6819	0.3835	0	67	0.012	2	U	R.NTIVHLFEWK.W
28	706.4620	1410.9094	1410.6754	0.2340	0	17	2.3e+03	1	U	R.WEPVFHMVEFR.N
44	885.0500	1768.0854	1767.8138	0.2717	0	93	3.3e-05	1	U	K.WADIASKCEMFLSVK.N
84	523.7530	1868.2372	1867.9104	0.3267	0	33	28	1	U	R.DSQIFLHYTNDKPYK.A
85	1077.1490	2152.2834	2153.0059	-0.7224	1	35	23	1	U	R.NCELTGLKDLDQSQDYVR.G
105	870.4870	2608.4392	2608.2306	0.2085	0	20	6.8e+02	2	U	R.NVVSGTELTNWNDNGSQQIAFSR.G
111	931.8910	2792.4512	2792.3745	0.2767	1	48	0.85	1	U	K.VCEKFLFQNDLGPAFRGENPLHYLK.N
112	936.5350	2806.5812	2806.3702	0.2130	1	58	0.097	1	U	R.AFIFQEVIDTSTDPVKNTEYTGFGE.V
113	945.1890	2832.5452	2832.2378	0.3076	0	81	0.00048	1	U	K.NWGTEWGLLDGGDTVSFVDNHDNER.D

2. gi|291232816 Mass: 60121 Score: 79 Matches: 1(1) Sequences: 1(1) emPAI: 0.06
PREDICTED: amylase-3 protein-like [Saccoglossus kowalevskii]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
24	644.0400	1286.0654	1285.6819	0.3835	0	79	0.00069	1	U	R.QSIVHLFEWK.W

Figure 41 Identification of peptide mass fingerprints of alpha-amylase protein that was induced with 1mM IPTG at 28°C for 4 hr by LC-MS. The protein have exactly matched to alpha-amylase of *Sitophilus oryzae*.

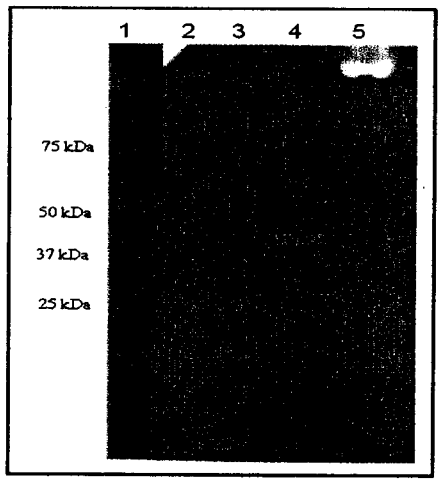


Figure 42 Zymogram analysis of recombinant pET-21b(+)-alpha-amylase that was induced with 1mM IPTG at 28°C for 4 hr. lane 1: Bio-Rad Precision Plus Protein™ Unstain Standards, lane 2: induced pET-21b(+)-alpha-amylase cell and lane 3: non-induce pET-21b(+)-alpha-amylase cell, lane 4: purified alpha-amylase after DEAE sepharose of adult *Sitophilus oryzae* (positive control) and Saliva from human (positive control).

Table 4 Activity of alpha-amylase as determined by the DNS assay

Sample	Alpha-amylase from *Sitophilus oryzae* (U/ml)
Induced pET21b(+) cell at 28°C for 4 hr	0.021
Non-induce pET-21b(+)-alpha-amylase cell at 28°C for 4 hr	0.021
Induced pET-21b(+)-alpha-amylase cell at 28°C for 4 hr	**0.021**
Purified alpha-amylase after DEAE sepharose of adult *Sitophilus oryzae* (positive control)	3.638
Saliva from human (positive control)	5.766

U/ml of enzyme activity was calculated using the formula: U/ml = (A_{550} control - A_{550} sample) ÷ A_{550}/ mg maltose ÷ 10 min ÷ 0.025 ml, where A_{550} control is the absorbance obtained from the substrate without the addition of enzyme, A_{550} sample is the absorbance for the starch digested with enzyme, A_{550}/ mg maltose is the absorbance for 1 mg of maltose as derived from the standard curve in Figure 18, 10 min is assay incubation time and 0.025 ml is the volume of the enzyme used in the assay.

Table 5 Activity of alpha-amylase as determined with the 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate

Sample	Alpha-amylase from *Sitophilus oryzae* (U/l)
Induced pET21b(+) cell at 28°C for 4 hr	19
Non-induce pET-21b(+)-alpha-amylase cell at 28°C for 4 hr	13
Induced pET-21b(+)-alpha-amylase cell at 28°C for 4 hr	**19**
Purified alpha-amylase after DEAE sepharose of adult *Sitophilus oryzae* (positive control)	75
Saliva from human (positive control)	100

U/l of enzyme activity was calculated using the formula: U/l = (ΔAbs sample x TV x 1000) ÷ (MMA x SV x LP), where ΔAbs sample is the absorbance for the starch digested with enzyme, TV is the total assay volume, 1000 is the conversion of U/ml to U/L, MMA is the millimolar absorptivity of 2-chloro-p-nitrophenol (12.9), SV is the sample volume, LP is the light path (1 cm).

Discussion

Cloning and analyzing nucleotide sequence of alpha-amylase genes from rice weevil (*Sitophilus oryzae*)

Alpha-amylase from insect plays a central role in carbohydrate metabolism with a starch-rich diet depend on the effectiveness of their amylases (Titarenko and Chrispeels, 2000). Rice weevil can cause considerable damage to rice grain and other economically important seeds. Previous reports have shown nucleotide sequences of insect's alpha-amylases such as *Zabrotes subfasciatus* (Grossi DE SA and Chrispeels, 1997), *Diabrotica virgifera virgifera* (Titarenko and Chrispeels, 2000), *Scirpophaga incertulas* Walker (Sharma, et al., 2009), and *Tenebrio molitor* (strobl, et al., 1997). In this study, the primers were designed from conserve region of nine known insect alpha-amylases to obtain the nucleotide of *Sitophilus oryzae* alpha-amylase. For nucleotide sequences, the results show nucleotide sequences of partial alpha-amylase contains 776 bp open reading frame encoding a predicted protein of 234 amino acids starting from the first ATG. The obtained nucleotide sequences were compared with GenBank database of National Center for Biotechnology Information (NCBI). The result shows sequence similar with alpha-amylase of Coleopteran which was almost 70%.

The full coding sequences of alpha-amylase gene were amplified with specific primers. The specific primers were designed based on our previous primary fragment. Then, we obtained the full coding sequence fragment by extended the primary fragment in both directions by RACE-PCR. The complete coding sequences gene contains 1,458 bp open reading frame encoding a predicted protein of 485 amino acids. This protein was predicted the molecular weight of 53,051.75 Daltons and theoretical pI of 4.69. The GenBank accession number of our alpha-amylase gene is HQ158012. This protein has putative signal peptide of 17 amino acid residues in length by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The amino acid sequences of this alpha-amylase have 71%, 62% and 60% sequence identity to *Anthonomus grandis*, *Diabrotica virgifera virgifera* and *Tribolium castaneum*, respectively. Our results and other studies lead to the conclusion that the active site of this enzyme is situated in a large groove or cleft that contains the catalytic site with three conserved residues Asp, Glu and Asp. Moreover, this important catalytic site is

found in *Sitophilus oryzae* alpha-amylase at Asp 202, Glu 238 and Asp 303 (Figure 28). On the other hand, porcine pancreatic alpha-amylase contains Asp 197, Glu 233 and Asp 300 (The numbering in porcine pancreatic excludes the signal peptide) (Grossi DE SA and Chrispeels, 1997) and *Ephestia kueniella* alpha-amylase contains Asp 193, Glu 230 and Asp 295 (Pytelkova, et al., 2009). In addition, Strobl, et al. (1998) analyzed the 3D structure of the yellow mealworm (*Tenebrio molitor*) alpha-amylase and indicated the similar residues (Asp 185, Glu 222, Asp 287). Furthermore, importance of these three amino acid residues has been confirmed by site-directed mutagenesis (Grossi DE SA and Chrispeels, 1997).

Biochemical property investigation of alpha-amylase from rice weevil (*Sitophilus oryzae*)

In this study, the alpha-amylase gene of *Sitophilus oryzae* was expressed in *E.coli* strain BL21(DE3)pLysS by used pET21b(+) vector. The result of protein expression was visualised by Coomassie staining at approximately 54 kDa. The *Sitophilus oryzae* alpha-amylase gene was expressed by transcription of the T7 promoter from pET21b(+) plasmid. The activity of alpha-amylase protein was determined by Zymogram, DNS assay and 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate. Unfortunately, the activity of *Sitophilus oryzae* alpha-amylase expressed in *E.coli* strain BL21(DE3)pLysS cannot be detected by these methods. Obtained alpha-amylase protein should be purified before further activity assessment. In addition, N-glycosylation of putative amino acid sequences was predicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetN Glyc/). The result shows that a putative alpha-amylase amino acid sequences have N-glycosylation sites, which localize at 403 amino acid residues. Furthermore, a post-translational protein modification was determined in the *Tenebrio molitor* (TMA) (Stobl, et al., 1997 and Stobl, et al., 1998), Bruchid (*Zabrotes Subfasciatus*) (Grossi DE SA and Chrispeels, 1997) and *Ephestia kueniella* (Pytelkova, et al., 2009). They found that the alpha-amylase has been post-translational modified. In addition, there are the predictions of O-glycosylation site on alpha-amylase from different insects based on protein and DNA sequence data. The study of Effio, et al. (2003) found that the insect alpha-amylase of *Zabrotes subfasciatus*, *Phaedon cochleriae, Lutzomyia longipalpis* and *Anopheles merus* have O-glycosylation site.

In the expression of alpha-amylase gene in *E.coli*, there is the study that expressed alpha-amylase of *Lipomyces starkeyi* in *E.coli* strain BL21(DE3)pLysS. The obtained protein can be determined the enzyme activity (Kang, et al., 2004). Moreover, Hyperthermophilic Archaeon *Pyrococcus* sp. was successfully cloned and expressed in *E.coli* and the obtained protein can be determined the enzyme activity (Tachibana, et al, 1996).

Recently, there are studies that reported the expression of insect alpha-amylase in insect cell lines with a baculovirus vector (Grossi DE SA and Chrispeels, 1997; Titarenko and Chrispeels, 2000). Furthermore, *Zabrotes subfasciatus* alpha-amylase was expressed in *Spodoptera frugiperda* (SF9) cells. The molecular mass of *Zabrotes subfasciatus* alpha-amylase was estimated to be 50 kDa and the expression produced the active alpha-amylase (Fatma and Maarten, 1997). Moreover, alpha-amylase of Western corn rootworm (*Diabrotica virgifera vigifera* (Dval)) was also successfully expressed in Sf9 insect cells (Titarenko and Chrispeels, 2000). Therefore, the expression of *Sitophilus oryzae* alpha-amylase gene should be expressed in the eukaryotic cells such as insect cells, yeast cells. After that, the activity of this alpha-amylase will be confirmed by Zymogram, DNS assay and 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate.

CHAPTER V

CONCLUSION

The full coding sequence of alpha-amylase gene contains 1,458 bp open reading frame encoding a predicted protein of 485 amino acids starting from the first ATG. The amino acid sequence of this clone shows almost 70% identities to Coleopteran alpha-amylases. The GenBank accession number of our alpha-amylase gene is HQ158012. The catalytic residues of *Sitophilus oryzae* alpha-amylase are Asp 202, Glu 238 and Asp 303.

Sitophilus oryzae alpha-amylase gene was expressed in *E.coli* strain BL21(DE3)pLysS using pET21b (+) vector. The expressed protein was analyzed by SDS-PAGE and visualized by Coomassie blue staining. The protein band at approximately 54 kDa was observed. The activity of alpha-amylase protein was determined by Zymogram, DNS assay and 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate. Unfortunately, the activity of *Sitophilus oryzae*'s alpha-amylase cannot be detected by these methods. Obtained alpha-amylase protein was determined a putative amino acid sequences using predicted N-glycosylation. The N-glycosylation localizes at 403 amino acid residues. However, *E.coli* cannot generate post-translational protein modifications. Therefore, in further study, the *Sitophilus oryzae* alpha-amylase protein should be purified before further activity or the gene should be expressed in eukaryote cell because of post-translational protein modifications. Then, this alpha-amylase enzyme will be investigated its biochemical properties against rice alpha-amylase inhibitor.

การวิเคราะห์หาลำดับนิวคลีโอไทด์ของยีน alpha-amylase จากมอดข้าวสาร

Nucleotide sequencing analysis of alpha-amylase gene from rice weevil (*Sitophilus oryzae* L.)

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รศ.ดร. สุขกิจ ยะโสธรศรีกุล[4]

บทคัดย่อ

ข้าวเป็นหนึ่งในสินค้าเกษตรที่สำคัญของประเทศไทย และมีความสำคัญต่อชีวิตความเป็นอยู่ของคนไทยเป็นอย่างมาก อย่างไรก็ตามปัญหาหลักของการปลูกข้าวคือแมลงศัตรูข้าว ซึ่งมอดเป็นหนึ่งในแมลงศัตรูข้าว โดยมอดจะสร้างเอ็นไซม์ alpha-amylase ซึ่งมีคุณสมบัติในการเร่งปฏิกิริยาการย่อยพันธะ alpha-(1,4) glycosidic bonds ใน amylose และ amylopectin ซึ่งเป็นองค์ประกอบของข้าว เพื่อใช้เป็นแหล่งอาหาร ดังนั้นการทราบคุณสมบัติทางชีวเคมีของเอ็นไซม์ alpha-amylase ในมอดจะเป็นประโยชน์ในการศึกษาหาตัวยับยั้งที่จำเพาะต่อ alpha-amylase ในมอด เพื่อเป็นการปกป้องข้าวจากการถูกทำลายโดยมอดได้ต่อไป งานวิจัยทำการโคลนและวิเคราะห์หาลำดับนิวคลีโอไทด์ของ alpha-amylase จากมอดข้าวสาร โดยสกัด RNA จากมอดข้าวสาร และเพิ่มปริมาณยีน alpha-amylase ด้วยเทคนิค RT-PCR โดยใช้ primer ที่ออกแบบมาจากส่วนที่ conserved ของยีน alpha-amylase ของแมลงในกลุ่ม Coleopteran จากนั้น PCR product ที่ได้ จะถูกโคลนเข้าสู่เวคเตอร์ pTZ57R/T จากนั้นลำดับนิวคลีโอไทด์ของยีนที่ถูกโคลนเข้าสู่เวคเตอร์จะถูกยืนยันโดยการทำ DNA sequencing ผลการทดลองแสดงให้เห็นว่า สามารถสกัด RNA จากมอดข้าวสารได้ นอกจากนั้น PCR product ที่ได้จากการทำ RT-PCR มีขนาดตามที่ได้ออกแบบไว้ คือ ประมาณ 950 bp สำหรับผลของการหาลำดับนิวคลีโอไทด์ เมื่อนำไปเปรียบเทียบกับฐานข้อมูลของ National Center for Biotechnology Information (NCBI) พบว่า มีความเหมือนกับ alpha-amylase ของแมลงในกลุ่ม Coleopteran โดยมีเปอร์เซ็นต์ Identities เท่ากับ 68 เปอร์เซ็นต์ จากนั้นผู้วิจัยทำการหา full-length ยีนของ alpha-amylase ของมอดข้าวสาร เพื่อทำการศึกษาถึงคุณสมบัติทางชีวเคมีของเอ็นไซม์นี้ต่อไป

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ABSTRACT

Rice is one of the important agricultural products of Thailand and also very important for Thai's life. However, the major problem of rice production is insect pest. Rice weevil is a pest that can produce alpha-amylase enzyme in order to utilize starch from rice grains as carbohydrate source for their growth and development. The alpha-amylase produced from the insect can hydrolyze alpha (1,4) glycosidic bonds of amylose and amylopectin. Understanding biochemical properties of this enzyme will help us to control the damage of stored rice grains by this insect in the future. Therefore, we aim to clone and analyse nucleotide sequences of alpha-amylase gene from rice weevil. Total RNA was extracted from rice weevil and RT-PCR was performed using primers which were designed from the conserved regions of Coleopteran alpha-amylase genes. The resulted PCR product was cloned into pTZ57R/T vector. Then, the nucleotide sequences of inserted gene were confirmed by DNA sequencing analysis. Our results showed the expected size of approximately 950 bp of the PCR product obtained from RT-PCR. The nucleotide sequences were blasted in the GenBank database. The result showed 68% identity with coleopteran alpha-amylases. We, therefore, will perform further experiments to obtain the full-length clone so that its biochemical properties can be studied.

บทนำ

ข้าวเป็นหนึ่งในสินค้าเกษตรที่สำคัญของประเทศไทย (Srinives et al. 2005) และมีความสำคัญต่อชีวิตความเป็นอยู่ของคนไทยอย่างสูง เนื่องจากคนไทยบริโภคข้าวเป็นอาหารหลัก และข้าวยังเป็นสินค้าส่งออกที่สำคัญของไทยตั้งแต่อดีตถึงปัจจุบัน (บุญหงษ์ จงคิด. 2547 : 11-17 ; วิมลพรรณ ปีตธวัชชัย. 2548 : 25) จากการสำรวจพื้นที่ปลูกข้าวในประเทศไทยช่วงปี พ.ศ. 2545-2546 พบว่ามีพื้นที่ปลูกข้าวประมาณ 78,250,960 ไร่ (กรมส่งเสริมการเกษตร. 2546) สามารถผลิตข้าวได้ 26.63 ล้านตัน มีการส่งออกข้าวประมาณ 7,345,971 ตัน คิดเป็นมูลค่ากว่า 76,699.16 ล้านบาท (สำนักงานเศรษฐกิจการเกษตร. 2551) การปลูกข้าวพบว่ามีปัญหาเกิดขึ้นหลายประการ เช่น พันธุ์ไม่บริสุทธิ์ ปัญหาดินเค็ม ดินเปรี้ยว เป็นต้น นอกจากนี้ยังมีปัญหาที่สำคัญของการปลูกข้าวเรื่องหนึ่ง คือ ศัตรูข้าว (สำนักบริการส่งออก. 2546) ซึ่งเป็นผลมาจากการขยายพื้นที่ปลูกข้าวและนำเทคโนโลยีใหม่ ๆ มาใช้เพื่อช่วยเพิ่มผลผลิต ซึ่งเป็นการเปลี่ยนแปลงสภาพนิเวศน์การเกษตรทำให้แมลงศัตรูข้าวที่มีอยู่เกิดการปรับตัว และก่อให้เกิดการระบาดของศัตรูข้าว ทำให้สูญเสียผลผลิต (จุฑารัตน์ อรรถจารุสิทธิ์. 2546)

แมลงศัตรูข้าวสามารถผลิตเอ็นไซม์ alpha-amylase ออกมาย่อยแป้งที่เป็นองค์ประกอบของข้าว ซึ่งเอ็นไซม์ alpha-amylase มีคุณสมบัติในการเร่งปฏิกิริยาการย่อยพันธะ alpha-(1,4) glycosidic bonds ในอะไมโลส (amylose) และอะไมโลเพกทิน (amylopectin) ซึ่งเป็นองค์ประกอบของแป้ง เพื่อใช้เป็นแหล่งอาหาร (MacGrego. 1993) นอกจากนี้ยังมีบทบาทในกระบวนการ metabolism ของคาร์โบไฮเดรต (carbohydrate) ด้วย (Stefan et al. 1997) จากคุณสมบัติของเอ็นไซม์ alpha-amylase นี้ ส่งผลกระทบต่อผลผลิตข้าว ทำให้ได้รับความเสียหายจาก

การเจาะทำลายของมอด หรือแมลงศัตรูข้าว ซึ่งการศึกษาเกี่ยวกับเอ็นไซม์ alpha-amylase ในมอดข้าวสารที่มีการแพร่ระบาดในประเทศไทยยังมีน้อยอยู่ ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อทำการวิเคราะห์หาลำดับนิวคลีโอไทด์ของยีน alpha-amylase จากมอดข้าวสาร (*Sitophilus oryzae*) เพื่อเป็นข้อมูลพื้นฐานในการศึกษาคุณสมบัติของเอ็นไซม์ alpha-amylase ในมอดข้าวสาร รวมถึงศึกษายีนที่ยับยั้งการทำงานของเอ็นไซม์ alpha-amylase ที่จำเพาะเจาะจงต่อกลุ่มแมลงเป้าหมายนี้ต่อไป และเพื่อเป็นประโยชน์ในการป้องกันกำจัดศัตรูพืชที่จำเพาะเจาะจง

วัตถุประสงค์การวิจัย

โคลนและวิเคราะห์หาลำดับนิวคลีโอไทด์ของเอ็นไซม์ alpha-amylase จากมอดข้าวสาร (*Sitophilus oryzae*)

วิธีการดำเนินการวิจัย

1. การเตรียมแมลงที่ใช้ในการทดลอง

โดยนำมอดข้าวสาร (*Sitophilus oryz*ae) ที่ได้มาจากแหล่งธรรมชาติ มาเลี้ยงในสภาวะแวดล้อมเช่นเดียวกับที่พบตามแหล่งธรรมชาติ โดยจะเลี้ยงมอดประมาณ 28 วัน จนโตเต็มวัย เมื่อได้ตัวเต็มวัยของมอดข้าวสารตามที่ต้องการแล้ว จากนั้นแยกมอดตัวเต็มวัยออกจากข้าวสาร โดยจัดเก็บใส่ tube โดยเก็บ tube ละ 200 มิลลิกรัม แล้วนำมอดที่แยกได้มาเก็บไว้ที่ -80 องศาเซลเซียส เพื่อใช้สำหรับสกัด RNA

1.2 การสกัด RNA จากมอดข้าวสาร (*Sitophilus oryzae*)

สกัด RNA จากมอดข้าวสาร โดยนำมอดตัวเต็มวัยปริมาณ 200 มิลลิกรัม บดในไนโตรเจนเหลวโดยใช้โกร่ง จากนั้นสกัด RNA โดยใช้ Trizol reagent เมื่อบดตัวอย่างในไนโตรเจนเหลวแล้วเติม Trizol reagent ทิ้งไว้ 5 นาที ที่อุณหภูมิห้องแล้วเติม Chloroform นำไปปั่น เพื่อแยกเอา RNA ซึ่ง RNA จะปนอยู่ใน Trizol reagent แล้วก็ทำการตกตะกอน RNA ด้วย Isopropanol ต่อมาจะล้างตะกอน RNA ด้วย ethanol 75 เปอร์เซ็นต์ และสุดท้ายละลาย RNA ด้วย RNase-free water แล้วนำ RNA ที่ได้ไปเป็นแม่แบบในการสังเคราะห์ cDNA ต่อไป

1.3 การสังเคราะห์ cDNA

นำ Total RNA ที่ได้จากการสกัด RNA จากมอดข้าวสารมาเป็นแม่แบบในการสังเคราะห์ cDNA ด้วยวิธี Reverse transcriptase polymerase chain reaction (RT-PCR) โดยอาศัยคุณสมบัติของเอ็นไซม์ reverse transcriptase ซึ่งสามารถสร้างสายของ cDNA ขึ้นมาโดยอาศัย RNA เป็นต้นแบบ และจะใช้ Oligo(dT) เป็น primer ซึ่งเป็น primer ที่จำเพาะต่อ poly A tail ของสาย RNA สำหรับการสังเคราะห์ cDNA โดยใช้ RevertAid™ First Strand cDNA Synthesis kit

1.4 การเตรียม DNA สำหรับการโคลน

นำ cDNA ที่ได้จากการสังเคราะห์เป็นแม่แบบ เพื่อทำการเพิ่มจำนวนด้วยเทคนิค polymerase chain reaction (PCR) โดยใช้ primer ที่ถูกออกแบบมาจากตำแหน่ง conserved region ของยีนที่กำหนดการสร้างเอ็นไซม์ alpha-amylase ของแมลงในกลุ่ม Coleopteran (แมลงปีกแข็ง) ซึ่งการออกแบบ primer จะใช้แมลงทั้งหมด 9 ชนิด ประกอบด้วย *Anthonomus grandis* (Amylag2) (gi: 25992515), *Anthonomus grandis* (Amylag1) (gi: 25992513), *Tribolium castaneum* (gi: 167466184), *Zabrotes subfasciatus* (gi: 8132974), *Callosobruchus chinensis* (gi: 57157295), *Diabrotica virgifera virgifera* (gi: 6644299), *Blaps mucronata* (Amy2) (gi: 27525633), *Blaps mucronata* (Amy1) (gi: 27450389), *Phaedon cochleariae* (GI: 4210797) โดยการนำลำดับนิวคลีโอไทด์ของแมลงแต่ละชนิดไป align ในฐานข้อมูล National Center for Biotechnology Information (NCBI) ซึ่ง primer ที่ใช้จะเป็น Degenerate primer โดย Forward Primer จะมีลำดับนิวคลีโอไทด์ดังนี้ 5'-TGGTGGGARAVRTACCA-3' และ Reverse Primer จะมีลำดับนิวคลีโอไทด์ 5'-GGATGVGCYADC ATRAA-3' โดย D คือ A+T+G, R คือ A+G, V คือ A+C+G และ Y คือ C+T เมื่อได้ PCR product แล้ว จะตรวจดูขนาดของ PCR product ด้วยเทคนิค Gel electrophoresis และ Elute เอา PCR product ออกจาก gel โดยใช้ชุด Kit FXTM PCR DNA and Gel Band Purification

1.5 การโคลน cDNA โดยใช้ TA cloning

นำ PCR product ที่ Elute จาก gel มาทำ PCR เพื่อทำให้ยีนมีปลาย 3'-dA overhangs สำหรับเชื่อมต่อกับเวคเตอร์ ที่มีปลาย ddT ในการโคลนยีนนี้ใช้ชุด TA cloning เพื่อเพิ่มจำนวน DNA หรือ พลาสมิด ซึ่งในการทำ TA cloning นั้นจะเริ่มจากการทำ ligation ซึ่งเป็นการเชื่อมยีน (alpha-amylase) กับเวคเตอร์ (pTZ57R/T) ที่มากับชุด kit จากนั้นนำเวคเตอร์ ที่มีการ insert ของยีนที่ต้องการ ทำการ transform เข้าไปใน host cell (*Escherichia coli* DH5α) ด้วยวิธี heat-shock แล้วนำเซลล์ไปเลี้ยงต่อใน SOC medium เป็นเวลา 1.5 ชั่วโมง จากนั้นทำการคัดเลือกโคลน หรือ DNA Recombinant โดยใช้วิธีคัดเลือกแบบ blue-white colony บนอาหารเลี้ยงเชื้อที่มี Isopropyl β-D-thiogalactopyranoside (IPTG) และ X-Gal แล้วนำไปบ่มข้ามคืนที่อุณหภูมิ 37 องศาเซลเซียส โดยคัดเลือกโคโลนีที่เป็นสีขาว ซึ่งเป็นโคโลนีที่มียีนที่ insert เข้าไป และคัดเลือกโคโลนีสีขาวไปเลี้ยง เพื่อเพิ่มจำนวนในอาหารเหลว และทำการสกัดพลาสมิดด้วย Pure YieldTM Plasmid Miniprep System เพื่อใช้ในการศึกษาหาลำดับนิวคลีโอไทด์ โดยการทำ DNA sequencing ต่อไป

1.6 การหาลำดับนิวคลีโอไทด์

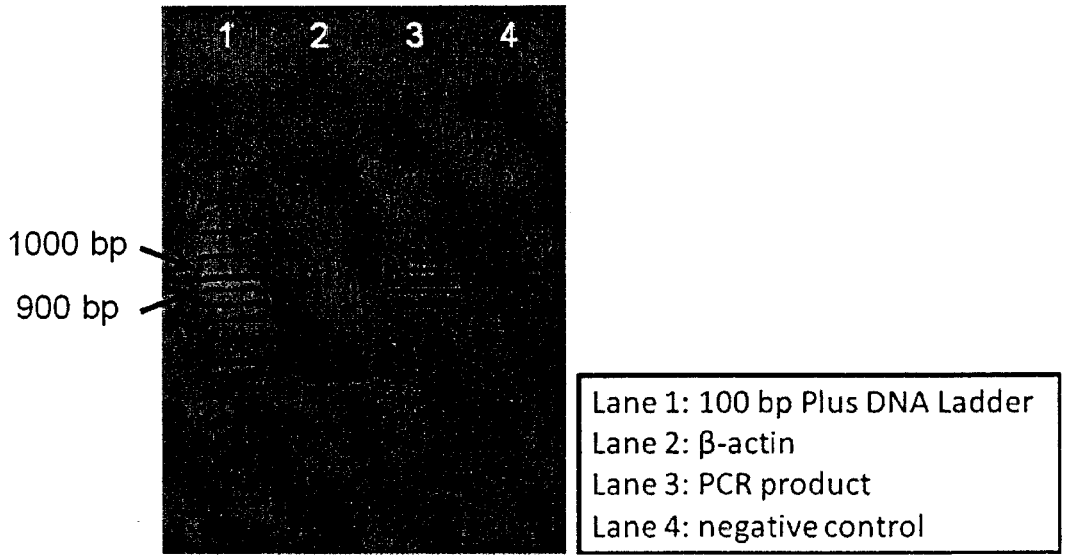
หาลำดับนิวคลีโอไทด์ด้วยเครื่อง Automatic Sequencer นำผลที่ได้มาวิเคราะห์และเปรียบเทียบกับฐานข้อมูลใน National Center for Biotechnology Information (NCBI)

ผลการวิจัย

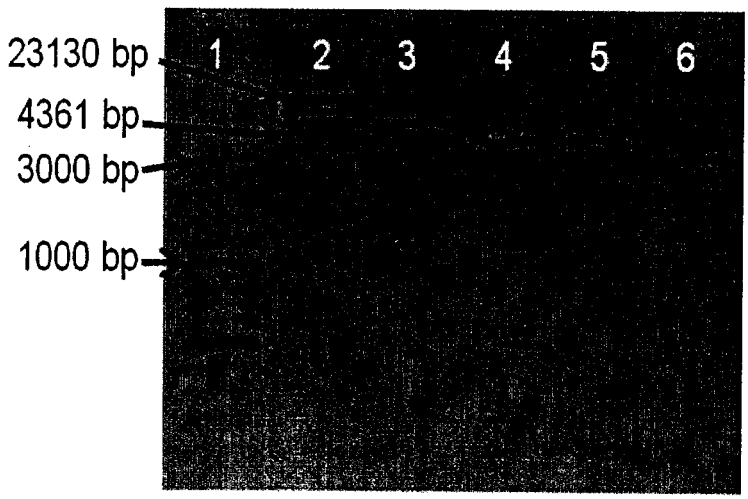
การโคลนยีน alpha-amylase

เมื่อทำ RT-PCR โดยใช้ primer ที่ออกแบบมาจากตำแหน่ง conserved region ของยีนที่กำหนดการสร้างเอ็นไซม์ alpha-amylase ของแมลงในกลุ่ม Coleopteran (แมลงปีกแข็ง) ทั้งหมด 9 ชนิด โดยขนาดของ PCR product ที่ได้จากการเพิ่มจำนวนมีขนาดประมาณ 950 bp เมื่อตรวจสอบผลของ PCR product ด้วย Gel electrophoresis (ภาพที่ 1) จากนั้นตัด band แล้วทำการ Elute เอา PCR product ออกจาก gel แล้วนำไปโคลนเข้าในเวคเตอร์ (pTZ57R/T) เมื่อได้โคลนมาแล้ว ทำการตรวจสอบโคลน โดยการตัดด้วยเอ็นไซม์ตัดจำเพาะ ซึ่งจะใช้เอ็นไซม์ *Xba* I และ *Hind* III พบว่า Product ที่ได้จากการตัดจะมีขนาดประมาณ 2800 bp และ 1000 bp (ภาพที่ 2)

ภาพที่ 1 ผลของ PCR product ที่ได้จาก primer ที่ออกแบบมาจากตำแหน่ง conserved region ของแมลงในกลุ่ม Coleopteran โดย PCR product มีขนาดประมาณ 950 bp



ภาพที่ 2 ผลการตรวจสอบโคลน โดยการตัดด้วยเอ็นไซม์ตัดจำเพาะ

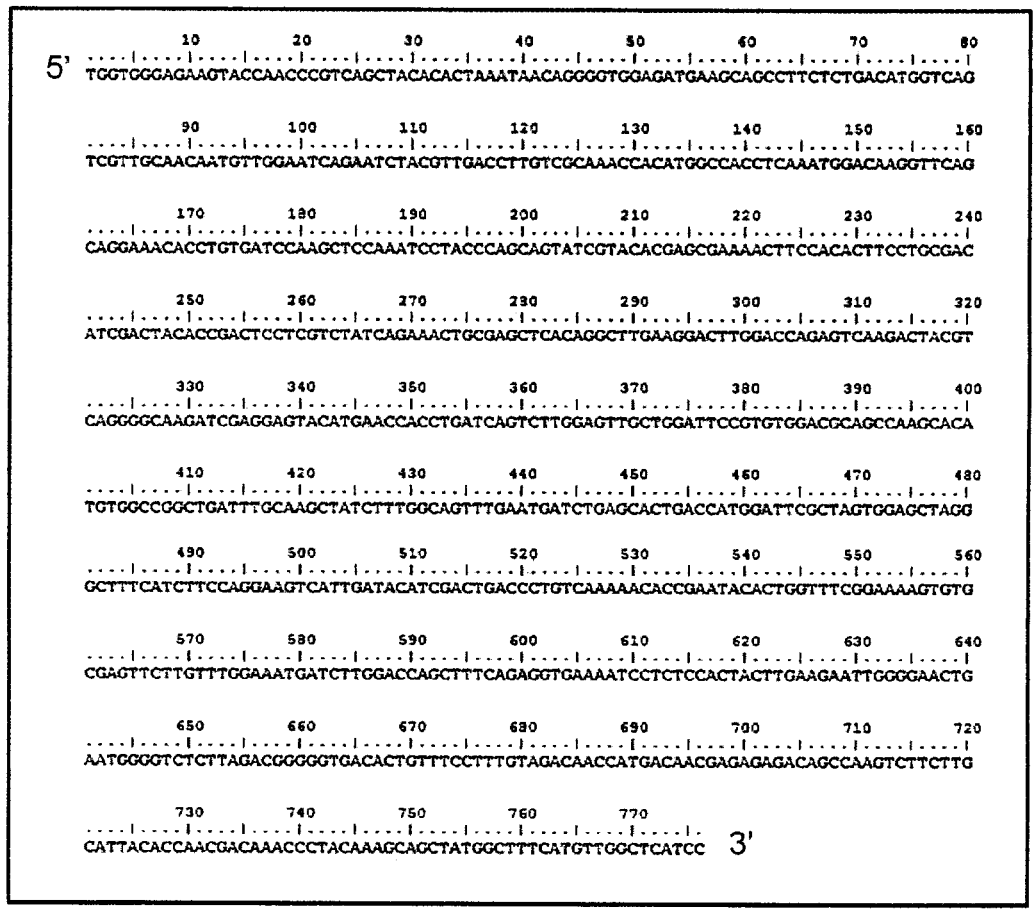


Lane 1: 100 bp Plus DNA Ladder
Lane 2: λ*Hind* III marker
Lane 3: Undigest plasmid
Lane 4: *Xba* I (~3800 bp)
Lane 5: *Hind* III (~3800 bp)
Lane 6: *Xba* I and *Hind* III (~2800 and ~1000 bp)

การหาลำดับนิวคลีโอไทด์ของยีน alpha-amylase

เมื่อนำโคลนที่มีการ insert ยีน ไปหาลำดับนิวคลีโอไทด์ โดยการทำ DNA sequencing พบว่ามี 776 นิวคลีโอไทด์ (ภาพที่ 3) ซึ่ง open reading frame มี 259 กรดอะมิโน และเมื่อนำไปเปรียบเทียบกับฐานข้อมูลของ National Center for Biotechnology Information (NCBI) พบว่า มีความเหมือนกับ alpha-amylase ของแมลงในกลุ่ม Coleopteran ซึ่งจะเหมือน *Anthonomus grandis* (Amylag1) (gi: 25992513) โดยมีเปอร์เซ็นต์ Identities เท่ากับ 68 เปอร์เซ็นต์, *Anthonomus grandis* (Amylag2) (gi: 25992515) มีเปอร์เซ็นต์ Identities เท่ากับ 67 เปอร์เซ็นต์, *Tribolium castaneum* (gi: 167466184) มีเปอร์เซ็นต์ Identities เท่ากับ 73 เปอร์เซ็นต์ และ *Phaedon cochleariae* (GI: 4210797) มีเปอร์เซ็นต์ Identities เท่ากับ 69 เปอร์เซ็นต์ (ภาพที่ 4) แล้วนำลำดับนิวคลีโอไทด์ที่ได้ไป submit ใน GenBank ซึ่ง accession number คือ bankit1256890

ภาพที่ 3 ลำดับนิวคลีโอไทด์บางส่วนของยีน alpha-amylase



ภาพที่ 4 ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ที่ได้กับฐานข้อมูล National Center for Biotechnology Information (NCBI)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF527876.1	Anthonomus grandis alfa-amylase (Amylaç1) mRNA, complete cd:	311	311	99%	4e-81	68%
Y17902.1	Phaedon cochleariae mRNA for alpha-amylase	179	179	65%	5e-51	69%
AF527877.1	Anthonomus grandis alfa-amylase (Amylaç2) mRNA, complete cds	134	134	58%	6e-28	67%
XM_964297.2	PREDICTED: Tribolium castaneum similar to alpha amylase, mRNA	111	111	25%	6e-21	73%

อภิปรายผล

เอ็นไซม์ alpha-amylase มีบทบาทสำคัญในกระบวนการย่อยสารอาหารพวกคาร์โบไฮเดรตของแมลงหลายชนิด โดยเฉพาะอย่างยิ่งพวกมอด ที่ชอบกินพืชที่มีแป้งเป็นองค์ประกอบในระหว่างที่เป็นดักแด้และตัวเต็มวัย ซึ่งเอ็นไซม์ alpha-amylase มีความสำคัญต่อการอยู่รอดของมอด (Oliveira-Neto et al. 2003) โดยจะเร่งปฏิกิริยาการย่อยพันธะ alpha-1,4 glycosidic ซึ่งพบในอะไมโลส และอะไมโลเพกทิน ที่เป็นส่วนประกอบหลักของแป้ง และพวกพอลิแซ็กคาไรด์ (polysaccharides) (Nahoum et al., 2000) สำหรับการศึกษาเอ็นไซม์ alpha-amylase จากมอดข้าวสาร โดยการวิเคราะห์หาลำดับนิวคลีโอไทด์ของยีน alpha-amylase โดยหาจากตำแหน่ง conserved region ของยีนที่กำหนดการสร้างเอ็นไซม์ alpha-amylase ของแมลงในกลุ่ม Coleopteran ทั้งหมด 9 ชนิด ซึ่งลำดับนิวคลีโอไทด์ที่ได้มี 776 นิวคลีโอไทด์ ซึ่ง open reading frame มี 259 อะมิโนแอซิด จากลำดับนิวคลีโอไทด์ที่ได้มีความเหมือนกับ alpha-amylase ในแมลงที่อยู่ในกลุ่มเดียวกัน คือ *Anthonomus grandis* (Amylag1) (gi: 25992513), *Anthonomus grandis* (Amylag2) (gi:25992515), *Tribolium castaneum* (gi:167466184) และ *Phaedon cochleariae* (GI:4210797) โดยมีเปอร์เซ็นต์ Identities เท่ากับ 68, 67, 73 และ 69 เปอร์เซ็นต์ตามลำดับ เมื่อนำไปเปรียบเทียบกับฐานข้อมูลของ NCBI และจะมีคุณสมบัติการเร่งปฏิกิริยาการย่อยพันธะ alpha-1,4 glycosidic ของแป้ง การที่ทราบลำดับนิวคลีโอไทด์บางส่วนของยีน alpha-amylase จะทำให้สามารถนำไปใช้ในการหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ต่อไป และนำไปใช้เป็นข้อมูลพื้นฐานในการศึกษาคุณสมบัติของเอ็นไซม์ alpha-amylase ในมอดข้าวสาร รวมถึงศึกษายีนที่ยับยั้งการทำงานของเอ็นไซม์ alpha-amylase ที่จำเพาะเจาะจงต่อกลุ่มแมลงเป้าหมายนี้ต่อไป และเพื่อเป็นประโยชน์ในการป้องกันกำจัดศัตรูพืชที่จำเพาะเจาะจง

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