CLONING, EXPRESSION AND CHARACTERIZATION OF ALPHA- AMYLASE INHIBITOR FROM KDML 105 THAI RICE (ORYZA SATIVA L.)

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This thesis entitled "Cloning, Expression and Characterization of Alpha-Amylase Inhibitor from KDML105 Thai Rice (Oryza sativa L.)" submitted by Natthawut Poomsila in partial fulfillment of the requirements for the Master of Science Degree in Biochemistry is hereby approved.

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important crops and a staple food for almost half of world's population. However, stored rice seeds are vulnerable to insect pests that feed on rice grain and cause loss of its value and quality. There are alpha-amylase inhibitors in plants, which can inhibit insect alpha-amylases, leading to undergrowth of insect pests. Nevertheless, there is no report on the nucleotide sequences and expression of alpha-amylase inhibitor genes from Thai rice, especially cultivar Kaw Dok Mali 105 (KDML 105). Moreover, specificity and selectivity between the insect alpha-amylases and inhibitors from plants have not yet been elucidated. Therefore, the aim of this study is to clone the full coding sequence of alpha-amylase inhibitor genes from KDML105 rice in order to further study their expression and inhibition activity. In this study, we have cloned a cDNA from young leaves of KDML105 rice. The clone consists of 603 nucleotides, encoding an alpha-amylase inhibitor. Its nucleotide and deduced amino acid sequences are identical to those of japonica rice alpha-amylase/subtilisin inhibitor. The 603 nucleotides of gene were expressed in BL21 *E.coli*. The expressed protein was peptide confirmed using LC-MS/MS and was partially purified using ion exchange chromatography. Inhibition activities was assayed using 2-Chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3), resulted in functional alpha-amylase inhibitor which decreased 68.82% and 83.52% of alpha-amylase activities of human saliva and *Bacillus subtilis*, respectively. In

contrast, it did not inhibit alpha-amylase recombinant activity from *Tribolium castaneum*. However, these results revealed the sequence of KDML105 alpha-amylase inhibitor differs from the indica Homjan alpha-amylase inhibitor, another clone from our group, indicating a complex family of proteinaceous inhibitors in rice that could lead to complex interactions of alpha-amylases. Moreover, the KDML105 alpha-amylase inhibitor can be reduced amylase activity from human saliva and microorganism, suggesting possible application of the KDML105 alpha-amylase inhibitor in microbial control and medicine.

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ABBREVIATIONS

µg = Microgram
µl = Microliter
AI = Amylase inhibitor
APS = Ammonium persulfate
BASI = Barley amylase/subtilisin ihibitor
bp = Base pair
BSA = Bovine serum albumin
^{o}C = Degree Celsius
$CaCl_2$ = Calcium chloride
cDNA = Complementary deoxyribonucleic acid
CM = Carboxymethyl
CNPG3 = 2-Chloro-4-Nitrophenyl-α-Maltotrioside
DEPC = Diethylpyrocarbonate
DNA = Deoxyribonucleic acid
dNTPs = Deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DM = Diabetes mellitus
E.C. = Enzyme commission
EDTA = Ethylenediaminetetraacetic acid
hr = Hour
IPTG = Isopropyl β-D-1-thiogalactopyranoside
kDa = Kilodaltons
KDML105 = Kaw Dok Mali 105
mA = Milliampare
mM = Millimolar
$MgCl_2$ = Magnesium chloride
NaCl = Sodium chloride
NCBI = National Center for Biotechnology Information
nm = Nanometer
min = Minute
mm = Millimeter

ABBREVIATIONS (CONT.)

mRNA	=	Messenger ribonucleic acid
OD	=	Optical density
ORF	=	Open reading frame
pH	=	Power of hydrogen ion concentration
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
pmol	=	Picomole
PPA	=	Porcine pancreatic amylase
PT	=	Pathumthani 1
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcriptase polymerase chain reaction
sec	=	Second
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMA	=	*Tenebrio molitor* amylase
v/v	=	Volume by volume
U	=	Unit
w/v	=	Weight by volume
WASI	=	Wheat amylase/subilisin inhibitor

CHAPTER I

INTRODUCTION

Rationale of this study

Rice (*Oryza sativa* L.) is one of the most important crops and a staple food for almost half of world's population (Kanawapee, et al., 2011). It has played economically important role in Thailand throughout its history (Choeun, et al., 2006). However, rice production of Thailand has been decreased by insect pests. Several insects can destroy the plants by feeding on plants and they are also vectors for rice viruses. After cropping, the grains which are stored in the warehouse can be attacked by weevil and other insects (Heinrichs, et al., 1985). The weevil larvae can excavate and penetrate into the seeds and the weevil commonly continue to propagate during seed storage. If the seeds are gathered in the warehouse for long periods, it may cause numerous losses (Franco, et al. 2002).

The rice insect pests can produce hydrolytic enzyme, α-amylase (α-1, 4-glucan-4-glucanohydrolases, EC 3.2.1.1) the α-amylase enzyme plays an important role for metabolism of carbohydrate, to hydrolyze α-1,4 glycosidic linkage of carbohydrate in rice grains for growth survival and development (Franco, et al., 2002; Strobl, et al., 1998).

In general, chemical insecticides are widely used in agricultural fields for controlling insect pests. Nevertheless, there is serious public debate because of environmental pollution and food safety concerns. The other alternative choice of insect control is the use of gene encoding insect resistance proteins in transgenic crops. It is beneficial to the agricultural production, the environment and the consumers because insecticidal proteins will be only targeted to insect pests (Morton, R.L., et al., 2000), for example, the Bt toxin, a protein from *Bacillus thuringiensis* has been used as insecticide in many countries (Tabashnik, B.E., 1994, Roush, R.T. and Shelton, A.M., 1997). In general, there is a part of plant defense mechanisms that resists against insect predation. Plant defense compounds consisting of many kind of proteins, for example, enzyme inhibitors, protease and α-amylases inhibitors, found in seeds and vegetative organ, play an important role in plant defense mechanism by disturbing the digestive system of insect and are able to control phytophage number as regulators (Ryan, C.A., 1990; Konarev, A.V., 1996). The plant α-amylase inhibitors (α-AI) are heat-stable proteins which function as specific inhibitors of insect α-amylase enzymes. They can form a complex with amylase and inhibit α-amylase causing decrease in digestion of starch. For example, wheat α-amylase inhibitors specifically inhibit only insect α-amylase but poorly inhibit or cannot inhibit α-amylases from mammals (Guo Hua Feng, 1996).

Currently, there are several studies focus on α-amylases and α-amylase inhibitors in plants. However, there are no researchers reporting nucleotide sequences of alpha-amylase inhibitor genes of Kaw Dok Mali 105 (KDML 105) and Pathumthani 1 (PT) Thai rice, famous and exported rice of Thailand. We aim to a clone full coding sequence of α-amylase inhibitor from Kaw Dok Mali 105 and Pathumthani 1 rice in order to further study its expression, specificity and selectivity towards certain insect pests.

Objective of this study

1. To clone the full coding nucleotide sequence of an alpha amylase inhibitor gene from Kaw Dok Mali 105 and Pathumthani 1 Thai rice (*Oryza sativa* L.).
2. To express and investigate inhibitory activity of alpha amylase inhibitor against selected alpha-amylases.

Hypotheses

Rice productions have been decreased by insect pests using alpha amylase enzyme which can hydrolyze alpha 1,4 glycosidic bond in starch. However, full coding nucleotide sequence, specificity and biochemical characteristics of alpha amylase inhibitor from KDML105 and PT indica rice are determined and understood. This can be applied and developed to prevent rice damaging from rice insect pests.

Scope of this study

1. The rice used in this experiment was Kaw Dok Mali 105 (KDML105) and Pathumthani 1 (PT) Thai rice (*Oryza sativa* L).
2. The RNA was extracted from young leaves of rice (*Oryza sativa* L).
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 coding nucleotide sequence of an alpha-amylase inhibitor was determined by using specific primer according to Japonica rice alpha amylase inhibitor sequence.
6. The protein expression and biochemical studies were investigated.

Keywords

alpha-amylase inhibitor, *Oryza sativa*, KDML105 rice, alpha-amylase, Insect pests

Anticipated outcome

1. To provide full coding nucleotide sequence of an alpha amylase inhibitor gene from KDML105 and PT (*Oryza sativa* L.) Thai rice and information for study an alpha amylase inhibitor in the other Thai rice.
2. To understand the inhibitory activity of KDML105 alpha amylase inhibitor.

CHAPTER II

LITERATURE REVIEWS

Rice

Rice (*Oryza sativa* L.) is monocot plant, one of the most important crops and a staple food for almost half of world's population (Kanawapee, et al., 2011). There are many varieties of rice. However, indica rice and japonica rice are two major varieties of rice. Differences between these rice varieties are the size of rice grains, the taste, and the rice flour composition. The japonica rice grains are usually more oval shape and shorter than indica rice grains and it is well known that after cooking indica rice is more aromatic and texture is quite different.

The indica rice is extensively cultivated in tropical regions such as Southeast Asia. On the other hand, the japonica rice, which is adapted to cooler regions, is widely cultivated in cooler countries such as central and northern China, Korea, and Japan. (Yoshida, S., 1981)

(a) Nipponbare (Japonica) (b) Khao Dawk Mali (indica) rice

Figure 1 The rice appearances between Japonica (a) and indica rice (b)

Source: Kasai, M., et al., 2007

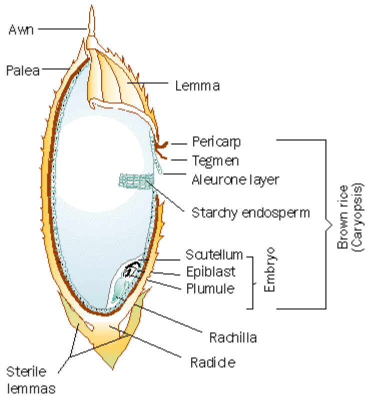


Figure 2 Morphology of rice seed

Source: http://www.ikisan.com/Crop%20Specific/Eng/links/ap_ricemorp.shtml

Components of rice seed

Rice grains are consists of several nutrition such as protein, carbohydrate, lipids, minerals, and other constituents. The most components of rice seed are starch, in milled rice, it has about 84 - 90% starch. Starch composes of two major types of carbohydrate molecules, amylose and amylopectin (Figure 3). Amylose is a linear α-1,4-linkage glucan, nevertheless amylopectin has various branch points that form by α-1,6 linkages joining linear chains (Yao, Zhang and Ding, 2002; Samuel, 1991).

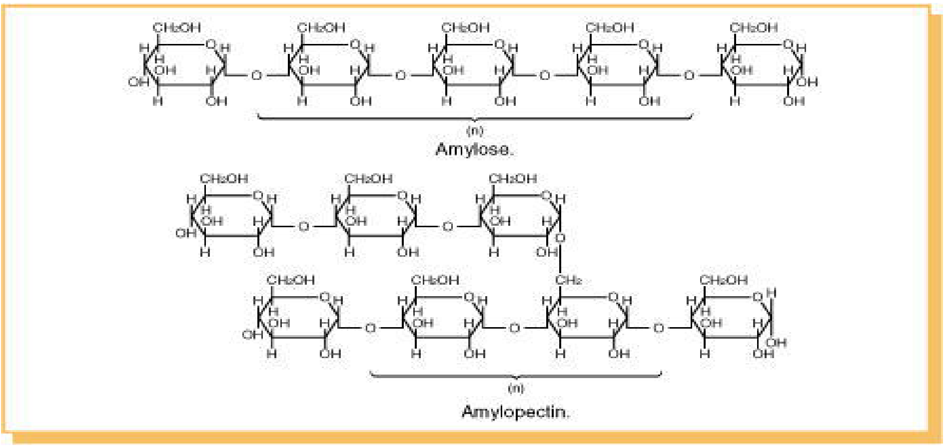


Figure 3 Amylose and amylopectin structure: 2 major components of rice seed

Source: http://www.nichidene.com/eng-approach.htm

Rice in Thailand

Rice is important crops and main food for Thai's population. It has played economically significant role in Thailand throughout its history (Choeun, et al., 2006). In 2011, Thailand, rice production had cultivated more than 27 million tons (Thai rice exporter association, 2013). Major variety of rice in Thailand is indica rice such as Pathumthani 1 rice and Jasmine rice.

1. Pathumthani 1 rice

Pathumthani 1 rice is new strains of fragrant rice that was developed in Pathumthani Rice institute. It is suitable harvested in central part of Thailand. However, the distinguished characteristics of Pathumthani 1 rice are brown planthopper (*Nilaparvata lugens* (Stål)) and whitebacked planthopper (*Sogatella furcifera* (Horvath)) resistant rice and also tolerant to plant disease such as blast disease (Khonkaen rice seed center, 2013).

Moreover, high yield production and similarity to aroma fragrant as Jasmine rice will promote Pathumthani 1 rice to be considered new strains of economically Thai rice in the future.

2. Jasmine rice or Kaw Dok Mali 105 (KDML105 rice)

Jasmine rice or Kaw Dok Mali 105 (KDML105 rice) is most popular rice in Thailand and has distinguished characteristics; aromatic, soft and delicious. The suitable areas for cultivated are Northern and Northeastern regions in Thailand and also well grows in rainy season However, KDML105 rice is weakly sensitive to photoperiod. The morphology of KDML105 rice, the tall plant is 140 centimeters height with 33 centimeters panicle length. The culm is erect with green leaves and leaf sheath, and light yellow internodes. The leaf is pubescent and the leaf angle is droopy. The ligules shape is acute and 2 white clefts. The color of the auricle and collar are light green. In flowering stage, the color of the apiculi and short sterile lemma are white. The stigma is also white. In harvesting stage, the apiculus and sterile lemma of grain will turn in to straw color with short hairs. The stem is moderately stood still with some lodging. The panicle is long, moderately compact with heavy secondary branching and a well exerted base. The leaves under flag leaf are mostly senescence (easy dry). One thousand grain weight is 27.9 grams.

KDML105 is distinguished rice; it is excellent grains, can grow in upland area and also acid sulfate soil, saline and drought tolerant. But this variety of rice is vulnerable attacked by rice disease and insect pests (Bureau of rice research and development, 2010).

Figure 4 Jasmine or KDML105 rice

3. Cooking and eating quality of KDML105 rice

KDML105 Rice is most famous cooked rice in Thailand. Because of cooked KDML105 rice is tender white long grain and aromatic smell. Moreover, KDML105 rice cooking and eating quality depend on the following properties;

1. Amylose content: Rice starch consists of 2 major fraction, amylose and amylopectin. Starch in non-glutinous rice contains 10-34% of amylose but glutinous rice starch consists of amylopectin or may not have a few of amylose. Therefore, the ratio of amylopectin and amylose is much important factor that identifies the characteristics of cooked rice such as sticky and harder texture. However, KDML105 rice contain 15-17 % of amylose, is classified as low amylose rice which make cooked KDML105 rice is soft and sticky.
2. Amount of cooking water: Amount of suitable water for cooked rice is depends on amount of amylose content. High amylose rice requires water added more than rice with low amylose content. KDML105 rice is rice with low amylose. Therefore, it needs less water for cooking.

3. Cooking time: Cooking time means range of time to cook rice until all starch was completely gelatinized. However, cooking time of rice depends on the gelatinization temperature. KDML105 rice is low gelatinization temperature rice that starch can be gelatinized at temperature below 70°C and cooking time should be cooked at 17 minutes or less.

4. Aroma: KDML105 rice has a natural aromatic fragrance from volatile chemical named 2-Acetyl-l-pyrroline (2AP). This substance can be found in rice, pandan leaves, biscuit, canned corn kernels and bread flowers.

5. Storage rice: KDML105 rice is photoperiod sensitive rice and can be harvested once a year. Thus, rice storage is much important for consumption and trading. High temperature will be rapidly turn new rice to old rice and will be reduced aromatic smell faster than low temperature. Moreover, high temperature can also develop a rancid smell. There is investigation show that keeping paddy and white rice in cold room (15°C) for 10 months can be kept rice quality as new rice. In the other hand, rice is in plastic bag, if it is opened, it should not be kept more than 4 months (Bureau of Rice Research and Development, 2010).

Rice insect pests

1. Rice weevil

In warm climate areas, Rice weevil (*Sitophilus oryzae*) is a primary stored-grain insect which causing important losses to stored grains, especially cereals such as rice and maize (Batta, 2004). They are originated in India and have been spread to many countries by commerce.

Both the adults and larvae bore and feed on whole grains, reducing them to hollow husk. 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 eats a cavity into a seed and then deposits a single egg in the cavity, sealing in the egg with secretions from her ovipositor. The larva develops within the seed, hollowing it out while feeding. The larva then pupates within the hollow husk of the grain kernel (Koehler, 2012).

Life cycle of rice weevil, the adults lay their eggs approximately 4 eggs a day for 4-5 months. The full cycle of rice weevil probably take only 26-32 days in summer but require a much longer period in cooler weather.

The rice weevil appearance is small and stout. However, the color of rice weevil is reddish-brown to black with four light yellow or reddish spots on the corners of the elytra (the hard protective forewings). The snout is 1 mm which is almost 1/3 of the whole 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 rice weevil is no leg in larvae and stays inside the hollowed grain kernel (Koehler, 2012).

Figure 5 Rice weevil (*Sitophilus oryzae*)

Source: http://edis.ifas.ufl.edu/ig120

2. Lesser grain borer (*Rhyzopertha dominica*)

Lesser grain borer is a primary and dangerous insect pest for stored agricultural product. It can cause numerous lost for cereal such as barley, wheat, maize and rice. Both larvae and adults can attack whole grain of cereal. It can excavate into the cereal grain, survive and develop inside the kernel stored product grains (Bashir, et al., 2003; Wright and Morton, 1995).

The adult of lesser grain borer is small (2-3 mm) slender and dark brown to black beetle.The head is hidden under the slightly knobby, pitted prothorax. The elytra (hard, shell-like forewings) have distinct rows of pits running their length. The antennae have 10 segments with the 3 enlarged segmented clubs.

For its life cycle, the female adult beetle can lay its eggs 1-30 eggs on the outside or inside of grains. The development of egg to adult depends on temperature. It probably takes 30 days in summer but basically average is approximately 58 days (Koehler and Pereira, 2012).

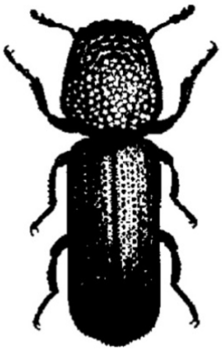


Figure 6 Lesser grain borer (*Rhyzopertha dominica*)

Source: http://edis.ifas.ufl.edu/ig117

3. Red flour beetle (*Tribolium castaneum* (Herbst))

Red flour beetle is one of the most important pests in warehouse and grocery. Both larvae and adult can primarily attack stored grain product like cereal grain and flour. Moreover, this beetle can secrete a benzoquinone mixture, (Villaverde, Juárez and Mijailovsky, 2007) which makes stingy smell to stored grain product and also decrease quality and value of products.

The red flour beetle is reddish brown small and flat beetle. The measured size of it is approximately 1/10 to 1/8 inch. This beetle lays 300-400 slender shaped eggs on damage grain or flour. The beetle propagation depend on temperature, lower 18°C, this beetle will not breed or develop (Bennett, 2003).

Figure 7 Red flour beetle (*Tribolium castaneum* (Herbst))

Source: http:// agspsrv34.agric.wa.gov.au/ento/pestweb/Images/redflour1degesch.jpg

Amylase

Amylases (E.C: 3.2.1.0) are enzyme which hydrolyze starch molecules and related saccharide to smaller polymers composed of glucose units (Reddy, Nimmagadda and Sambasiva, 2003). They can be widely found in several sources such as insect, plants, animals (Zverlov, Holl and Schwarz, 2003) and also microorganisms such as *Bacillus* species and *Aspergillus* species (Gouda and Elbahloul, 2008; Pandey, et al., 2000). Starch component contains two major types of glucose polymer; amylose and amylopectin. Amylose is linear glucose polymer with α-1,4glycosidic linkages. Another saccharide is amylopectin, short linear glucose with α-1,4glycosidic linkages and α-1, 6 linked side chain. This enzyme act as carbohydrate degrading enzyme by specifically cleave the glycosidic bonds in starch, a storage polysaccharide present in seeds, tubers etc. of various plants.

1. Application of amylase

Currently, Usage of Amylase enzyme can be applied in several industrial processes such as in the food, textiles, paper industries (Fogarty and Kelly, 1979), bakery (Cheetham, 1980), syrups from glucose and fructose detergents, fuel ethanol from starches (Upadek and Kottwitz, 1997), fruit juices (Wiseman, 1980), alcoholic beverages (Macleod, 1979). Moreover, there are several reports involving in clinical applications (Viril and Klotz, 1963).

2. Amylase classification

Amylases compose of alpha, beta and gamma-amylases and can be classified into two categories, Endo-amylases and Exo-amylases. Endo-amylases are alpha-amylase that hydrolyzes starch molecules producing linear and branched oligosaccharides of various chain lengths. The other class is Exo-amylases, contain beta-amylase and gluco-amylase to catalyze the non-reducing end effectively resulting in short end product (Reddy, Nimmagadda and Sambasiva, 2003; Pandey, et al., 2000).

2.1 Alpha-amylase

Amylolytic enzymes named α-amylases (α-1, 4 - glucan – 4 - glucanydrolase, EC3.2.1.1) are a group of enzyme which broadly distributes in organisms such as bacteria, fungi, insects, plants, animals and humans. The function of this enzyme is to catalyst the hydrolysis of α-(1, 4) glycosidic bonds found in starch components and other related polysaccharides (Bompard-Grilles, et al., 1996).

In general, α-Amylases play an important role in metabolism of carbohydrates of several organisms. Especially in insects this enzyme is important for growth and development of insects (Feng, et al., 1995; Franco, et al., 2000). Therefore, inhibition of α-amylases can control growth and development of insect pest and also protect grains from insect pest attack.

2.2 Beta-amylase

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

2.3 Gamma-amylase (Glucoamylase)

Gamma-amylase (EC.3.2.1.3) is one of exo-amylase group that catalyze the α -1, 4-glucosidic bonds and α -1, 6-glucosidic bonds at amylose and amylopectin in starch molecules. This enzyme has been found in several microorganisms like bacteria and fungi.

Amylase inhibitor

Alpha-amylase inhibitors (α-AI) are one of the most significant inhibitor for carbohydrate metabolism, forming complex with alpha amylase enzyme, causing decreased starch digestion and carbohydrate metabolism.

Alpha-amylase inhibitors can be classified into 2 major classes: Non-proteinaceous alpha-amylase inhibitors and proteinaceous alpha-amylase inhibitors.

Non-proteinaceous alpha-amylase inhibitors

The group of non-proteinaceous inhibitors consists of various types of organic compounds such as acarbose, isoacarbose, acarviosine-glucose, hibiscus acid and the cyclodextrins (Franco, et al., 2002).

However, applications of non-proteinaceous alpha amylase inhibitors for plant transgenic modifications are not suitable because of Acabose or other plant organic compounds are very complicated and also many metabolic pathways are involved. Therefore, proteinaceous alpha amylase will be more suitable and effective.

Proteinaceous alpha-amylase inhibitors

Alpha-amylase inhibitors (α-AI), the heat-stable proteins that act as inhibitors of salivary, pancreatic, bacterial and insect α-amylase enzymes. They can form a complex with amylase and inhibits α-amylase causly decreasing in digestion of starch. Plant α-amylases such as wheat α-amylase inhibitors can inhibit amylase that can be targets of drug-design development for obesity and hyperlipidemia treatment (Layer, Carlson and DiMagno, 1985; Bischoff, et al., 1994) and decrease hyperinsulinemia and hyperglycemia in diabetes patients (Makkhar, Siddhuraju and Becker, 2007). Moreover, plant α-amylases inhibitors can inhibit pathogens and insect pests. This inhibitor resists digestive process of insect pests. Some plant α-amylases inhibitors such as wheat α-amylases inhibitors specifically inhibit just insect α-amylases but badly inhibit or cannot inhibit α-amylases from mammals (Feng, et al., 1996). Several years ago, plant α-amylase inhibitors, found in many higher plants such as rice, barley, wheat, bean and others (Garcia-Olmedo, et al., 1987; Barber, et al., 1989), have been broadly studied (Feng, et al., 1995; Franco, et al., 2000). Six classes of proteinaceous α-amylases inhibitors can be classified by Richardson following by their tertiary structure as describe (Richardson, 1990).

Table 1 The classes of plant α-amylase inhibitors

Structure class	Source	Residue numbers	Disulfide bonds	Names
Knottin type	Amaranth	32	3	AA 1
γ-Purothionin type	Sorghum	47-48	5	SI α1, SI α2, SI α3
Cereal type	Barley, Rye, Wheat, Ragi	124-160	5	RATI(RBI), 0.19,0.28,0.53, WRP26, WRP27, BMAI-1
Kunitz type	Barley, Wheat, Rice	176-181	1-2	BASI, WASI, RASI
Thaumatin type	Maize	173-235	5-8	Zeamatin
Lectin type	Common bean	240-250	5	α-AI 1, α-AI 2

Note: This data from Franco, et al., 2002; Svensson, et al., 2004

1. Knottin-type alpha-amylase inhibitors

The 32 amino acid residue inhibitors is the smallest α-amylase inhibitors from Mexican plants amaranth (*Amaranthus hypochondriacus*). Its known as Knottin-like inhibitor which contains three disulfide bridges and knottins fold (Franco, et al., 2002; Chagolla-Lopez, et al., 1994). The Structure of Knottin-like inhibitor was determined by NMR spectrometry (Martins, et al., 2001).This inhibitors have structure that most congruously with gurmarin, Diabetes Mellitus traditional medicine and the toxin of a funnel-web spider (Barbosa Pereira, et al.,1999).

The Knottin-like inhibitor can inhibit α-amylase from insects by block the catalytic site of insect α-amylase. The specific properties of AAI that resist insect α-amylase can bring it to be an insect resistant inhibitor for plants insect resistant development (Barbosa Pereira, et al., 1999; Chagolla-Lopez, et al., 1994).

2. γ-Purothionin-like alpha-amylase inhibitors

γ-Purothionin is 47-48 amino acids of α-amylase inhibitor protein that found in the γ-thionin superfamily. Functional effects of this superfamily concerned plant defense such as membrane permeability modification (Castro, et al., 1996; Thevissen, et al., 1996), inhibitory of proteases (Wijaya, et al., 2000) and inhibitory of protein synthesis (Mendez, et al., 1996).

SIα-1, SIα-2 and SIα3 are insect α-amylase inhibitors from sorghum (*Sorghum bicolor*), are important α-amylase inhibitors of insects such as cockroach (*P.americana*) and grasshopper (*S.americana*). But, mammalian α-amylases and α-amylases from *A. oryzae* cannot be inactivated or pretty badly inactivated by these molecules (Bloch Jr. and Richardson, 1991).

The structure of SIα-1 is determined by NMR but enzyme-substrate complex of this molecule have not been determined yet (Bloch Jr., et al., 1998).

3. Cereal type alpha-amylase inhibitors

Cereal-type α-amylase inhibitors are actually 120-160 amino acid residues with five disulfide bonds (Franco, et al., 2002) from higher plants such as wheat, barley and Indian finger millet. Another name of this class is CM-proteins. The most studied inhibitors of the class is 0.19 wheat α-amylase inhibitors that widely specific α-amylase inhibitor in many organisms (Franco, et al., 2000) and the double headed Ragi α-amylase/trypsin inhibitors (RATI). And others wheat- α-amylase inhibitors such as WRP24, WRP25, WRP26 and WRP 27 can inactivate different organisms depending on their properties (Feng, et al., 1995).However, some wheat α-amylase inhibitors can inhibit several organisms but some wheat α-amylase and barley inhibitors are also major allergen associated with baker's asthma (Gomez, et al., 1989; Barber, et al., 1989).

The structure of 0.19 wheat α-amylase inhibitor is crystallized and determined (Miyazaki, 1994) and RATI structure was solved by NMR (Strolb, et al., 1995) and crystallized (Strolb, et al., 1998) with TMA/RATI complex.

4. Kunitz-like alpha-amylase inhibitors

One of the proteinaceous α-amylase inhibitors from cereal, such as rice wheat and barley (Franco, 2002), is Kunitz-type α-Amylase inhibitors. This protein acts like a regulator in plants- defense processes and control endogenous α-amylase

(Alves, et al., 2009). Barley α-amylase/subtilisin inhibitor (BASI) is the best-characterized inhibitor from this class. It is similar to wheat α-amylase/subtilisin inhibitor (WASI) and ragi α-amylase/subtilisin inhibitor (RASI) (Franco, et al., 2002).

Interestingly, BASI can inhibit just subtilisin-type serine protease and amylase isozyme 2(AMY2) from barley seeds, but does not inhibit barley amylase isozyme 1(AMY1), which is 74% similarity to AMY2 (Mundy, Svendsen and Hejgaard 1983; Jones and Jacobsen, 1991; Nielsen, et al., 2004). Moreover, this inhibitor does not specifically inhibit α-amylase at the catalytic site, but it interacts at domain A and B of this enzyme, not far from catalytic site (Franco, et al., 2002).

BASI consists of approximately 20kDs and 2 disulfide bonds. The three-dimension structure of BASI is determined with BASI/AMY2 complex (Vallee, et al., 1998).

5. Thaumatin-like alpha-amylase inhibitors

The 22 kDs corn (*Zea mays*) proteins name Zeamatin is the best - distinguished thaumatin like inhibitors. Homologous proteins that are similar to sweet-tasted protein thaumatin and pathogenesis-related group 5 (PR5) proteins (O’Rourke, Richardson and Selitrennikoff, 2001). Interestingly, α-amylases cannot be inhibited by thaumatin and other-related proteins but thaumatin and other-related proteins contain antifungal property. However, Zeamatin can act as insect α-amylase inhibitors (Svensson, et al., 2004).

A structure of this protein inhibitor is determined (Batalia, et al., 1996) but the enzyme-inhibitor complex had not been solved yet (Svensson, et al., 2004).

6. Lectin-like alpha-amylase inhibitors

Lectin is commonly found in many plants. If it is accumulated, it might act as a defense against insect pests (Murdock and Shade, 2002). Lectin type α-amylase inhibitors, 120-160 amino acid residues, are commonly presented in common bean (*P.vulgaris*) and also found in a group of kidney bean (white, red, black kidney bean) (Kasahara, et al., 1996; Marshall and Lauda, 1975; Wilcox and Whitakers, 1984; Le Berre-Anton, et al., 1997). α-AI1 is the best-characterized isoform of this class of inhibitor. It can perfectly inhibit porcine pancreatic α-amylase (PPA) and can also inhibit *C.maculatus* and *C.chinensis* α-amylases. On the other hands, another isoform of α-AI1, such as α-AI2 acts contrastingly to α-AI1. It can only inhibit *Z.subfasciatus* but it cannot inhibit other α-amylases like α-AI1 can (Grossi de S̓a, et al., 1997). However, there are another isoforms of this type: α-AIL (α-AI3) (Finardi-Filho, Mirkov and Chrispeels, 1996), α-AI4 and α-AI5 (Lee, Gepts and Whitaker, 2002).

The structure of α-AI1, the best-characterized in complex forms with TMA and PPA are determined and described. (Bompard-Grilles, et al., 1996; Nahoum, et al., 1998)

The class of inhibitor can be applied to DM and obesity controller, and a biotechnological pesticide that protect insects and control growth development of insect pests and plant pathogens (Franco, et al., 2002).

Significances and applications of α-amylase inhibitors

The α-Amylase inhibitors can be applied in the field of nutrition and also biological technologies such as industrial and agricultural application. There are researches reported that α-amylase inhibitors can act as weapons that resist insect pests. The α-Amylase inhibitors in common bean (*Phaseolus vulgaris*) affect as plants defense mechanism that can inhibit seeds legumes from cowpea weevil (*Callosobruchus maculatus*) and Azuki bean weevil (*Callosobruchus chinensis* L.) (Ishimoto and Kitamura, 1989; Ishimoto, et al., 1996; Shade, et al., 1994). Moreover, the other reports showed that wheat α-amylase inhibitors, WRP 25, WRP 26, can inhibit α-amylase activity of rice weevil (*Sitophilus oryzae*), red flour beetles (*Tribolium castaneum*) and yellow meal worm (*Tenebrio molitor*) (Feng, et al., 1995).

For medical uses, these enzyme inhibitors can inhibit α-amylases cause control post-prandial hyperglycemia, PPHG, which is the risk factor of non-insulin dependent diabetes mellitus (NIDDM/type II), in NIDDM patients (Notkin, 2002; Gin and Rigalleau, 2000). There is a report confirmed that Indian herb extracts, *M. koenigii* and *O. tenuflorum*, have ability to decrease glucosidase (amylase) activity of porcine pancreatic α-amylase. These can be used to manage PPHG in NIDDM type II patients (Bhat, et al., 2008). Furthermore, obesity can be treated by commercial *P.vulgaris* α-amylase inhibitors (Tormo, et al., 2004). Some study found that wheat α-amylase inhibitors can decrease intake of foods and weight gain but no mal-absorption (Kataoka and Dimagno, 1999).

On the other hands, Wheat α-amylase inhibitors might be related to allergic protein for hypersensitivity by both ingestion and inhalation. This knowledge can be applied to design allergic diagnose specifically for wheat protein (James, et al., 1997).

CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Rice

Kaw Dok Mali 105 and Pathumthani 1 Thai rice seeds (*Oryza sativa*) were obtained from Rice Department of Thailand, Ministry of Agricultural and Cooperatives. Thailand.

2. Equipment

2.1 Thermal cycle (My genie 96 thermal block, Bioneer, Korea)
2.2 Horizontal gel electrophoresis (Sunrise Garment Co., LTD., USA)
2.3 Gel documentation system (Bio Imaging Systems Co., LTD., England)
2.4 Incubator (ShelLab Co., LTD., USA)
2.5 Incubator shaker (Zhicheng Instrument, China)
2.6 Hot air oven (MMM Medcenter Einrich-tungen GmbH Co., LTD., Germany)
2.7 Autoclave (Sanyo electric Co., LTD., Japan)
2.8 Micro-centrifuge (Labnet International, Inc., USA)
2.9 Refrigerated Centrifuge (Tomy kogyo Co., LTD., Japan)
2.10 Balance (Mettler-Toledo International Inc. Switzerland)
2.11 Water bath (Julabo Labortechnik GMBH Co., LTD., Germany)
2.12 pH meter (Mettler-Toledo International Inc., Switzerland)
2.13 Heating Block (Labnet International, Inc., USA)
2.14 UV/Vis Spectrophotometer (Beckman Coulter Co., LTD., USA)
2.15 Microwave oven (Sharp, Japan)
2.16 NanoDrop (ND-1000, NanoDrop Technologies, USA)
2.17 AKTA-prime plus (GE Healthcare, USA)
2.18 High speed refrigerated centrifuge (2236R Speed Scan, Labogene, Denmark)
2.19 Microplate Reader (Synergy HT, BioTek, USA)
2.20 Sonicator (VCX130, Sonics and Materials Inc., USA)
2.21 Dialysis tubing (Snake skin, Thermo scientific, USA)

- 2.22 Micro pipette
- 2.23 Pipette tips
- 2.24 Beakers
- 2.25 Magnetic Bar
- 2.26 Culture dish
- 2.27 Spreader
- 2.28 Loop
- 2.29 Tube
- 2.30 Duran bottle
- 2.31 Erlenmeyer flasks
- 2.32 Volumetric flasks
- 2.33 Cylinder
- 2.34 Mortar and pestle
- 2.35 Vortex

3. Chemicals

- 3.1 RNA extraction
  - 3.1.1 Liquid nitrogen
  - 3.1.2 Chloroform (BDH,England)
  - 3.1.3 TRI-reagent (MRC, USA)
  - 3.1.4 Isopropanol (TEDIA, USA)
  - 3.1.5 Ethanol (Merck, Germany)
  - 3.1.6 DEPC-treated water (Fermentas, USA)
- 3.2 cDNA synthesis
  - 2x RT-PCR kit, (Fermentas, USA)
- 3.3 DNA amplification
  - 2X PCR Master Mix (Fermentas, USA)
- 3.4 Agarose gel electrophoresis
  - 3.4.1 Agarose (Usb, USA)
  - 3.4.2 Tris base (Usb, USA)
  - 3.4.3 Boric acid (Usb, USA)
  - 3.4.4 *Ethylenediaminetetraacetic acid* (BIO-RAD, USA)
  - 3.4.5 Ethidium bromide (BIO-RAD, USA)

3.4.6 Generuler^{TM} Low-range DNA ladder (Fermentas, USA)
3.4.7 VC 100bp plus DNA ladder (Vivantis, Malaysia)

3.5 DNA Purification

Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan)

3.6 Cloning

3.6.1 DifcoLB broth, Lennox (Becton, Dickinson and Company, USA)
3.6.2 Agar powder (Himedia Laboratories Pvt. Ltd., India)
3.6.3 Calcium chloride (Ajax Finechem Pty Ltd., New Zealand)
3.6.4 Glycerol (MERCK, Germany)
3.6.5 TA cloning kit (Fermentas, USA)
3.6.6 Isopropyl β-D-1-thiogalactopyranoside (IPTG) (USbiological, USA)
3.6.7 NecleoSpin plasmid Kit (Macherey-Nagel, Germany)
3.6.8 Amplicilin
3.6.9 Restriction enzyme (Invitrogen, USA)

3.7 SDS-PAGE

3.7.1 Tris-Base
3.7.2 Glycine (BIO-RAD, USA)
3.7.3 Acrylamide/Bis Solution 19:1 (BIO-RAD, USA)
3.7.4 Ammonium persulfate (BIO-RAD, USA)
3.7.5 Sodium dodecyl sulfate (SDS) (Amersham, USA)
3.7.6 TEMED (Fisher Scientific, USA)
3.7.7 2-Mercaptoethanol (BIO-RAD, USA)
3.7.8 Chromatein prestained protein ladder (Vivantis, Malaysia)
3.7.9 Broad way dual protein ladder (Intron biotechnology, Korea)
3.7.10 PRO-STAIN^{TM} Prestained Protein Marker (Intron biotechnology, Korea)

3.8 Protein staining and de-staining

3.8.1 Coomassie Billiant Blue G-250 (BIO-RAD, USA)
3.8.2 Acetic Acid (Merck, Germany)
3.8.3 Methanol (LAB-SCAN, Ireland)

3.9 Protein Determination

Quick Start^{TM} protein Bradford assay (BIO-RAD, USA)

3.10 Protein Purification

3.10.1 Sodium acetate (Sigma, UK)

3.10.2 Sodium chloride (Ajax Finechem, Australia)

3.10.3 Hitrap^{TM} IEXselection column (GE Healthcare, UK)

3.10.4 Sephadex G-100 (Amersham, USA)

3.11 Amylase Inhibitor Assay

3.11.1 2-chloro-4-nitrophenyl-a-d-maltotrioside (Sigma, UK)

3.11.2 Bacillus amylase (Sigma, UK)

3.11.3 Human saliva amylase (Sigma, UK)

4. Oligonucleotides

Table 2 List of oligonucleotide and sequence

Primer name	Nucleotide Sequence (5' to 3')	Tm (°C)	Size (bp)
α-AI	F: CCCCATATGGTTAGCCTCCGCCTC R: CCCCTCGAGTTACTCTGGTGGGCTTG	55	603
Rice-EF	F: CAC CGA GTG GCA TGT CGG R: GAG TCC CTG CAC GAC ACC	55	118

Note: Underline as recognized of restriction enzyme

Methods

1. Rice culture

Rice seeds of KDML105 and Pathumthani 1 were obtained from Rice Department, Ministry of Agriculture and Cooperation, Thailand. Seeds of KDML105 and Pathumthani 1 indica rice were germinated on natural soil for 3 weeks at room temperature. The leaves of indica rice were harvested and immediately stored at -50°C.

2. Primer design

To obtain full coding nucleotide sequence of alpha-amylase inhibitor from indica rice, pairs of primer were designed according to the rice bifunctional alpha amylase/subtilisin inhibitor gene sequence (Accession number: AY166458.1) found in japonica rice, Forward primer: 5’CCCCATATGGTTAGCCTCCGCCTC 3’ and Reverse primer: 5’CCCCTCGAGTTACTCTGGTGGGCTTG3’. The rice elongation factor primer was also designed to amplify rice elongation factor as housekeeping gene control.

The recognition site of restriction enzyme was added to 5' end as linker for facilitation of cloning. The AI-specific primer was designed in such a way that the PCR product contains *Nde*I restriction endonuclease recognition sequence upstream of start codon and *Xho*I restriction endonuclease recognition sequence downstream of the stop codon of α-AI gene. The DNA sequences of primers were shown in Table 2.

3. RNA extraction from rice

For total RNA isolation, young leaves of KDML105 and Pathumthani 1 indica rice were used for RNA extraction by TRI-reagent (MRC, USA). The 100 mg of rice leaves were homogenized by grinding with a mortar and pestle in liquid nitrogen. Homogenized samples were transferred into a micro-centrifuge tube containing 700 µl of TRI-reagent, vigorously mixed by vortex, and incubated for 5 min at room temperature. 200 µl of chloroform was added into samples, gentle mixed by hand inversion, incubated for 3 min at room temperature, and centrifuged at 12,000 g for 15 min at 4°C. After centrifugation, upper aqueous layer of mixture containing RNA was transferred into new sterile micro-centrifuge tube. Total RNA was precipitated using 500 µl of isopropanol, mixed and incubated at room temperature for 10 min. Total RNA was pelleted by centrifuged at 12,000 g for 10 min at 4°C, and then the supernatant was removed. Total RNA pellet was washed by 1 ml of 75% ethanol (V/V), centrifuged at 12,000 g for 10 min at 4°C, and then supernatant was removed. Total RNA was dried by incubated at 55°C. Finally, Total RNA pellet was dissolved using DEPC-treated water. The RNA solution was immediately used to synthesize first strand cDNA as described in the next step.

The concentration of RNA solution was determined by measuring the UV absorbance using NanoDrop (ND-1000, NanoDrop Technologies, USA) at 260 nm (specific for nucleic acids), 280 nm (specific for proteins). An OD 260/280 ratio better than 1.8 is usually considered a good RNA quality.

4. cDNA synthesis

Total RNA extracts were used as templates for the synthesis of first-strand cDNA by using reverse transcriptase polymerase chain reaction (RT-PCR) technique.

Firstly, 1-10 µg total RNA was mixed with oligo-$(dT)_{18}$ primer (10 µg/µl) and sterile distilled water up to 10 µl, incubated at 65°C for 5min and quickly placed on ice. The cDNA was synthesized by reverse transcriptase (2x RT-PCR kit, Fermentas, USA). This mixture was transferred into new sterile micro-centrifuge tube containing 10 µl 2X RT-PCR mixture, mixed by pipetting and synthesized by incubating at 42°C for 60 min. The reaction was terminated by heating at 70°C for 10 min. The cDNA was used as a template for amplification by PCR.

5. DNA amplification

To obtain the full-coding sequencing of α-AI gene, polymerase chain reaction (PCR) was performed by using first-strand cDNA as template The PCR mixture composed of 5µl 2X PCR Master Mix (Fermentas, USA) containing, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 0.05 units/µl Taq DNA Polymerase in reaction buffer, 1µl of 10 µmole forward primer, 1µl of 10 µmole reverse primer, 2 µl of nuclease free water and 1 µl of cDNA template. The rice elongation factor gene was used as housekeeping gene control.

The PCR amplification was performed using PCR thermocycle machine follow this condition as describe in Table 3.

Table 3 PCR condition

Primer	PCR condition		
Alpha-Amylase Inhibitor	Pre-denaturation	95 °C, 5 min	1 cycle
	Denaturation	95 °C, 30 sec.	30 cycles
	Annealing	55 °C, 30 sec	
	Extension	72 °C, 30 sec	
	Final-extension	72 °C, 10 min	1 cycle
Rice - elongation factor	Pre-denaturation	95 °C, 5 min	1 cycle
	Denaturation	95 °C, 30 sec.	30 cycles
	Annealing	55 °C, 30 sec	
	Extension	72 °C, 30 sec	
	Final-extension	72 °C, 10 min	1 cycle

Finally, The PCR product was analyzed by 1% agarose gel electrophoresis.

6. Gel electrophoresis

The PCR products were detected using electrophoresis on agarose gel. The amplified PCR mixtures were re-suspended with loading dye and loaded to agarose gel in 0.5 TBE buffer. Electrophoresis was run at 100 volts for 30-60 min depending on the size of amplified PCR samples. After that, the agarose gel was dissolved in 10 µg/ml ethidium bromide solution for 10 min and de-stained with distilled water for 5 min. The PCR bands were visualized under UV light by gel documentation system (Bio Imaging Systems Co., LTD., England). The PCR band size was approximated by comparing to standard DNA ladder.

7. DNA Purification

For DNA purification, the DNA product was extracted using Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan). The DNA band was excised from the agarose gel and transferred to new sterile tube containing 500 µl of DF buffer, mixed by vortex, incubated at 55°C for 10 min until the gel has been completely dissolved, inverted tube every 2-3 min to make gel faster dissolve. After that, sample was cooled down at room temperature, during sample cooling, the DF column was placed into the collection tube. After that, binding step, 800 µl of sample mixture was added into DF column, centrifuged at 8,000 rpm for 30 sec and the flow-though was discarded, the column was placed back in the collection tube. If sample mixture is more than 800 µl, repeat binding step again. Washing step, 500µl of wash buffer with ethanol was added into DF column, centrifuged at 8,000 rpm for 30 sec and the flow-though was discarded, column was placed back in the collection tube. Column drying step, DF column was centrifuged at 14,000 rpm and transferred dried column into a new sterile tube. Finally, elution step, 15-20µl of elution buffer was added into the center of column matrix, incubated at room temperature for 2 min until elution buffer was absorbed to column matrix and centrifuged at full speed to elute purified DNA.

8. Cloning

8.1 Preparation of competent cells

A single colony of DH5α and BL21 cells was picked into 3 ml LB medium and incubated 16-18 hours at 37°C with 200 rpm shaking condition. On the following day, 1 ml of the cells was transferred into 100 ml LB broth and shaking incubated at 37°C for 2-3 hours until OD_{600} was approximately 0.4-0.6. After that, the growing cells were transferred to 50 ml new sterile centrifuged tube and the pellets were collected by centrifuged at 4,000 rpm for 3 min at 4°C. 1/20 of the original culture volume of cold 0.1M $CaCl_2$ was added and gently re-suspended, incubated the re-suspended cells on ice for 5 min. After that, the cells were pelleted by centrifuged at 4,000 rpm for 3 min at 4°C. 1/20 of the original culture volume of cold 15% glycerol in 0.1M $CaCl_2$ was added and gentle re-suspended. The cells were incubated on ice for 1 hour. Finally, 50 µl of the competent cells were aliquot into new sterile micro-centrifuged tube and storage at -80°C until use.

8.2 Ligation

Ligation of α-AI gene purified PCR product into pTZ57R/T vector (Fermantas, USA) was performed according to the plasmid supplier's recommendation. The reaction mixture was composed of 1 µl of pTZ57R/T vector, 3 µl of purified DNA fragment (50-200 ng), 2 µl of 5X T4 ligase buffer, and 0.3 µl of T4 DNA ligase (5 U/µl). Sterile water was added to mixture up to 10 µl of total volume reaction. The mixture was incubated at 4 °C overnight.

8.3 Transformation

For transformation step, the competent cells were thawed on ice. The ligation product was pipetted into thawed competent cells, gently mixed by hand and incubated on ice for 30 min. The cells were heat-shocked transformed at 42°C for 90 sec and then immediately transferred to ice box for 2 min. After that, 450 µl of LB medium was added to transformed cells and continuously cultured at 37ºC for 1 hour with shaking condition. The cultured cells were spread on LB agar contain 100 µg/ml ampicillin, and incubated at 37 °C overnight. For confirmation, the recombinant clones were amplified using colony PCR techniques and restriction enzyme digestion.

8.4 Screening of recombinant clones

The colonies were picked from transformation plate by using sterile toothpicks. An individual colony was pick-up to master cultured plate to safe the recombinant cloned for re-propagation and then suspended in 10 µl of 2X PCR mixtures. PCR was performed using PCR condition as described in PCR method and analyzed on agarose gel electrophoresis. The corrected clone was selected from the master plate for further characterization.

8.5 Isolation and purification of plasmid DNA

The recombinant DNA plasmid was isolated using NecleoSpin plasmid (Macherey-Nagel, Germany). The recombinant clone from master plate was cultured into 5 ml of LB broth containing 100 µg/ml of ampicillin at 37°C for overnight with shaking condition. The culture cells were transferred to new sterile micro-centrifuge tube and pelleted by centrifuged at 11,000 g for 30 sec and supernatant was discarded. After that, cell lysis step, 250 µl of Buffer A1 was added into the cell pellet and then 250µl of Buffer A2 was added, mixed gently by inversion, incubated at room temperature for 5 min. After that, 300 µl of Buffer A3 was added, mixed gently by inversion and centrifuged at 11,000 for 5 min at room temperature. DNA binding step, the NucleoSpin column was placed in the collection tube. The 750 µl of supernatant from cell lysis step was added into the column, centrifuged at 11,000 g for 1 minute and then flow-through was removed and placed the column back. Washing step, 500µl of 50°C pre-heated Buffer AW was added, centrifuged at 11,000 g for 1 min and then 600µl of Buffer A4 with ethanol was added, centrifuged at 11,000 g for 1 min and then flow-through was discarded and placed the column back.

For column drying, Centrifugation was used at 11,000 g for 2 min. Finally, elution step, the column was placed in new sterile micro-centrifuge tube and 50 µl of Buffer AE was added, incubated for 1 min at room temperature until buffer was absorbed and finally centrifuged at 11,000 g for 1 min. The DNA plasmid concentration was determined by measuring absorbance at 260 nm. The DNA plasmid purity was investigated by 260/280 absorbance ratio, the good DNA plasmid purity should be 1.8-2.0. To confirm DNA plasmid result, agarose gel electrophoresis was performed.

8.6 DNA sequencing

The recombinant clone, carrying rice α-AI gene was determined by DNA sequencer (1st Base, Malaysia). The obtained nucleotide sequences were analyzed by Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI), a free software program available on the internet: http://www.ncbi.nlm.nih.gov/BLAST/. The sequence alignment of the nucleotide sequence and deduced amino acid sequence of full-coding sequence α-AI were obtained by searching on available website: http://www.ebi.ac.uk/Tools/msa/clustalw 2. The theoretical isoelectric point and molecular weight of α-AI were investigated using the 'Compute pI/Mw tool' on the ExPasy server (http://www.expasy.ch/tools/pitool.html). The signal peptide of α-AI predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP).

9. Protein expression assay

9.1 Construction of KDML105 α-AI clone/pET17b in expression vector

KDML105 α-amylase inhibitor was expressed in pET system, which is one of the most effective methods for producing recombinant protein in *E. coli*. The pET-17b(+) expression vector and KDML105 α-amylase inhibitor recombinant gene were double digested by *Nde*I and *Xho*I restriction enzyme. The restriction mixture was consists of 4 µl of DNA, 1 µl of *Nde*I (10 unit/µl), 1 µl of *Xho*I (10 unit/µl), 2 µl of 10X Tango buffer and 12 ml of distilled water. The mixture was incubated at 37°C for overnight. The double digestion plasmids were analyzed by 1% agarose gel electrophoresis and plasmids were gel purified as describe in section 2.7.5. The KDML105 α-amylase inhibitor was ligated to pET-17b(+) expression vector. The ligated mixture was composed of 3 µl of pET17b(+) vector, 3 µl of α-AI ligated product, 2 µl of 5X T4 ligase buffer, and 0.3 µl of T4 DNA ligase (5 U/µl). Sterile water was added to mixture up to 10 µl of total volume reaction. The mixture was incubated at 4 ºC for overnight. The KDML105 α-AI/pET17b(+) recombinant plasmid was transformed to *E.coli* strain BL21(DE3) pLysS using heat shocked transformation method as describe in section 2.7.3. The cultured cells were spread on LB agar plate with 100 µg/ml of ampicillin and incubate 37°C for overnight. The recombinant clones were confirmed by DNA sequencing**.**

9.2 Protein induction

KDML105 α-AI clone was transformed into *E.coli* expression host strained BL21 (DE3) pLysS expression host for α-AI protein induction. Firstly, 10 µl of α-AI cloned was inoculated in 5 ml of LB medium containing 100 µg/ml of ampicillin antibiotic and was shaking incubated at 37°C, 16-18 hours. Secondly, after 16-18 hours, 500 µl of cultured cells were added into sterile flask containing 50 ml of freshly LB medium and 100 µg/ml of ampicillin. The starter cells were 200 rpm shaking cultured at 37°C approximately 2-3 hours until logarithm phase or OD 600 was 0.4-0.6. When the starter cells ready to used, 1mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG), allolactose mimicry, was added into cultured cells for protein expression. The cells were continuing cultured and were collected for 1, 2, 3, 6, 9 and 24 hours. For analysis, those of expressed protein and α-AI non-induced protein were compared.

10. Sodium Dodecyl Sulfate polyacrylamide electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate polyacrylamide electrophoresis technique was performed to determine molecular weight of interested protein.

First step is SDS-PAGE preparation. SDS-PAGE contains 2 layers: Stacking gel and separating gel. Separating gel is on the lower layer of gel, consist of Water, 10-15 % acrylamide, 1.5 M Tris-HCl pH 8.8, 10% sodium dodecyl sulfate (10%SDS), 10% ammonium persulfate (10%APS) and TEMED. For stacking gel preparation, Stacking gel is on the upper layer of gel include sample wells, consist of Water, 10-15 % acrylamide, 0.5 M Tris-HCl pH 6.8, 10% sodium dodecyl sulfate (10%SDS), 10% ammonium persulfate (10%APS) and TEMED. 10X running buffer

(Tris-HCl, glycine and SDS) were prepared and 1X running buffer was used for electrophoresis.

Second step is protein preparation. Protein pellet was re-suspended by 1XPBS buffer, centrifuged at 10,000 RPM for 5 min. 1X PBS buffer was absolutely removed and sample buffer with β-mercaptoethanol was added and mixed. Samples were heat at 95 °C for 5 min and centrifuged at 10,000 rpm for 5 min.

Final step, samples were loaded into stacking gel. Samples were run follow by this condition: 120 volts and 25 mA. Running time was approximately 1.30 hours or front dye of sample buffer was released from gel to running buffer. To determine molecular weight of interest protein, protein bands were compared to standard protein ladder.

Table 4 Separating gel recipe

Solution	15%	10%
H_2O	3.5 ml	4.75 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml
10% (w/v) SDS	0.1 ml	0.1 ml
40% Acrylamide	3.75 ml	2.5 ml
10% (w/v) ammonium persulfate (APS)	50µl	50µl
TEMED	10 µl	10 µl
Total	10 ml	10 ml

Table 5 Stacking gel recipe (4 % Acrylamide)

Solution	Volume
H_2O	3.1 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	50 µl
Acrylamide	500 µl
10% (w/v) ammonium persulfate (APS)	50 µl
TEMED	5 µl
Total	5 ml

11. Coomassie Blue Staining

After SDS-PAGE process, stacking polyacrylamide gel was excised and only separating polyacrylamide gel was stained by coomassie brilliant blue R-250 (BIO-RAD, USA) for 30 min. After that, coomassie brilliant blue R-250 was removed and the gel was de-stained by de-staining solution containing water: methanol: acetic acid (5:4:1) for 1 hour or until expressed protein band was appeared.

12. Cell lysis

After protein expression by IPTG induction, 50 ml of cultured cell was transferred to sterile centrifuge tube. The cultured cell was centrifuged by high speed centrifugation (Labogene, Denmark) at 4°C 10,000 rpm for 10 min. After that, the supernatant was removed from tube and 5ml of 1X PBS solution was added and re-suspended for pellet dissolving. Repeat centrifuged step with same condition. After that, the supernatant was absolutely removed from tube and 10ml of 50 mM sodium acetate buffer pH 5.5 was added and dissolved the pellet. However, before cell sonication, 1X protease inhibitor was added for protein prevention from protease which released during cell lysis step. The cell was lysed by sonicator (Sonics and Materials Inc., USA) following by this condition; sonicate on ice with 60% pulse, 15 sec turn on and 5 sec turn off for 5 min.

13. Protein Determination

Protein determination was assay by Quick Start™ protein Bradford assay (BIO-RAD, USA). Bovine Serum Albumin (BSA) was used as standard protein. The range of BSA was 125-1,000 μg/ml. 5 μl of crude protein was triplicate pipetted into microplate wells and buffer was used for blank (0 μg/ml). After that, 250 μl of 1X dye reagent was added into each well. Samples were incubated at room temperature for at least 5 min. Standard and crude proteins were measured by microplate reader (Synergy HT, BioTek, USA) at absorbent 595 nm. For analysis, crude protein samples were determined by using BSA standard protein curve.

14. Identification of protein by LC-MS/MS

The 22kDa protein band from SDS-PAGE was excised from gel 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 interested protein was dehydrated, alkylated, trypsin digested and extracted from gel.

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 minutes. 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. Protein purification

15.1 CM FF column

The α-AI recombinant proteins were purified by Cation exchange chromatography using weak cation exchanger, CM sepharose Fast Flow column. cation exchanger (GE Healthcare, UK), with binding buffer (50 mM sodium acetate pH 5.5), and then with elution buffer (50 mM sodium acetate, 1M NaCl, pH 5.5). The purification was monitored by AKTA prime plus (GE Healthcare, UK) at 280 nm.

15.2 SP FF column

The α-AI recombinant proteins were purified by Cation exchange chromatography using strong cation exchanger, SP sepharose Fast Flow column (GE Healthcare, UK), with binding buffer (50 mM sodium acetate pH 5.5), and then with elution buffer (50 mM sodium acetate, 1M NaCl, pH 5.5). The purification was monitored by AKTA prime plus (GE Healthcare, UK) at 280 nm.

15.3 Gel filtration chromatography

The α-AI recombinant proteins were purified by Gel filtration chromatography using Sephadex G-100 column. The Sephadex beads were dissolved in 50 mM sodium acetate buffer and were packed in 30 cm column long. The fractions contain interested protein were collect.

After that, all step of protein purification that containing α-AI recombinant proteins were identified on polyacrylamide gel electrophoresis, pooled, and dialyzed at 4°C and protein concentration using Viva Spin (GE Healthcare, UK).

16. Inhibitor Assay

Inhibition study, alpha amylase inhibitor was assayed using 2-Chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) (Sigma, USA) as substrate. The KDML105 amylase inhibitor was incubated with 3 sources of alpha amylase; Human saliva, *Bacillus subtilis* (Sigma, UK) and *Tribolium castaneum* (recombinant), at 37°C for 30 min. After that, 150 µl of CNPG3 was added to enzyme-inhibitor samples and incubated at 37°C for 30 min. Finally, the mixtures were measured the absorbance using microplate reader at 405 nm and the inhibition rates were calculated.

CHAPTER IV

RESULTS AND DISCUSSION

Cloning and nucleotide sequence analysis of alpha-amylase inhibitor genes from Kaw Dok Mali 105 and Pathumthani 1 indica rice (*Oryza sativa*)

To obtain the full coding sequences of alpha-amylase inhibitor gene from “Kaw Dok Mali 105” (KDML105) and “Pathumthani1” (PT) indica rice (*Oryza sativa*), Total RNA was isolated from young leaves of KDML105 and PT using Trizol reagent. The quality and concentration of total RNA was determined by measuring the UV absorbance using Nanodrop. An OD 260/280 absorbance ratio of total RNA was 1.8-2.0, which indicated acceptable quality of RNA.

The first strand complementary DNA (cDNA) was synthesized using reverse transcriptase polymerase chain reaction (RT-PCR) techniques. The cDNA fragments obtained from young leaves of KDML105 and PT indica rice were used as template. The rice elongation factor, housekeeping gene in rice was amplified to confirm quality of cDNA (figure 8).

The full coding nucleotide sequences of the rice-bifunctional alpha-amylase/subtilisin inhibitor from “Nipponbare” japonica rice (GenBank: AY166458.1) was used to design oligonucleotide primers. The size of PCR products were electrophoretic analyzed on agarose gel as shown in figure 9.

The PCR products were excised and eluted from an agarose gel for DNA purification using Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan). The purified DNA products were ligated to pTZ57R/T vector (Fermantas, USA) and ligated products were heat shocked transformed into DH5α *E.coli* competent cell as mention in Methods. The colony PCR technique was performed to confirm positive clone as mention in methods. The results of positive clone confirmation were shown in figure 10. The positive clones were nucleotide sequenced.

The 4 positive clones; KDML105, PT clone 1, PT clone 2 and PT clone 3 were confirmed with sequencing analysis. The nucleotide sequences were shown in figure11, 12, 13 and 14, respectively. The nucleotide sequences consist of 603 base pairs (bp) and predicted to 200 amino acid sequence. All of obtained nucleotide sequences were blasted with the GenBank database, the result revealed that these nucleotide sequences encode to alpha amylase inhibitor. All of deduced amino acid sequences from indica rice were multiple aligned using ClustalW, available tools on the website: http://www.ebi.ac.uk/Tools/msa/clustalw2 as shown in figure15. The multiple alignment result indicated that 3 clone obtained nucleotide and deduced amino acid sequence (KDML105, PT clone 1 and PT clone 3) are identical to those of japonica rice alpha-amylase/subtilisin inhibitor (GenBank accession number AY166458.1) whereas, PT clone 2 was slightly difference sequence, Its nucleotide and deduced amino acid sequence are 99% identity to rice alpha-amylase/subtilisin inhibitor. Moreover, rice alpha-amylase inhibitor from KDML105 and PT differs from the indica Homjan alpha-amylase inhibitor as shown in figure16.

The nucleotide sequences of full-coding sequence of α-AI have been deposited into the GenBank database. Accession numbers of the obtained rice α-amylase inhibitor genes were shown in Table 6.

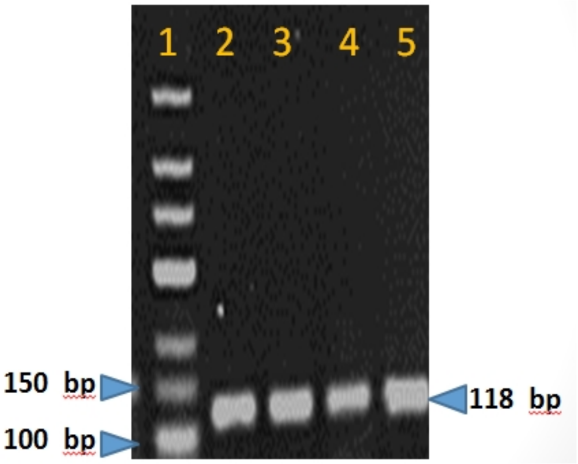


Figure 8 The RT-PCR of the rice-EF, housekeeping gene of rice from KDML105 rice and Pathumthani rice on agarose gel electrophoresis, Lane 1: Low-range DNA ladder, Lane 2-3: rice EF gene from KDML105 rice, Lane 4-5: rice EF gene from PT rice

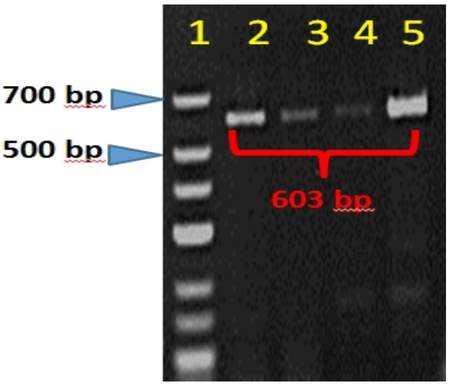


Figure 9 The Amplification of the full-coding sequence of α-AI from KDML105 rice and Pathumthani rice on agarose gel electrophoresis, Lane 1: Low-range DNA ladder Lane 2-3: α-AI from KDML105 rice Lane 4-5: gene α-AI from PT rice

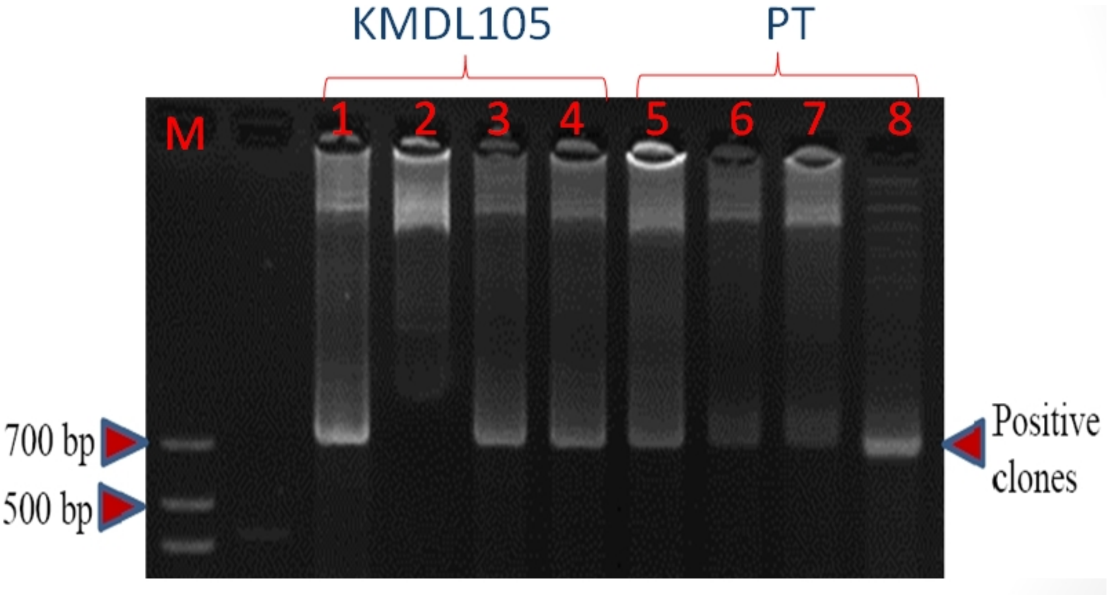
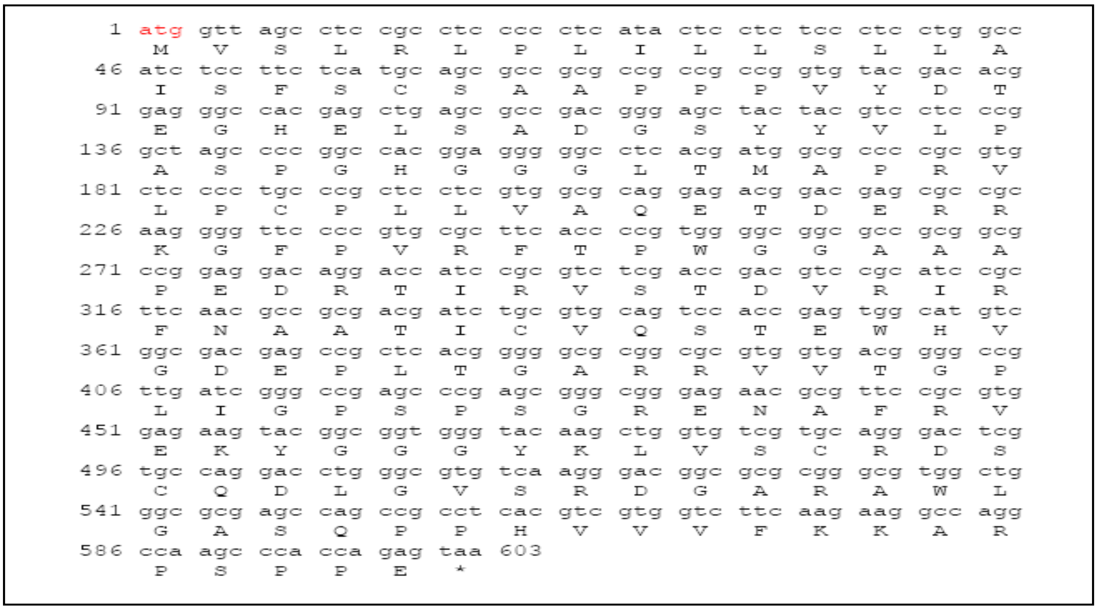


Figure 10 The agarose gel electrophoresis of recombinant clones screening using colony PCR technique, Lane M: Low-range DNA ladder, Lane 1-4: KDML105 α-AI clones, Lane 5-8 PT α-AI clones



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     I   S   F   S   C   S   A   A   P   P   P   V   Y   D   T
 91 gag ggc cac gag ctg agc gcc gac ggg agc tac tac gtc ctc ccg
     E   G   H   E   L   S   A   D   G   S   Y   Y   V   L   P
136 gct agc ccc ggc cac gga ggg ggc ctc acg atg gcg ccc cgc gtg
     A   S   P   G   H   G   G   G   L   T   M   A   P   R   V
181 ctc ccc tgc ccg ctc ctc gtg gcg cag gag acg gac gag cgc cgc
     L   P   C   P   L   L   V   A   Q   E   T   D   E   R   R
226 aag ggg ttc ccc gtg cgc ttc acc ccg tgg ggc ggc gcc gcg gcg
     K   G   F   P   V   R   F   T   P   W   G   G   A   A   A
271 ccg gag gac agg acc atc cgc gtc tcg acc gac gtc cgc atc cgc
     P   E   D   R   T   I   R   V   S   T   D   V   R   I   R
316 ttc aac gcc gcg acg atc tgc gtg cag tcc acc gag tgg cat gtc
     F   N   A   A   T   I   C   V   Q   S   T   E   W   H   V
361 ggc gac gag ccg ctc acg ggg gcg cgg cgc gtg gtg acg ggg ccg
     G   D   E   P   L   T   G   A   R   R   V   V   T   G   P
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     L   I   G   P   S   P   S   G   R   E   N   A   F   R   V
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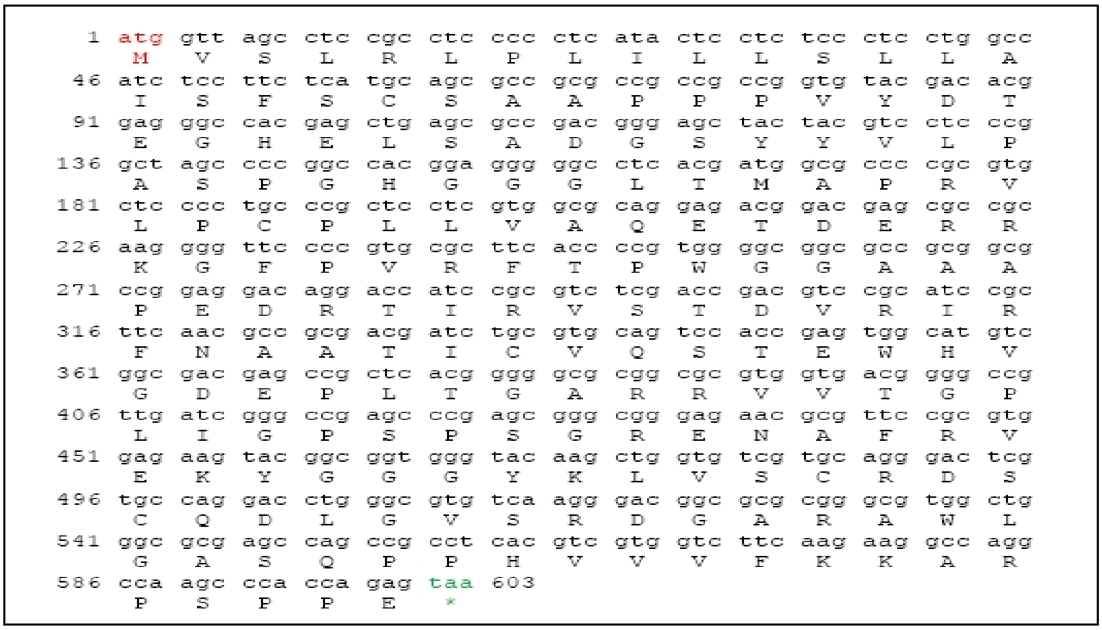
     E   K   Y   G   G   G   Y   K   L   V   S   C   R   D   S
496 tgc cag gac ctg ggc gtg tca agg gac ggc gcg cgg gcg tgg ctg
     C   Q   D   L   G   V   S   R   D   G   A   R   A   W   L
541 ggc gcg agc cag ccg cct cac gtc gtg gtc ttc aag aag gcc agg
     G   A   S   Q   P   P   H   V   V   V   F   K   K   A   R
586 cca agc cca cca gag taa 603
     P   S   P   P   E   *
```

Figure 11 The nucleotide sequence and deduced amino acid sequences of full coding alpha amylase inhibitor gene from KDML105 rice



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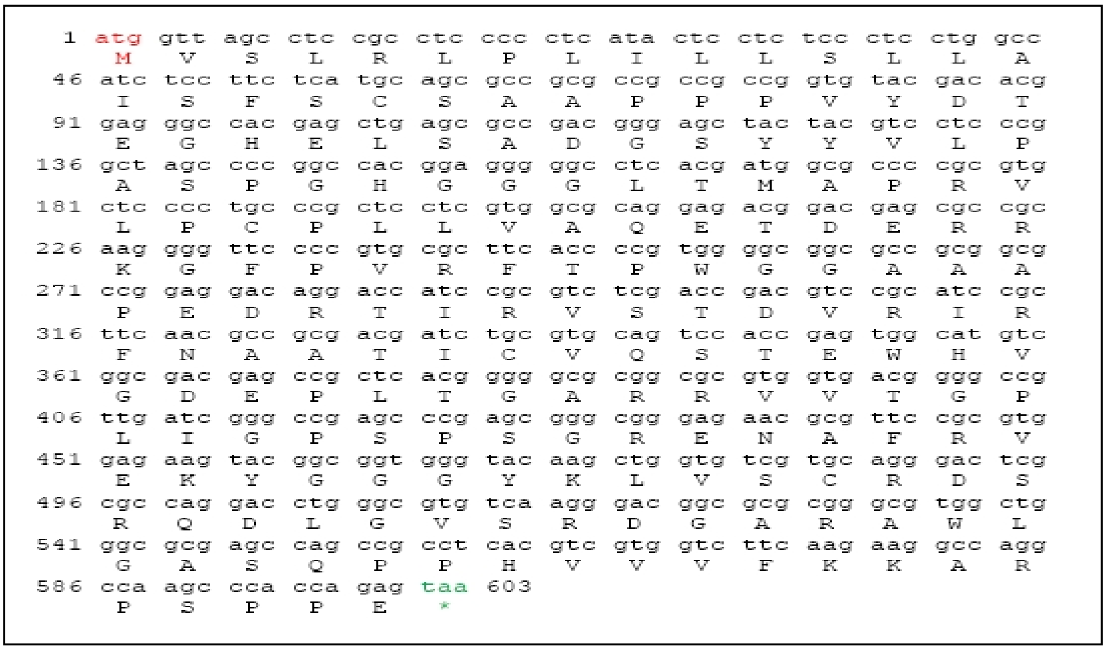
     I   S   F   S   C   S   A   A   P   P   P   V   Y   D   T
 91 gag ggc cac gag ctg agc gcc gac ggg agc tac tac gtc ctc ccg
     E   G   H   E   L   S   A   D   G   S   Y   Y   V   L   P
136 gct agc ccc ggc cac gga ggg ggc ctc acg atg gcg ccc cgc gtg
     A   S   P   G   H   G   G   G   L   T   M   A   P   R   V
181 ctc ccc tgc ccg ctc ctc gtg gcg cag gag acg gac gag cgc cgc
     L   P   C   P   L   L   V   A   Q   E   T   D   E   R   R
226 aag ggg ttc ccc gtg cgc ttc acc ccg tgg ggc ggc gcc gcg gcg
     K   G   F   P   V   R   F   T   P   W   G   G   A   A   A
271 ccg gag gac agg acc atc cgc gtc tcg acc gac gtc cgc atc cgc
     P   E   D   R   T   I   R   V   S   T   D   V   R   I   R
316 ttc aac gcc gcg acg atc tgc gtg cag tcc acc gag tgg cat gtc
     F   N   A   A   T   I   C   V   Q   S   T   E   W   H   V
361 ggc gac gag ccg ctc acg ggg gcg cgg cgc gtg gtg acg ggg ccg
     G   D   E   P   L   T   G   A   R   R   V   V   T   G   P
406 ttg atc ggg ccg agc ccg agc ggg cgg gag aac gcg ttc cgc gtg
     L   I   G   P   S   P   S   G   R   E   N   A   F   R   V
451 gag aag tac ggc ggt ggg tac aag ctg gtg tcg tgc agg gac tcg
     E   K   Y   G   G   G   Y   K   L   V   S   C   R   D   S
496 tgc cag gac ctg ggc gtg tca agg gac ggc gcg cgg gcg tgg ctg
     C   Q   D   L   G   V   S   R   D   G   A   R   A   W   L
541 ggc gcg agc cag ccg cct cac gtc gtg gtc ttc aag aag gcc agg
     G   A   S   Q   P   P   H   V   V   V   F   K   K   A   R
586 cca agc cca cca gag taa 603
     P   S   P   P   E   *
```

Figure 12 The nucleotide sequence and deduced amino acid sequences of full coding alpha amylase inhibitor gene from PT rice clone 1



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     I   S   F   S   C   S   A   A   P   P   P   V   Y   D   T
 91 gag ggc cac gag ctg agc gcc gac ggg agc tac tac gtc ctc ccg
     E   G   H   E   L   S   A   D   G   S   Y   Y   V   L   P
136 gct agc ccc ggc cac gga ggg ggc ctc acg atg gcg ccc cgc gtg
     A   S   P   G   H   G   G   G   L   T   M   A   P   R   V
181 ctc ccc tgc ccg ctc ctc gtg gcg cag gag acg gac gag cgc cgc
     L   P   C   P   L   L   V   A   Q   E   T   D   E   R   R
226 aag ggg ttc ccc gtg cgc ttc acc ccg tgg ggc ggc gcc gcg gcg
     K   G   F   P   V   R   F   T   P   W   G   G   A   A   A
271 ccg gag gac agg acc atc cgc gtc tcg acc gac gtc cgc atc cgc
     P   E   D   R   T   I   R   V   S   T   D   V   R   I   R
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     F   N   A   A   T   I   C   V   Q   S   T   E   W   H   V
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     G   D   E   P   L   T   G   A   R   R   V   V   T   G   P
406 ttg atc ggg ccg agc ccg agc ggg cgg gag aac gcg ttc cgc gtg
     L   I   G   P   S   P   S   G   R   E   N   A   F   R   V
451 gag aag tac ggc ggt ggg tac aag ctg gtg tcg tgc agg gac tcg
     E   K   Y   G   G   G   Y   K   L   V   S   C   R   D   S
496 cgc cag gac ctg ggc gtg tca agg gac ggc gcg cgg gcg tgg ctg
     R   Q   D   L   G   V   S   R   D   G   A   R   A   W   L
541 ggc gcg agc cag ccg cct cac gtc gtg gtc ttc aag aag gcc agg
     G   A   S   Q   P   P   H   V   V   V   F   K   K   A   R
586 cca agc cca cca gag taa 603
     P   S   P   P   E   *
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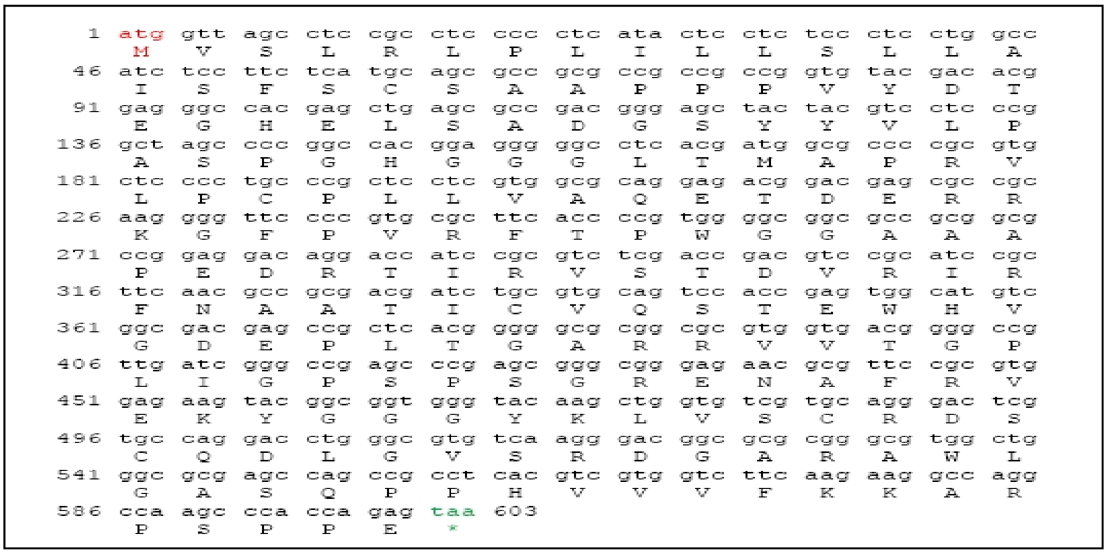
Figure 13 The nucleotide sequence and deduced amino acid sequences of full coding alpha amylase inhibitor gene from PT rice clone 2



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     E   G   H   E   L   S   A   D   G   S   Y   Y   V   L   P
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     K   G   F   P   V   R   F   T   P   W   G   G   A   A   A
271 ccg gag gac agg acc atc cgc gtc tcg acc gac gtc cgc atc cgc
     P   E   D   R   T   I   R   V   S   T   D   V   R   I   R
316 ttc aac gcc gcg acg atc tgc gtg cag tcc acc gag tgg cat gtc
     F   N   A   A   T   I   C   V   Q   S   T   E   W   H   V
361 ggc gac gag ccg ctc acg ggg gcg cgg cgc gtg gtg acg ggg ccg
     G   D   E   P   L   T   G   A   R   R   V   V   T   G   P
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     L   I   G   P   S   P   S   G   R   E   N   A   F   R   V
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     E   K   Y   G   G   G   Y   K   L   V   S   C   R   D   S
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     C   Q   D   L   G   V   S   R   D   G   A   R   A   W   L
541 ggc gcg agc cag ccg cct cac gtc gtg gtc ttc aag aag gcc agg
     G   A   S   Q   P   P   H   V   V   V   F   K   K   A   R
586 cca agc cca cca gag taa 603
     P   S   P   P   E   *
```

Figure 14 The nucleotide sequence and deduced amino acid sequences of full coding alpha amylase inhibitor gene from PT rice clone 3

```
Japonica        MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 60
KDML105         MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 60
PT clone1       MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 60
PT clone2       MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 60
PT clone3       MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 60
                ************************************************************

Japonica        LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 120
KDML105         LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 120
PT clone1       LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 120
PT clone2       LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 120
PT clone3       LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 120
                ************************************************************

Japonica        GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSCQDLGVSRDGARAWL 180
KDML105         GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSCQDLGVSRDGARAWL 180
PT clone1       GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSCQDLGVSRDGARAWL 180
PT clone2       GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSRQDLGVSRDGARAWL 180
PT clone3       GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSCQDLGVSRDGARAWL 180
                ********************************************* **************

Japonica        GASQPPHVVVFKKARPSPPE 200
KDML105         GASQPPHVVVFKKARPSPPE 200
PT clone1       GASQPPHVVVFKKARPSPPE 200
PT clone2       GASQPPHVVVFKKARPSPPE 200
PT clone3       GASQPPHVVVFKKARPSPPE 200
                ********************
```

Figure 15 Multiple alignments of the amino acid sequences of the α-amylase inhibitor from the japonica rice, the 'KDML105' indica rice, the α-amylase inbitor clone1, clone 2 and clone 3 from Pathumthani 1 indica rice

```
KDML105 AI     MVSLRLPLILLSLLAISFSCSAAPPPVYDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 60
Homjan AI      ---------------------MAPPPVHDTEGHELSADGSYYVLPASPGHGGGLTMAPRV 39
                                     *****:********************************

KDML105 AI     LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 120
Homjan AI      LPCPLLVAQETDERRKGFPVRFTPWGGAAAPEDRTIRVSTDVRIRFNAATICVQSTEWHV 99
               ************************************************************

KDML105 AI     GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSCQDLGVSRDGARAWL 180
Homjan AI      GDEPLTGARRVVTGPLIGPSPSGRENAFRVEKYGGGYKLVSCRDSCQDLGVSRDGARAWL 159
               ************************************************************

KDML105 AI     GASQPPHVVVFKKARPSPPE 200
Homjan AI      GASQPPHVVVFKKARPSPPE 179
               ********************
```

Figure 16 Alignment of the amino acid sequences of the α-amylase inhibitor from the 'KDML105' indica rice and α-amylase inhibitor from the Homjan indica rice

Table 6 Summary of alpha amylase inhibitor accession number that provided by Genbank Database

	Name	Accession number
1	*Oryza sativa* Indica Group cultivar Kaw Dok Mali 105 alpha amylase inhibitor mRNA, complete cds	JX426136
2	*Oryza sativa* Indica Group cultivar Pathumthani Thai clone 1 alpha-amylase inhibitor mRNA, complete cds	KF219883
3	*Oryza sativa* Indica Group cultivar Pathumthani Thai clone 2 alpha-amylase inhibitor mRNA, complete cds	KF219884

Expression and Characterization of alpha - amylase inhibitor gene from KDML105 indica rice

1. Construction of pET17b(+)- KDML105 alpha amylase inhibitor gene.

The pTZ57R/T- KDML105 alpha-amylase inhibitor (α-AI) recombinant plasmid was double digested with *Nde*I and *Xho*I restriction enzymes and was analyzed by electrophoresis, as shown in Figure17. The digested α-AI DNA fragment was ligated to the pET-17b(+) expression vector and was heat shock transformed to *E.coli* strain BL21(DE3) pLysS as mentioned in Methods. The recombinant plasmid clones were confirmed by double digestion with restriction enzyme and DNA sequencing.

2. Expression of recombinant pET17b(+)-KDML105 alpha-amylase inhibitor protein in *E.coli* strain BL21(DE3) pLysS.

2.1 Optimization of the expression time for the recombinant pET-17(+)-KDML105 alpha-amylase inhibitor protein in *E.coli* expression system

In order to optimize time expression of the KDML105 α-AI, the interested clone carrying KDML105α-AI gene was transformed to *E.coli* BL21 (DE3) pLysS for α-AI protein induction using 1mM IPTG at 37°C and harvested at various times (1, 2, 3, 6, 9 and 24 hrs.). The α-AI induced proteins were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE). The result in figure 18 shows SDS-PAGE analysis of KDML105 α-AI recombinant protein expression. The approximately 22 kDa of α-AI protein from KDML105 rice can be expressed in 1, 2, 3, 6, 9 and 24 hours at 37°C and 3 hrs IPTG inductions provided higher level of 22 kDa α-AI protein.

2.2 Optimization of the expression temperature for the recombinant pET-17(+)-KDML105 alpha-amylase inhibitor protein in *E.coli* expression system

In order to optimize temperature expression of the KDML105 α-AI, the protein was induced with 1mM IPTG at various expression temperatures (28°C, 37°C and 42°C). The cultures were induced for 3 hours. The result in figure19 showed the expression levels at 37°C provided better condition amount of expression temperatures.

2.3 Optimization of the culture medium for the recombinant pET-17(+)-KDML105 alpha-amylase inhibitor protein in *E.coli* expression system

In order to optimize culture medium in expression system, the KDML105α-AI was culture in LB medium and minimal medium. The α-AI protein was induced with 1mM IPTG at 37°C for 3 hours. The comparison result of expression levels between LB and Minimal showed the expression level is not different. Therefore, the

Therefore, the condition for the recombinant pET17b(+)- KDML105 α-AI expression is culture in LB medium and induced with 1mM IPTG for 3 hours.

3. Identification of protein by LC-MS/MS

The 3 hours IPTG induced protein was determined by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The 22 kDa protein band was cut out from acrylamide gel, then trypsinized and identified by LC-MS/MS. Analyzing of recombinant protein band was searched using MASCOT (http://www. matrixscience.com). The result showed the matched peptide to alpha amylase inhibitor from *Oryza sativa* as shown in figure 22 and 23.

4. KDML105 α-AI protein purification

The KDML105 α-AI recombinant proteins were purified by Gel filtration chromatography using 30 cm-long Sephadex G-100 column (Amersham, USA) with 1ml/min flow rate. The protein fractions were collected every 1 min and were analyzed by SDS-PAGE. The protein patterns were shown in figure24.

The KDML105 α-AI recombinant proteins were purified by Cation exchange chromatography using Cation exchanger, SP sepharose and CM sepharose Fast Flow column (GE Healthcare, UK). The protein fractions collected by ATKA prime plus (GE Healthcare, USA) were electrophoretic analyzed by SDS-PAGE. The protein patterns were shown in figure25 and figure26, respectively.

Combination purification of Cation exchange chromatography and gel filtration was performed for better purification. The result (data not shown) showed KDML105 α-AI protein cannot detect by the 2step combined purification method.

Therefore, the KDML105 α-AI partial purified from CM sepharose was used for inhibition study.

5. Inhibition assay

For inhibition study, alpha amylase was assayed 2-Chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) (Sigma, USA) as substrate. The inhibitory study was assayed as follows: KDML105 alpha-amylase inhibitor (α-AI) was pre-incubated with alpha amylases from Human saliva, *Bacillus subtilis* and *Tribolium castaneum*. After that, enzyme/inhibitors were added into reaction (buffer and substrate) and incubated for 30 min and absorbance measurement at 405 nm. The results of inhibitory activity showed in figure25, the KDML105 alpha-amylase inhibitor can decrease amylase activity from human saliva and *Bacillus subtilis* at 68.82 %and 83.52.1% inhibition, respectively, at *B.subtilis* amylase: α-AI molar ratio of 35:1 and at human saliva: α-AI molar ratio of 3:1 (Figure 25 and Table 7). In the other hand, the KDML105 alpha-amylase inhibitor cannot effectively inhibit recombinant alpha-amylase from red flour beetle (*Tribolium castaneum*).

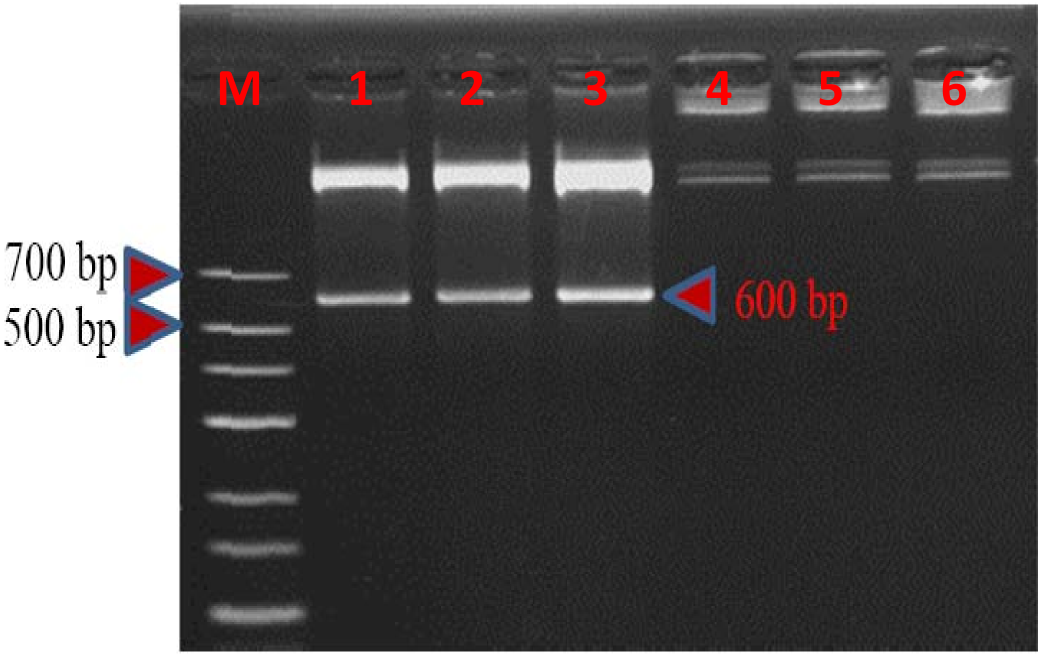


Figure 17 The 1% agarose gel electrophoresis of double digestion of α-AI recombinant plasmids with *NdeI* and *XhoI* restriction enzymes, Lane M: Low-range DNA ladder, Lane 1: KDML105 α-AI recombinant plasmid digested with NdeI and XhoI restriction enzyme, Lane 2-3 : PT α-AI recombinant plasmid digested with NdeI and XhoI restriction enzyme, Lane 4: non-digestion KDML105 α-AI recombinant plasmid Lane 5-6: non-digestion PT α-AI recombinant plasmid

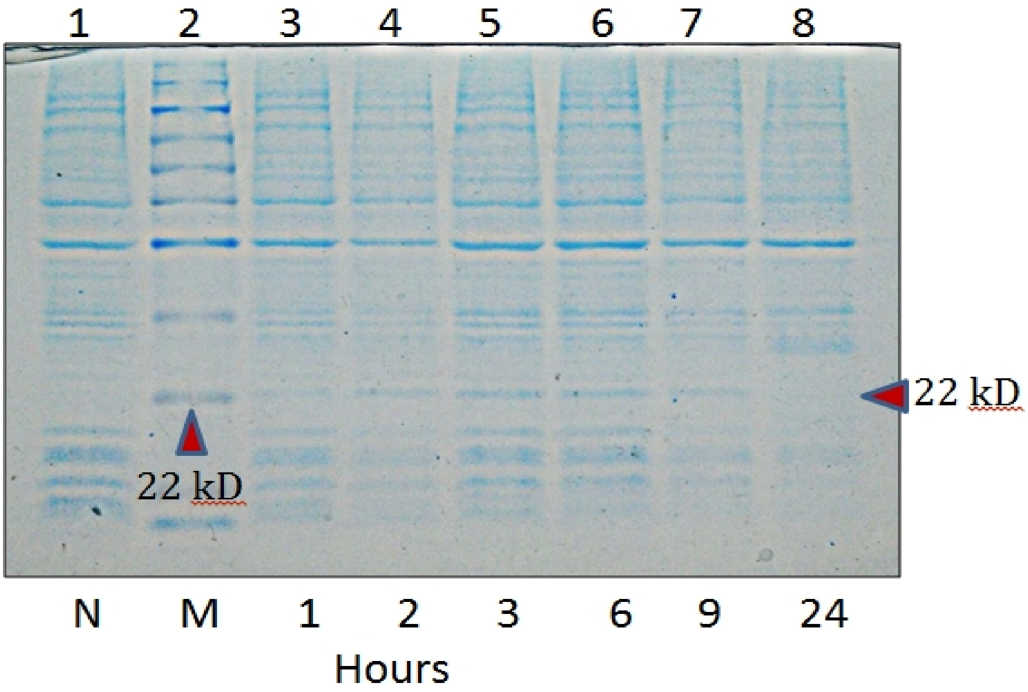


Figure 18 15% SDS-PAGE gel of recombinant pET-17b(+)-KDML105 alpha-amylase inhibitor was induced with 1mM IPTG at 37°C in the various time, lane 1: pET17b(+) expression vector, lane 2: Chromatein prestained protein ladder, Lane 3-8: induced pET-17b(+)-KDML105 alpha-amylase inhibitor for 1, 2, 3, 6, 9 and 24 hours, respectively

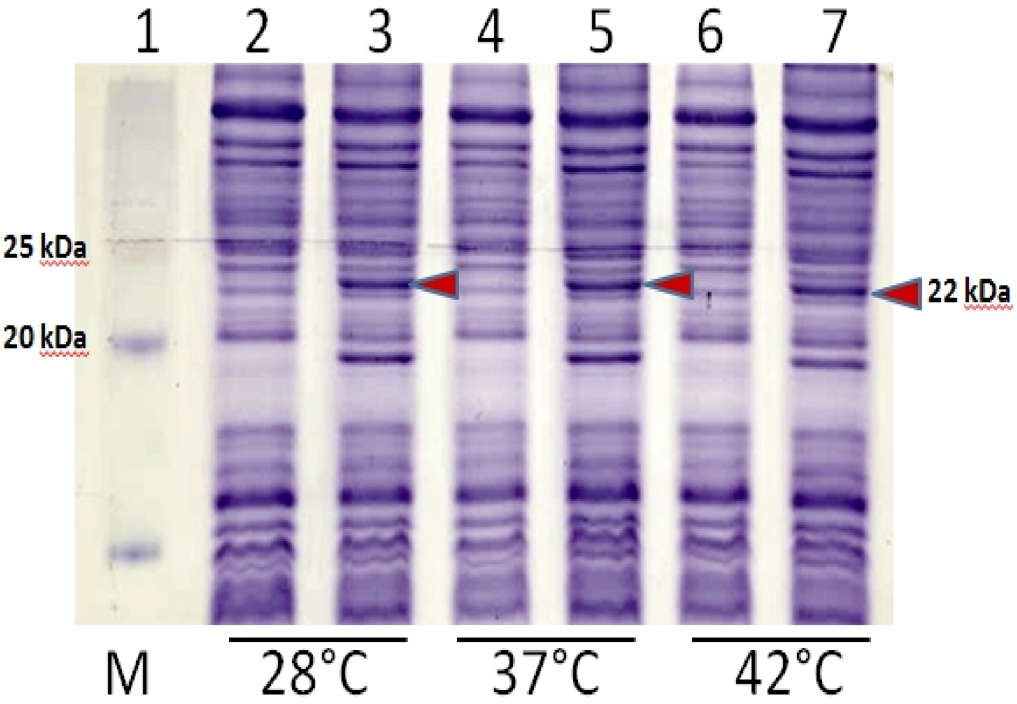


Figure 19 15% SDS-PAGE gel of recombinant pET-17b(+)-KDML105 alpha-amylase inhibitor was induced with 1mM IPTG at the various temperature. Lane 1: Chromatein prestained protein ladder, Lane 2, 4, 6: pET17b, Lane 3, 5, 7: KDML105 alpha-amylase inhibitor recombinant protein.

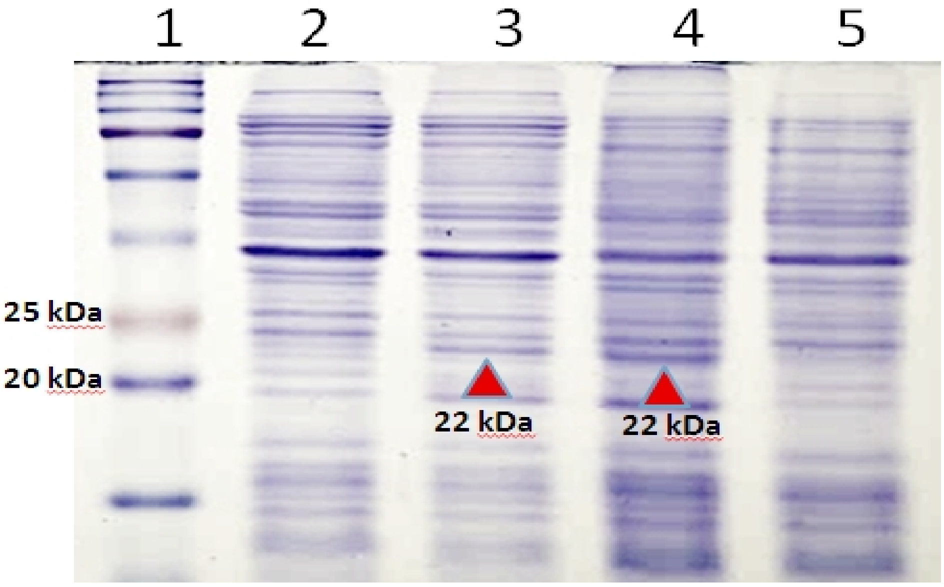


Figure 20 15% SDS-PAGE gel of ITPG induction recombinant pET-17b(+)-KDML105 alpha-amylase inhibitor in different medium. Lane 1: Chromatein prestained protein ladder, Lane 2: Minimal media cultured pET17b, Lane 3: Minimal media cultured KDML105 alpha-amylase inhibitor recombinant protein, Lane 4: LB media cultured KDML105 alpha-amylase inhibitor recombinant protein, Lane 5: LB media culturedpET17b

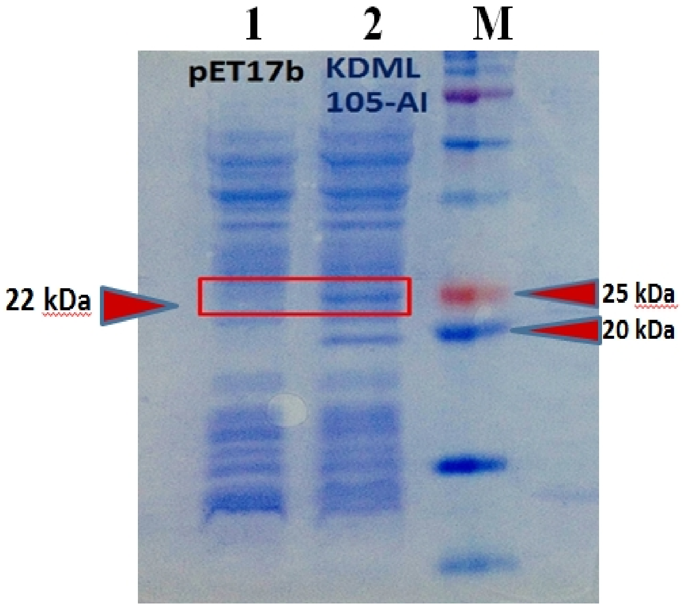
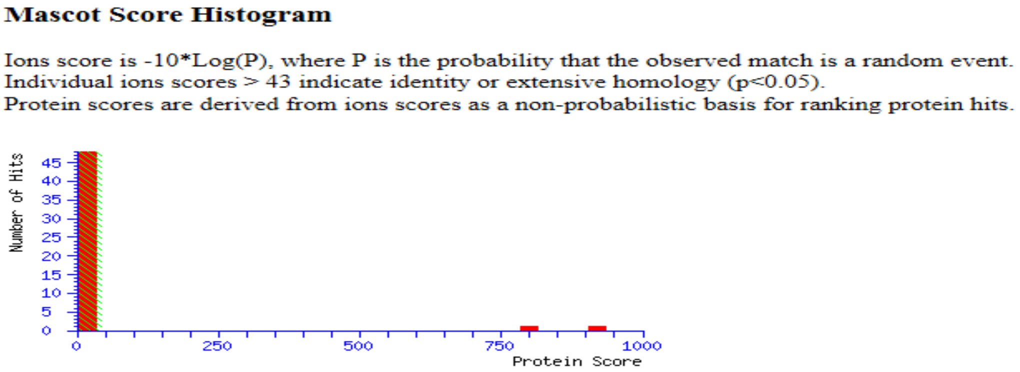


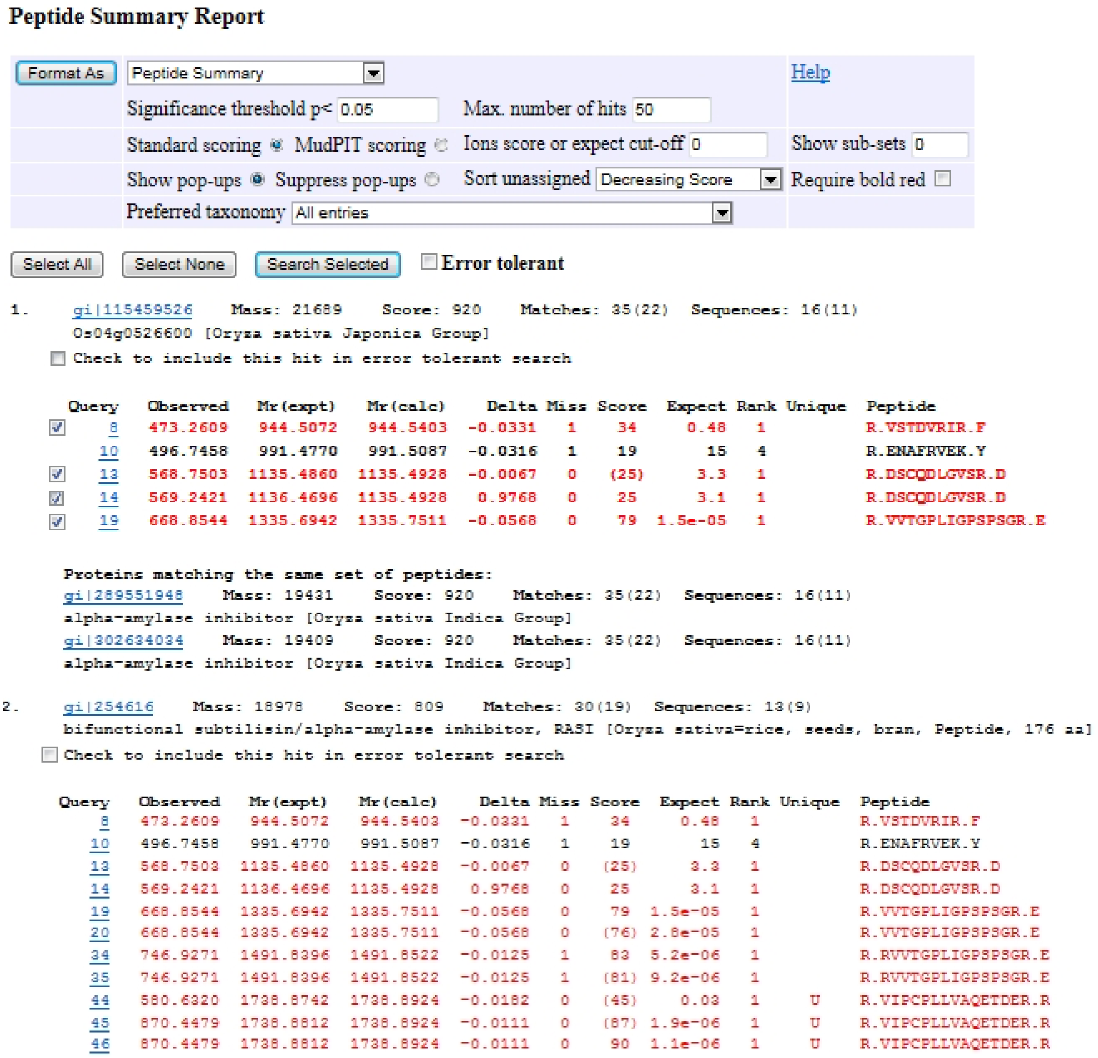
Figure 21 15% SDS-PAGE gel of recombinant pET-17b(+)-KDML105 alpha-amylase inhibitor was induced with 1mM IPTG at 37°C, lane 1: pET17b(+) expression vector, lane 2: 1mM-IPTG induced pET-17b(+)-KDML105 alpha-amylase inhibitor for 3 hours Lane M : intron pre-stained protein ladder



Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 43 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Figure 22 Mascot search of LC-MS/MS result showed that the tryptic digests of 22 kDa protein have exactly matched to alpha-amylase inhibitor from rice *(Oryza sativa)*



Peptide Summary Report

Format As: Peptide Summary

Significance threshold p< 0.05 Max. number of hits 50

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 ☐

Preferred taxonomy All entries

☐ Error tolerant

```
1.    gi|115459526    Mass: 21689    Score: 920    Matches: 35(22)   Sequences: 16(11)
      Os04g0526600 [Oryza sativa Japonica Group]
    ☐ Check to include this hit in error tolerant search

      Query   Observed    Mr(expt)   Mr(calc)    Delta Miss Score  Expect Rank Unique  Peptide
    ☑     8   473.2609    944.5072   944.5403  -0.0331    1    34    0.48    1         R.VSTDVRIR.F
         10   496.7458    991.4770   991.5087  -0.0316    1    19      15    4         R.ENAFRVEK.Y
    ☑    13   568.7503   1135.4860  1135.4928  -0.0067    0  (25)     3.3    1         R.DSCQDLGVSR.D
    ☑    14   569.2421   1136.4696  1135.4928   0.9768    0    25     3.1    1         R.DSCQDLGVSR.D
    ☑    19   668.8544   1335.6942  1335.7511  -0.0568    0    79 1.5e-05    1         R.VVTGPLIGPSPSGR.E

     Proteins matching the same set of peptides:
     gi|289551948    Mass: 19431    Score: 920    Matches: 35(22)   Sequences: 16(11)
     alpha-amylase inhibitor [Oryza sativa Indica Group]
     gi|302634034    Mass: 19409    Score: 920    Matches: 35(22)   Sequences: 16(11)
     alpha-amylase inhibitor [Oryza sativa Indica Group]

2.   gi|254616    Mass: 18978    Score: 809    Matches: 30(19)   Sequences: 13(9)
     bifunctional subtilisin/alpha-amylase inhibitor, RASI [Oryza sativa=rice, seeds, bran, Peptide, 176 aa]
   ☐ Check to include this hit in error tolerant search

     Query   Observed    Mr(expt)   Mr(calc)    Delta Miss Score  Expect Rank Unique  Peptide
         8   473.2609    944.5072   944.5403  -0.0331    1    34    0.48    1         R.VSTDVRIR.F
        10   496.7458    991.4770   991.5087  -0.0316    1    19      15    4         R.ENAFRVEK.Y
        13   568.7503   1135.4860  1135.4928  -0.0067    0  (25)     3.3    1         R.DSCQDLGVSR.D
        14   569.2421   1136.4696  1135.4928   0.9768    0    25     3.1    1         R.DSCQDLGVSR.D
        19   668.8544   1335.6942  1335.7511  -0.0568    0    79 1.5e-05    1         R.VVTGPLIGPSPSGR.E
        20   668.8544   1335.6942  1335.7511  -0.0568    0  (76) 2.8e-05    1         R.VVTGPLIGPSPSGR.E
        34   746.9271   1491.8396  1491.8522  -0.0125    1    83 5.2e-06    1         R.RVVTGPLIGPSPSGR.E
        35   746.9271   1491.8396  1491.8522  -0.0125    1  (81) 9.2e-06    1         R.RVVTGPLIGPSPSGR.E
        44   580.6320   1738.8742  1738.8924  -0.0182    0  (45)    0.03    1     U   R.VIPCPLLVAQETDER.R
        45   870.4479   1738.8812  1738.8924  -0.0111    0  (87) 1.9e-06    1     U   R.VIPCPLLVAQETDER.R
        46   870.4479   1738.8812  1738.8924  -0.0111    0    90 1.1e-06    1     U   R.VIPCPLLVAQETDER.R
```

Figure 23 Identification of peptide mass fingerprints result, alpha-amylase inhibitor protein was induced with 1mM IPTG at 37^{o}C for 3 hours and identified by LC-MS/MS. The KDML105 α-AI protein has exactly matched to alpha-amylase inhibitor from rice *(Oryza sativa)*

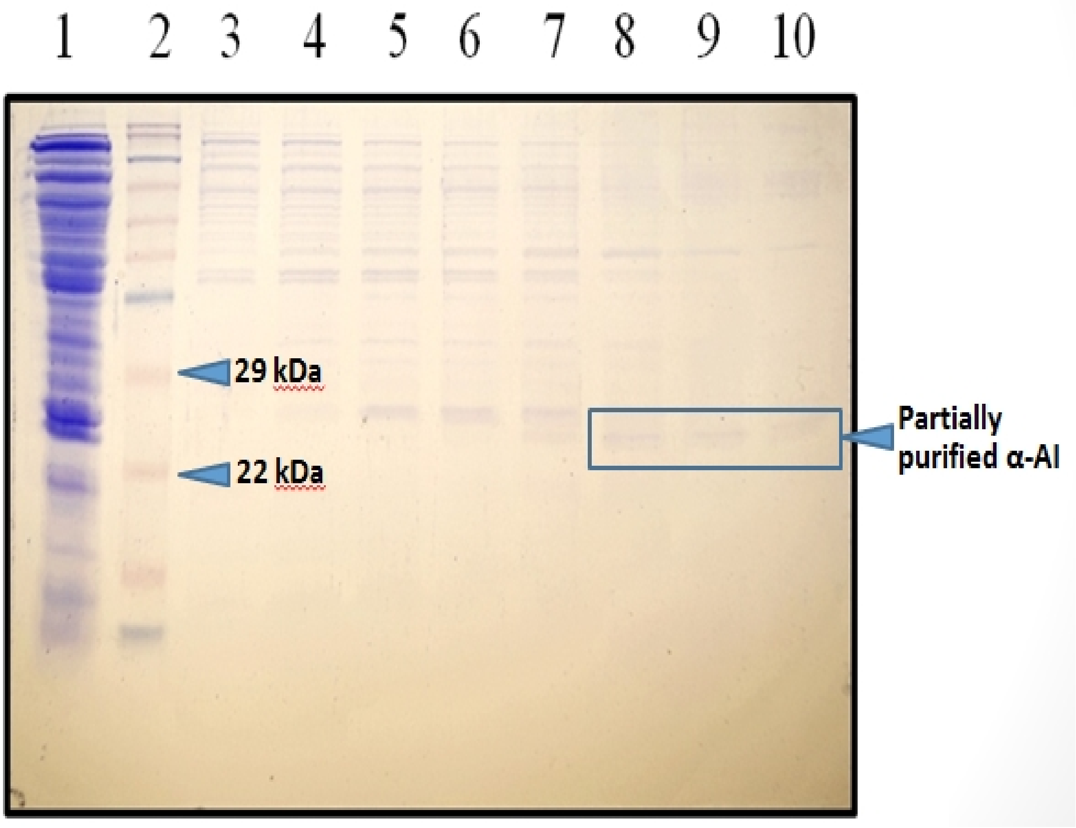


Figure 24 15% SDS-PAGE gel showed the pET17(b)+- KDML105 α-AI recombinant protein was purified using Sephadex G-100 gel filtration chromatography, Lane 1: Crude protein , Lane 2: Chromatein prestained protein ladder, Lane 3-10: fractionated protein from Sephadex G-100 gel filtration chromatography

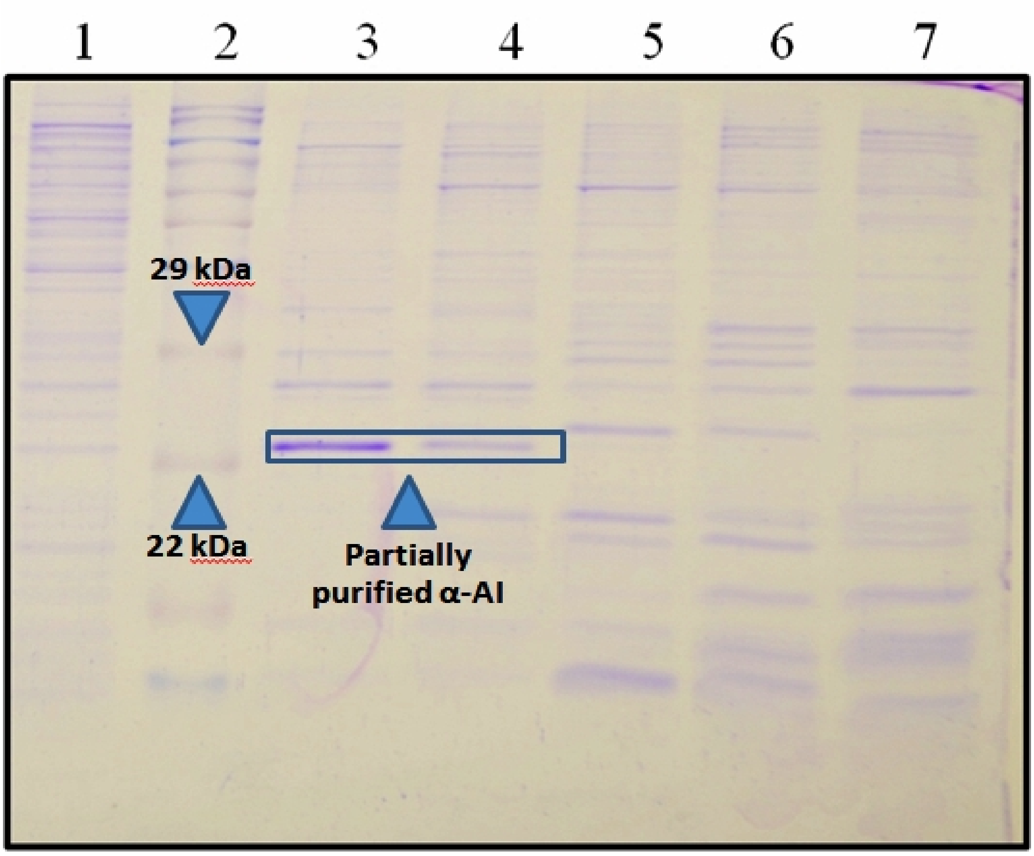


Figure 25 15% SDS-PAGE gel showed the pET17(b)+- KDML105 α-AI recombinant protein was purified using SP FF Cat-ion exchange chromatography, Lane 1: Crude protein, Lane 2: Chromatein prestained protein ladder, Lane 3-7: fractionated protein from SP FF Cat-ion exchange chromatography

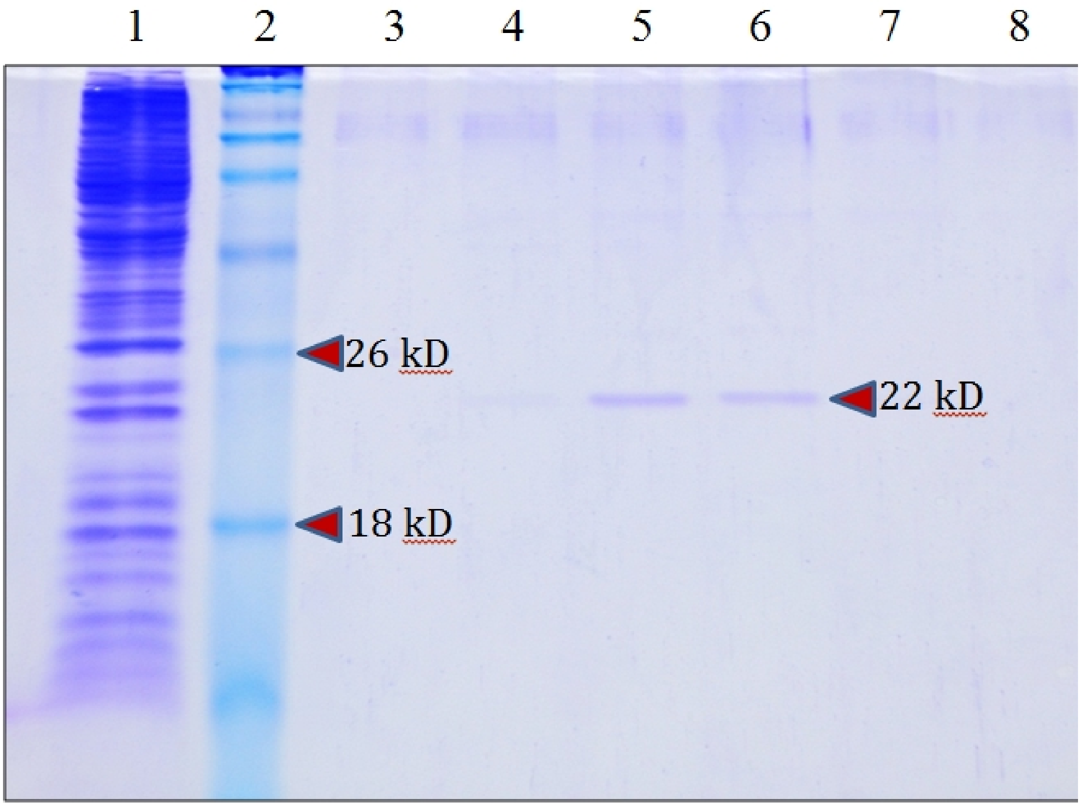


Figure 26 **15% SDS-PAGE gel showed the pET17(b)+- KDML105 α-AI recombinant protein was purified using CM FF Cat-ion exchange chromatography, Lane 1: Crude protein, Lane 2: PRO-STAIN^{TM} Pre-stained Protein Marker, Lane 3-8: fractionated protein from CM FF Cation exchange chromatography**

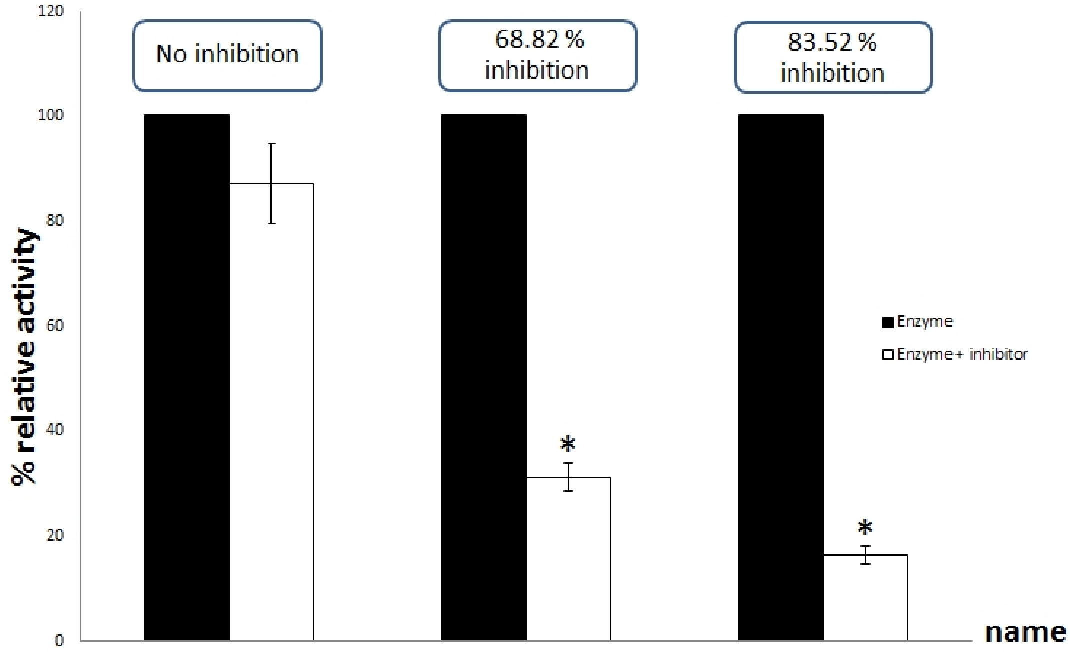


Figure 27 **The graph showed enzyme activity of several alpha amylase and its activity with the recombinant KDML105 alpha-amylase inhibitor (α-AI), the enzyme activity in the absent and present of α-AI was represent in black bar and white bar, respectively (Statistics data is analyzed by T-test statistics)**

Table 7 Summary of alpha-amylase activity with absent and present of KDML105 alpha –amylase inhibitor

	Enzyme **activity (unit/µl)**	Enzyme **and KDML105 α-AI activity (unit/µl)**	**% inhibition**	**Molar ratio enzyme : inhibitor**
Human saliva α-amylase	**69.88**	**21.79**	**68.82**	**3:1**
Bacillus subtilis α-amylase	**5.33**	**0.88**	**83.52**	**35:1**
*Tribolium casteneum*α-amylase	**0.034**	**0.029**	**No**	**1:8**

This table shows alpha-amylase activities and inhibitory activities by KDML105 alpha-amylase inhibitor against human saliva alpha- amylase and *Bacillus subtilis* alpha- amylase. The recombinant KDML105 alpha-amylase inhibitor can inhibit 68.82% and 83.52% enzyme activities of human saliva alpha- amylase and *Bacillus subtilis* alpha- amylase, respectively. On the others hand, the recombinant KDML105 alpha-amylase inhibitor cannot inhibit recombinant alpha- amylase from *Tribolium castaneum*.

Discussion

Cloning and analysis of nucleotide sequence of alpha-amylase inhibitor genes from Kaw Dok Mali 105 and Pathumthani 1 indica rice (*Oryza sativa*)

Alpha-amylase is an enzyme which plays an important role in carbohydrate metabolism in several organisms, especially in insect. They feed on plant seeds during the larval and/or adult stage and depend on their α-amylase for growth and development (Franco, et al., 2002). Because of their important biochemical roles, there are many researches interest in isolating and identifying plant proteinaceous inhibitors that inhibit several α-amylases. Cloning, expression and characterization of alpha-amylase inhibitor have been revealed in barley (Leah and Mundy, 1989), rye (Iulek, et al., 2000) and wheat (Maeda, 1986).

In this study, the full-length coding nucleotide sequence of the alpha amylase inhibitor (α-AI) genes from the Kaw Dok Mali 105 (KDML105) rice and Pathumthani 1 (PT) rice were obtained by PCR using the primers designed base on the α-AI sequence (Accession number: AY166458.1). These PCR products were purified, cloned and sequenced. The Nucleotide sequence results illustrated the clones; KDML105, PT clone 1 and 2 consist of 603 bp nucleotide sequences. This is the first report of nucleotide sequence of alpha amylase inhibitor from KDML105 and PT indica rice. The KDML105, PT clone 1 and 2 were deposited in GenBank database at National Center for Biotechnology Information (NCBI).

The obtained nucleotide sequence from KDML105 and PT clone 1 are 100% similar to the full coding nucleotide sequence of japonica rice alpha-amylase/subtilisin inhibitor (Accession number: AY166458.1). The nucleotide sequences were deduced to 200 amino acids. Sequence comparison illustrated that deduced amino acid sequence of the clone was identical to the japonica rice alpha-amylase/subtilisin inhibitor (Accession number: AAN86549) (Yamasaki, et al., 2006).The deduced amino acid was calculated to a molecular mass of 21,417 Dalton, and the N-terminus of its sequence contains signal peptide (1-22). Therefore, the predicted mature protein sequence comprise of 178 amino acids.

The obtained sequences and the deduced amino acid sequences are similar to the α-amylase inhibitor from japonica rice. Moreover, this protein supposed to be bifunctional α-amylase/subtilisin inhibitor which can inhibit both α-amylase and subtilisin in soybean Kunitz-type inhibitor family. For example, Rice bifunctional amylase/subtilisin inhibitor (RASI) (Yamasaki, et al., 2006), Barley bifunctional amylase/subtilisin inhibitor (BASI) (Rodenburg, et al., 1995) and Wheat bifunctional amylase/subtilisin inhibitor (WASI) (Gvozdeva, et al., 1993). This inhibitor group contains two disulfide bonds and approximately 180 amino acid residues. It is able to inhibit both α-amylase and serine protease named subtilisin (Franco, et al., 2002; Yamasaki, et al., 2006).

However, the obtained PT clone 2 sequence differs from the other clones. Its deduced amino acid sequence converts from Cys 166 to Arg 166, leading to disulfide bond formation change. Basically, the inhibitor in Kunitz family comprises of two-disulfide bond (Franco, et al., 2002). Thus, it must have 4 cysteines. Cysteine can be oxidized to form a dimer containing disulfide bridges between a pairs of cysteines. The functions of disulfide bond are protein stability and maintain protein integrity (Hogg, 2003). Therefore, conversion of amino acid from Cysteine to Arginine in this protein may losses disulfide bonds leading to protein destabilization.

Moreover, the KDML105 and PT clone alpha-amylase inhibitor differs from the indica Thai Homjan alpha-amylase inhibitor (accession number: FJ750521) indicating complex family of proteinaceous inhibitors in rice that could lead to complex interactions of alpha-amylase and the inhibitors.

Expression and biochemical characteristics of alpha amylase inhibitor gene from KDML105 indica rice

In this study, the alpha-amylase inhibitor (α-AI) gene of Kaw Dok Mali 105 indica rice was expressed in *E.coli* strain BL21 (DE3) pLysS using pET17b (+) expressed vector. The result showed approximately 22 kDa of recombinant α-AI protein at 37°C for 3 hours after 1 mM IPTG induction in LB cultured medium. After that, KDML105 α-AI protein was purified using ion exchange chromatography and identified using LC-MS/MS.

The activity of alpha-amylase inhibitor protein toward several alpha-amylases was assayed by 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate. Previous study by Yamasaki and colleagues mentioned that rice alpha amylase/subtilisin inhibitor from japonica rice did not inhibit alpha-amylase from larvae and adult at all. Moreover, this inhibitor did not inhibit alpha amylase from porcine pancreatic but weakly inhibit against microorganism such as *Bacillus subtilis* and *Bacillus globigii* and *Aspergillus oryzae* (Yamasaki, 2006). Interestingly, we found that the partial purified KDML105 α-AI protein strongly reduced alpha-amylase activities from *Bacillus subtilis* and effectively inhibited an alpha-amylase from human saliva. On the other hand, this inhibitor cannot affect to alpha-amylase from red flour beetle, *Tribolium castaneum*. The result on *Tribolium castanuem* is similar to Yamasaki (Yamasaki, et al., 2006).

Possibility of KDML105 recombinant alpha-amylase inhibitor does not inhibit recombinant alpha-amylase from *Tribolium castaneum* is thioredoxin fusion protein which tagged with recombinant alpha-amylase. Thioredoxin is tagged N-terminal protein which increase protein solubility (Terpe, 2003). Thioredoxin may help correct protein conformation by serving as covalent joined chaperone protein. This property of thioredoxin could be viewed as analogous to the covalent chaperone role proposed for the N-terminal propeptide regions (La Villie, et al., 2000; Shinde, Chatterjee and Inouye 1993; Silen, et al., 1989). Therefore, the conformation of thioredoxin fusion protein tagged with recombinant alpha-amylase probably interferes with reactive site of KDML105 alpha-amylase inhibitor leading to the inhibitory activity cannot detect. The other reason is molar ratio suitability between alpha-amylase and alpha-amylase inhibitor. The molar ratio of enzyme: inhibitor (1: 2) may not be sufficient to drive amylase activity for detection by CNPG3 method. We recommend that further inhibitory activity against alpha-amylase could adjust molar ratio to 1:1 (enzyme: inhibitor).

In addition, there are α-amylase inhibitors in Kunitz-like family such as barley alpha-amylase subtilisin inhibitor (BASI). It showed inhibitory activity against barley α-amylase isozyme 2 (AMY2) from barley seeds but does not inhibit barley amylase isozyme 1(AMY1). Interestingly, both isoforms of BASI share 74% sequence identity (Mundy, Svendsen and Hejgaard, 1983; Jones and Jacobsen, 1991; Nielsen, et al., 2004). Moreover, the enzyme/substrate complex study of BASI/AMY2 reported the inhibitor does not directly interact with α-amylase at the three catalytic acidic residues of enzyme like the other inhibitors. But, the inhibitor can reduce amylase activity by strongly interaction with domain A and B that locating near catalytic site (Valle, et al., 1998; Franco, et al., 2002).

The wheat alpha amylase inhibitor named WRP25 and WRP26, showed surprisingly result of specifically inhibition toward amylase from Mexican bean weevil, *Zabrotes subfasciatus* (ZSA). WRP25 can be an effectively inhibitor against ZSA, whereas the WRP26 wheat inhibitor is inability to inhibit ZSA while WRP25 and WRP26 share 98% sequence identity (Franco, et al., 2000). Even though the investigation of specificity between alpha-amylases and it inhibitors are not many publications. However, there are several researches reporting that electrostatic properties (Franco, et al., 2000; Rodenburg, et al., 2000) hydrogen bonding capacity (Pereira, et al., 1999) and disulfide bonding of cysteine (Franco, et al., 2000) are multifactor involving specificity of alpha-amylase and its inhibitor.

In general, the biotechnological applications of alpha amylase inhibitors have been investigated. There are several researches reported that the plant alpha amylase inhibitor such as α-AI from *T.aestivum* and *Z.mays* can inhibit alpha-amylase from red flour beetle, *Tribolium castaneum* (Franco, et al., 2002). In transgenic plant, α-AI from *Pisum sativum* effectively resisted pea weevil (*Bruchus pisorum*) under field conditions (Morton, et al., 2000). Moreover, Pusztai and colleague studied transgenic pea expressing high level of α-AI1 in animal model, 300 g/kg level of transgenic peas expressing bean a-AI gene was fed by rats. The result showed the short-term study has not significant effect on growth, metabolism and health of rats (Pusztai, et al., 1999).

There are other insecticidal proteins like cysteine protease inhibitor can suppress growth and development of *Tribolium castaneum* (Oppert, et al., 2003). Therefore, Combination using of alpha amylase inhibitor and protease such as the bifunctional alpha-amylase inhibitor will be effective inhibitor better than single inhibitor (Yamasaki, et al., 2006).

Moreover, the inhibition activity of alpha amylase inhibitor against *B.subtilis* alpha-amylase showed effective inhibitor in plant seed defense mechanism toward microorganisms. The bifunctional amylase subtilisin inhibitor such as RASI, BASI and WASI, their function likely protect endogenous alpha-amylase for regulation of starch digestion that important to premature sprouting prevention of the seed (Yamasaki, et al., 2006).

On the other hand, the application of plant α-amylase inhibitors in term of insect pests controlling are concerned, there are the other applications in medical as further describe. Basically, the α-amylase play a critical role in carbohydrate metabolism including starch and glycogen digestion, utilizing of α-amylase inhibitor as starch blocker in medical applications such as treatment of carbohydrate uptake disorder. several studies indicated these inhibitors can inhibit α-amylases leading to control post-prandial hyperglycemia, PPHG, which is the risk factor of non-insulin dependent diabetes mellitus (NIDDM/type II), in NIDDM patients (Notkin, 2002; Gin and Rigalleau, 2000). There are researches reported that traditional medicine plants, *S. cumini* seed and *P.guajava* leaves showed a dose-dependent inhibitory on α-amylase activity from porcine (Karthic, et al., 2008) *M. koenigii* and *O. tenuflorum*, Indian herb extracts have ability to decrease glucosidase (amylase) activity of porcine pancreatic α-amylase (Bhat, et al., 2008). Therefore, theα-amylase inhibitors are beneficial to manage and treatment of diabetes mellitus.

Furthermore, the commercial common bean, *P.vulgaris* α-amylase inhibitors has been used in treatment of obesity (Tormo, et al., 2004). The common bean αAI-1 is possible to be an extensive anti-obesity and anti-diabetes treatment (Wang, et al., 2011). Kataoka and Dimagno study found that wheat α-amylase inhibitors can decrease intake of foods and weight gain without mal-absorption (Kataoka and Dimagno, 1999).

Therefore, in the future, the knowledge of alpha amylase and its inhibitor will be beneficial to be a biological tool that resisted plant against insect and microbial pests. Moreover, it could be used in prevention or therapy of diabetes and obesity.

CHAPTER V

CONCLUSION

Cloning and analysis of nucleotide sequence of alpha-amylase inhibitor genes from Kaw Dok Mali 105 and Pathumthani 1 indica rice (*Oryza sativa*)

This study, we successfully cloned the full coding sequence of alpha-amylase inhibitor gene (α-AI) from both varieties of indica rice; Kaw Dok Mali 105 (KDML105) and Pathumthani 1(PT) Thai rice. The obtained nucleotide sequences were amplified using primer that design according to rice alpha-amylase/subtilisin from japonica rice. We obtained 4 clones; KDML105 and PT clone 1, 2 and 3, consist of 603 bp nucleotide sequences and 200 deduced amino acid sequences.

The obtained KDML105, PT clone 1 and clone 3 are identical to the full coding nucleotide sequence of japonica rice alpha-amylase/subtilisin inhibitor (Accession number: AY166458.1). Whereas, the obtained PT clone 2, only one amino acid change, is 99% identity to japonica rice alpha-amylase/subtilisin inhibitor.

The deduced amino acid was calculated to a molecular mass of 21,417 Dalton, and the N-terminus of its sequence contains a signal peptide (1-22) causing its predicted mature protein sequence to be 178 amino acids in length.

We deposit the nucleotide sequences of KDML105, PT clone 1 and 2 to GenBank database with accession number JX426136, KF219883 and KF219884, respectively.

Expression and biochemical characteristics of alpha amylase inhibitor gene from KDML105 indica rice

The KDML105 alpha-amylase inhibitor (α-AI) was constructed with pET17b(+) expression vector in *E.coli* strain BL21 (DE3) pLysS. The KDML105 α-AI was induced using 1mM IPTG at 37°C for 3 hours. The KDML105 α-AI expressed protein was analyzed by SDS-PAGE and detected by Coomassie blue staining. The result shows an approximately 22 kDa ofKDML105 α-AI expressed protein. The expected protein was identified using LC-MS/MS, the results show highly match to alpha amylase inhibitor from *Oryza sativa*.

For protein purification, the KDML105 α-AI recombinant proteins were purified using Gel filtration chromatography, Cat-ion exchange chromatography and 2 step combined purification. The result show CM-Sepharose Cat-ion chromatography is best purification among all step we have been purified. Therefore, the partial purified KDML105 α-AI from CM Sepharose Cat-ion purification was used for inhibition activity study.

Inhibition activity study, the KDML105 α-AI partial purified protein was incubated withα-amylase from human saliva and *Tribolium castaneum*, and was assay using 2-Chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3). The inhibition activity was compared to α-amylase and calculated to % inhibition. The results reveal that the KDML105 α-AI partial purified protein can be inhibited against α-amylase from human saliva and *Bacillus subtilis* with 68.82% and 83.52 % inhibition but no inhibitory effect toward red flour beetle *Tribolium castaneum* alpha-amylase.

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APPENDIX

APPENDIX A VECTOR FOR EXPERIMENT

Cloning vector

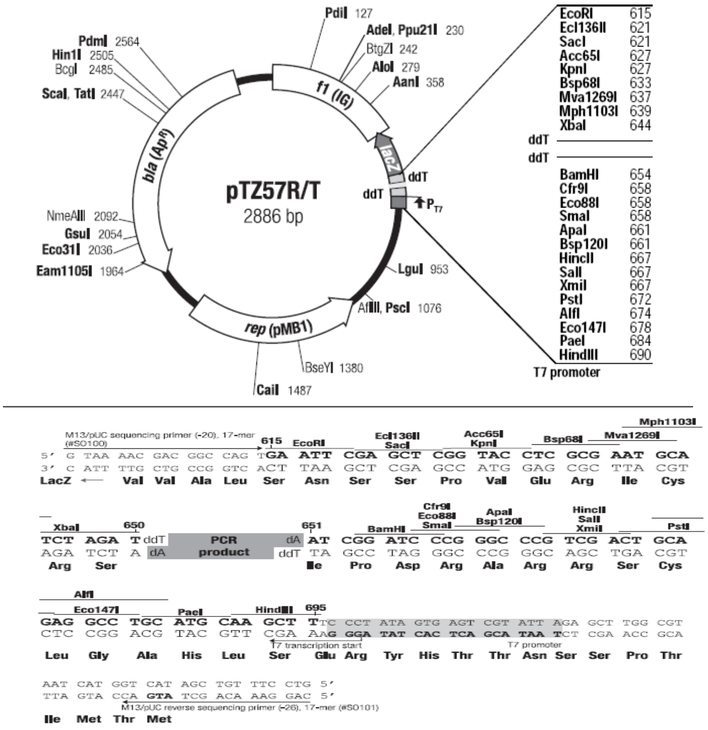

Figure 28 Map of the pT57R/T cloning vector and DND sequence of MCS region

Expression vector

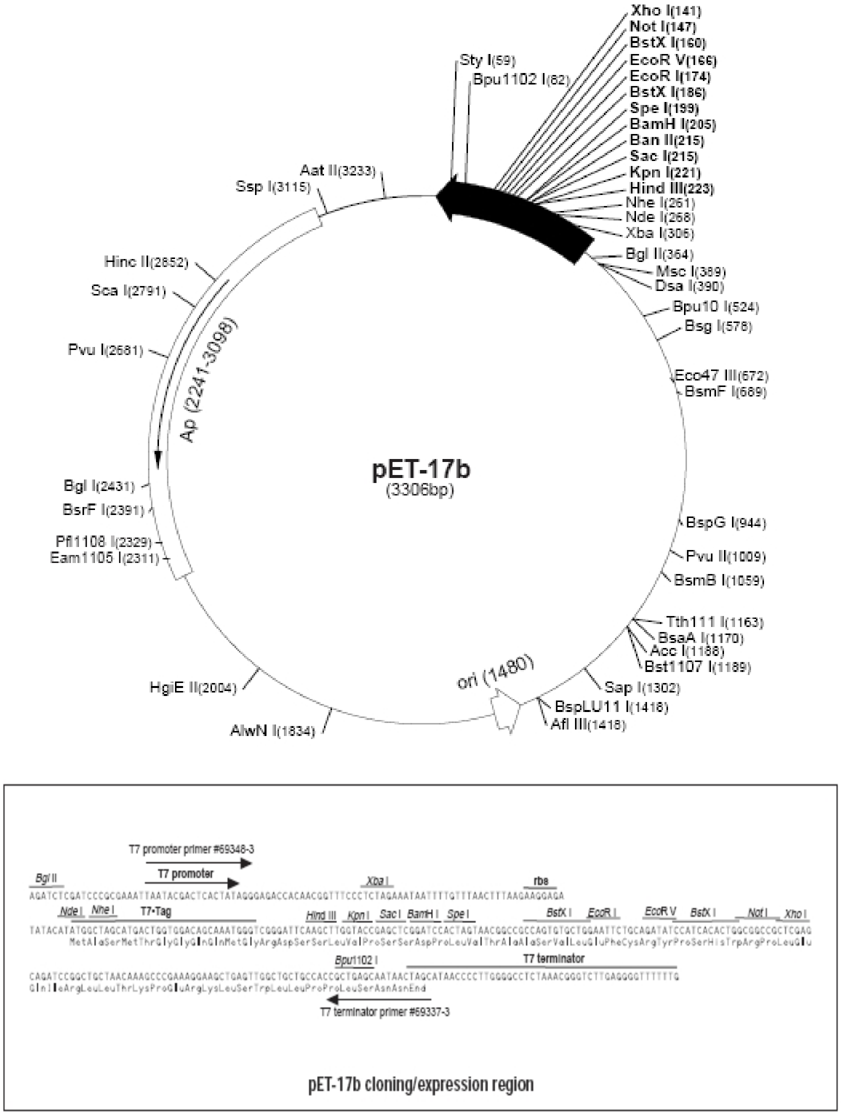

Figure 29 Map of the pET17b expression vector

APPENDIX B BACTERIAL STRAINS

Escherichia coli DH5α was used as host cells for amplification of recombinant plasmids (Madison, WI, USA)

Escherichia coli BL21(DE3)pLysS was used as host cells for expression (Novagen, USA)

Table 8 Strains Genotypes

Strain	Genotype
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA*-*argF*)U169, hsdR17(r_K^- m_K^+), λ–
BL21(DE3)pLysS	F^-*ompT hsdS*B(rB^- mB^-) *gal dcm* (DE3) pLysS (CamR)

APPENDIX C LIST OF SOLUTIONS AND BUFFER

10X TBE buffer solution (1000 ml)

a. 108 g Tris-base	0.89 M
b. 55 g Boric acid	0.89 M
c. 40 ml EDTA	20 mM
pH 8.3	

Ampicillin stock solution (50 ml)

a. 2.5 g ampicillin sodium salt in 50 ml H_2O	50 mg/ml

100 mM IPTG stock solution (50 ml)

a. 1.2 g IPTG (isopropyl-β-D-thiogalactopyranoside) in 50 mlH_2O

1.5 M Tris-HCl (pH 8.8), 100ml

a. 18.2 g Tris

b. Add HCl to pH 8.8

0.5 M Tris-HCl (pH 6.8), 100 ml

a. 6.0 g Tris

b. Add HCl to pH 6.8

10% SDS (w/v), 100 ml

a. 10 g SDS

10% Ammonium persulfate, 5 ml

a. 0.5g ammonium persulfate

Electrophoresis buffer for nondenaturing (1 liter)

a. 3.0 g Tris	25 mM
b. 14.4 g Glycine	192 mM

pH 8.8

Stock sample buffer (9.5 ml)

a. 1.2 ml 1 M Tris-HCl (pH 6.8)	0.06 M
b. 1 ml Glycerol	10%
c. 0.5 ml 0.5% bromophenol blue (W/V)	0.025%
d. 2 ml 10% SDS	2%
e. 4.8 ml H_2O	

Notice: store at room temperature, Adding 50 µl of 2-mercaptoethanol to 0.95 ml of stock sample buffer before use.

Electrophoresis buffer for denaturing (1 liter)

a. 3.0 g Tris	25 mM
b. 14.4 g Glycine	192 mM
c. 1 g SDS (W/V)	0.1%

Staining solution (500 ml)

a. 0.5 g Coomassie brilliant blue R-250	0.1%
b. 200 ml Methanol	40%
c. 50 ml Acetic acid	10%
d. 250 ml H_2O	

Destaining solution (500 ml)

a. 200 ml Methanol	40%
b. 50 ml Acetic acid	10%
c. 250 ml H_2O	

4% stacking gel (5 ml)

a. 0.5 ml 40% acrylamide

b. 1.25 ml 0.5 M Tris-HCl pH 6.8

c. 0.05 ml 10% ammonium persulfate

d. 0.05 ml 10% SDS

e. 0.005 ml TEMED

f. 3.1 ml H_2O

10% separating gel (10ml)

a. 2.5 ml 40% acrylamide
b. 2.5 ml 1.5 M Tris-HCl pH 8.8
c. 0.05 ml 10% ammonium persulfate
d. 0.1 ml 10% SDS
e. 0.005 ml TEMED
f. 4.75 ml H_2O

15% separating gel (10ml)

a. 3.75 ml 40% acrylamide
b. 2.5 ml 1.5 M Tris-HCl pH 8.8
c. 0.05 ml 10% ammonium persulfate
d. 0.1 ml 10% SDS
e. 0.005 ml TEMED
f. 3.5 ml H_2O

1X PBS (1 liter)

a. 8 g NaCl
b. 0.2 g KCl
c. 1.44 g Na2HPO4
d. 0.24 g KH2PO4
pH 7.4

Binding buffer for protein purification (500 ml)

a. 3.42 g sodium acetate	50 mM
b. 500 ml sterile H_2O	
pH 5.5	

Elution buffer for protein purification (500 ml)

a. 3.42 g sodium acetate	50 mM
b. 29.11 g sodium chloride	1 M
c. 500 ml sterile H_2O	
pH 5.5	

CNPG3 buffer (20 ml)

a. 1 ml 1M phosphate buffer	50 mM
b. 0.585 g NaCl	500 mM
c. 0.002 g$CaCl_2$	1 mM

Add ddH_2O until 20 ml

5X M9 salt (100 ml)

a. 3 g Na_2HPO_4
b. 1.5 g KH_2PO_4
c. 0.5 g NH_4Cl
d. 0.25 g $NaCl_2$

Add ddH_2O until 100 ml

Minimal media (100 ml)

a. 20 ml 5X M9 salt	
b. 0.2 ml $MgSO_4$	1 M
c. 2 ml glucose	20% (W/V)
d. 0.01 ml $CaCl_2$	1 M

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BIOGRAPHY

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