

## CHAPTER III

### RESEARCH METHODOLOGY

#### Materials

##### 1. Insect

Red flour beetles, *Tribolium castaneum*, were collected from the rice stored in Phitsanulok province and were identified by National biological control research center, Naresuan University.

##### 2. Equipment

- 2.1 Thermal cycle (Applied Biosystem Co., LTD., USA)
- 2.2 Horizontal gel electrophoresis (Sunrise Garment Co., LTD., USA)
- 2.3 Gel documentation system (Biolmaging Systems Co., LTD., England)
- 2.4 Vertical laminar flow cabinets (Faster, Italy)
- 2.5 Incubator (ShellLab Co., LTD., USA)
- 2.6 Incubator shaker (ZhichengInstrument,China)
- 2.7 Hot-air oven (Medcenter Einrichtung GmbH Co.,LTD., Germany)
- 2.8 Autoclave (Sanyo electric Co., LTD., Japan)
- 2.9 Microcentrifuge (Labnet International, Inc., USA)
- 2.10 Refrigerated Centrifuge (Tomy kogyo Co., LTD., Japan)
- 2.11 Balance (Mettler-Toledo International Inc. Switzerland)
- 2.12 Water bath (Julabo Labortechnik GMBH Co., LTD., Germany)
- 2.13 pH meter (Mettler-Toledo International Inc.,Switzerland)
- 2.14 Block Heater (Labnet International, Inc., USA)
- 2.15 UV/Vis Spectrophotometer (Beckman Coulter Co., LTD., USA)
- 2.16 Ultrasonic processor (Sonics & Materials Inc., USA)
- 2.17 Microwave oven (Sharp, Japan)
- 2.18 ÄKTAprime™ plus protein purification system
- 2.19 HiTrap™ Desalting (GE Healthcare, UK)
- 2.20 HisTrap FF (GE Healthcare, UK)

- 2.21 Synergy HT Multi-Mode Microplate Reader (Biotek, USA)
- 2.22 Beakers
- 2.23 Flasks
- 2.24 Cylinders
- 2.25 Duran bottles
- 2.26 Magnetic Bars
- 2.27 Petri dishes
- 2.28 Spreaders
- 2.29 Loop & Alcohol burner
- 2.30 Micro pipette, Pipette tips
- 2.31 Centrifuge Tube, Micro Centrifuge Tube, PCR tube

### **3. Chemicals**

#### **3.1 Insect maintenance**

- 3.1.1 Wheat flour
- 3.1.2 Yeast powder
- 3.1.3 Organic Thai Jasmine rice, Khao Dowk Mali 105 (KDML 105), powder (Moral rice project) ground by pestle and mortar

#### **3.2 RNA isolation**

- 3.2.1 Liquid nitrogen
- 3.2.2 RNeasy Mini Kit (Qiagen, Germany)
- 3.2.3 Trizol reagent (Invitrogen, USA)
- 3.2.4 Chloroform (BDH, England)
- 3.2.5 Isopropanol (TEDIA, USA)

#### **3.3 Complementary DNA (cDNA) synthesis**

- 3.3.1 RevertAid<sup>TM</sup> M-MuLV Reverse Transcriptase (200 U/ $\mu$ l) (Fermentas, USA)
- 3.3.2 RiboLock<sup>TM</sup> RNase Inhibitor (20 U/ $\mu$ l) (Fermentas, USA)
- 3.3.3 5X Reaction Buffer (Fermentas, USA)
- 3.3.4 Oligo(dT) Primer (Fermentas, USA)
- 3.3.5 DEPC-treated Water (Fermentas, USA)
- 3.3.6 DNase I (Invitrogen, USA)

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

- 3.4.1 10X PCR buffer
- 3.4.2 50 mM MgCl<sub>2</sub>
- 3.4.3 Taq DNA polymerase 5 U/μl
- 3.4.4 10 mM dNTP mix

**3.5 Agarose gel electrophoresis**

- 3.5.1 Tris base (Usb, USA)
- 3.5.2 Boric acid (Usb, USA)
- 3.5.3 Ethylenediamine tetra acetic acid (EDTA) (BIO-RAD, USA)
- 3.5.4 Agarose (Usb, USA)
- 3.5.5 Ethidium bromide (BIO-RAD, USA)

**3.6 illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK)**

- 3.6.1 Capture buffer type 2
- 3.6.2 Wash buffer type 1
- 3.6.3 Elution buffer type 4
- 3.6.4 Elution buffer type 6

**3.7 InsTAclone™ PCR Cloning Kit (Fermentas, USA)**

- 3.7.1 Vector pTZ57/T, 55 ng/μl
- 3.7.2 5X Ligation Buffer
- 3.7.3 T4 DNA Ligase, 5 U/μl
- 3.7.4 C-medium
- 3.7.5 T solution (A)
- 3.7.6 T solution (B)
- 3.7.7 Water, nuclease-free

**3.8 LB broth and LB agar**

- 3.8.1 Agar powder (Himedia Laboratories Pvt. Ltd., India)
- 3.8.2 Difco™ LB broth, Lennox (Becton, Dickinson and Company, USA)

**3.9 Preparation of competent cell**

- 3.9.1 Sodium acetate trihydrate (Riedel-de Haen, Germany)
- 3.9.2 Manganese chloride (Sigma, USA)

3.9.3 Sodium chloride (Ajax Finechem Pty Ltd., New Zealand)

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

3.9.5 Glycerol (MERCK, Germany)

### **3.10 Restriction enzyme**

3.10.1 BamHI / XhoI (Fermentas, USA)

### **3.11 DNA ladder**

3.11.1 100 bp Plus DNA ladder (Fermentas, USA)

3.11.2 200 bp DNA ladder (Bio basic inc., Canada)

3.11.3  $\lambda$ DNA/HindIII (Fermentas, USA)

3.11.4 VC 100 bp Plus DNA ladder (Vivantis, Malaysia)

### **3.12 Cloning & Protein expression**

3.12.1 pET 32a(+) plasmid vector (Novagen, Germany)

3.12.2 Isopropyl  $\beta$ -D-thiogalactopyranoside (Sigma, USA)

3.12.3 BL21(DE3) bacterial cells (Novagen, Germany)

### **3.13 Protein gel electrophoresis**

3.13.1 Glycine (BIO-RAD, USA)

3.13.2 Tris base (Usb, USA)

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

3.13.4 Bromophenol blue (BIO-RAD, USA)

3.13.5 2-Mercaptoethanol (BIO-RAD, USA)

3.13.6 Glycerol (MERCK, Germany)

3.13.7 40% Acrylamide/Bis Solution 19:1 (BIO-RAD, USA)

3.13.8 Tetramethylethylenediamine (TEMED) (Fisher scientific, UK)

3.13.9 Ammonium persulfate (Sigma, USA)

3.13.10 Coomassie Brilliant Blue G-250 (BIO-RAD, USA)

3.13.11 Methanol (LAB-SCAN, Ireland)

3.13.12 Acetic Acid (Merck, Germany)

### **3.14 Protein determination**

3.14.1 Quick Start™ Bradford Protein Assay (BIO-RAD, USA)

### **3.15 Protein purification**

3.15.1 Sodium phosphate (Sigma, USA)

3.15.2 Sodium chloride (Ajax Finechem Pty Ltd., New Zealand)

3.15.3 Imidazole (Sigma, USA)

3.15.4 Protease Inhibitor cocktail (sigma, USA)

### **3.16 Enzyme activity assay**

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

3.16.2 Sodium phosphate (Sigma, USA)

3.16.3 Sodium chloride (Ajax Finechem Pty Ltd., New Zealand)

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

3.16.5 Iodine (Sigma, USA)

3.16.6 Potassium iodide (Sigma, USA)

3.16.7 Iodine (Merck, Germany)

3.16.8 3,5-Dinitrosalicylic acid (Sigma, USA)

3.16.9 Sodium hydroxide (Ajax Finechem Pty Ltd., New Zealand)

3.16.10 Potassium sodium (+)-tartrate (Riedel-de Haen, Germany)

3.16.11 2-Chloro-4-nitrophenyl- $\alpha$ -D-maltotriose substrate  
(Sigma, USA)

## **Methods**

### **1. Sample preparation**

#### **Insect**

Beetles were reared on 95% wheat flour with 5% yeast powder in a plastic box covered with cheesecloth for ventilation. The culture was maintained in laboratory at  $28 \pm 2$  °C for 50-60 days. Only adults were used for this experiment.

#### **Effect of $\alpha$ -amylase gene expression on different starches**

Two hundred grams of wheat flour supplemented with 5% yeast powder and 200 g of Jasmine rice (KDML 105) powder were placed separately in each plastic box. Two hundred adult red flour beetles were added into each box covered with cheesecloth for ventilation. The culture was maintained in laboratory at  $28 \pm 2$  °C. Thirty milligrams of adult *Tribolium castaneum* were collected at week 1, 2, 3, 4, 6 and 8 for RNA extraction and cDNA synthesis

## **2. Cloning of *Tribolium castaneum* $\alpha$ -amylase gene**

### **Total RNA isolation and cDNA synthesis**

Samples of 30 mg of adult beetles were homogenized in liquid nitrogen by a mortar and pestle. Subsequently, the homogenized samples were lysed by RLT buffer (Qiagen) supplemented with 1% 2-mercaptoethanol. The total RNA was extracted using the QIA shredder spin-column and the RNeasy Mini Kit (Qiagen) prior to being treated with Dnase I according to the manufacture's instruction. Elution was performed with 50  $\mu$ l nuclease-free water. First-strand cDNA was synthesized using complementary DNA (cDNA) synthesis kit (Fermentas). Firstly, 1  $\mu$ g total RNA and 1  $\mu$ l Oligo (dt18) primer was incubated at 65 °C for 5 min. Then, the sample was added with the following components: 4  $\mu$ l 5X reaction buffer, 1  $\mu$ l of 20 units Ribolock Ribonuclease Inhibitor, 2  $\mu$ l of 10 mM dNTP mix and 1  $\mu$ l 200 units RevertAid M-MuLv Reverse transcriptase. Finally, the reaction mixture was incubated at 42 °C for 60 min prior to heating at 70 °C for 5 min to terminate the reaction. The reverse transcription reaction product was directly used in PCR amplification.

### **Amplification and cloning of *Tribolium castaneum* $\alpha$ -amylase gene**

The specific primers were designed based on *Tribolium castaneum*  $\alpha$ -amylase mRNA sequence (NCBI Reference Sequence: NM\_001114376.1). The forward primer contained *Bam*HI restriction site and the reverse primer contained *Xho*I restriction site. The insect  $\beta$ -actin gene primers were designed based on known sequences (Table 1). The PCR reaction mixture was composed of 0.5  $\mu$ l first-strand cDNA, 0.5  $\mu$ l of 10 pmol primer as a putative  $\alpha$ -amylase and insects  $\beta$ -actin (internal control), 1  $\mu$ l of 10X PCR buffer, 0.2  $\mu$ l of 10 mM dNTPs, 0.4  $\mu$ l of 50 mM MgCl<sub>2</sub>, 0.1  $\mu$ l of 5 U/ $\mu$ l Taq DNA Polymerase and distilled water to make the volume up to 10  $\mu$ l. *Tribolium castaneum*  $\alpha$ -amylase gene and insect  $\beta$ -actin gene were amplified by the following condition: 94 °C for 5 min; 30 cycles at 94 °C for 30 s; 54 °C for 30 s; 72 °C for 1 min; and 72 °C for 10 min. The PCR products were visualized by agarose gel electrophoresis. After electrophoresis, the gel was stained with 10  $\mu$ g/ml of ethidium bromide solution for 10 min and destained with distilled water. The band in the gel was visualized by Gel documentation system (BioImaging Systems Co., LTD., England).

**Table 1 Oligonucleotide primers used for DNA amplification of *Tribolium castaneum*  $\alpha$ -amylase gene and insect  $\beta$ -actin gene actin**

<i>Primer name</i>	<i>Direction</i>	<i>Sequence(5'→ 3')</i>
$\beta$ -actin primer	Forward	GTTCCCATCCATCGTAGGTCG
$\beta$ -actin primer	Reverse	GCAGAGCGTAACCTTCGTAGAT
TcasA primer	Forward	<u>GGATCC</u> ATGCATTTCAAACCCATCCTCG
TcasA primer	Reverse	<u>CTCGAGCA</u> ATTTGGCATT

**Note:** the underlines were the forward primer contained *Bam*HI and the reverse primer contained *Xho*I restriction site

#### **Purification of PCR product from agarose gels**

$\alpha$ -Amylase cDNAs were isolated from agarose gel by illustra™ GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare). PCR products were cut out of the gel and transferred into 1.5 ml microcentrifuge tubes. The sample was dissolved with 400  $\mu$ l capture buffer type 2 and incubated at 50 °C until the gel was completely dissolved. Then, 600  $\mu$ l of sample mixture was transferred to the column and incubated at room temperature for 1 min. After centrifugation at 12,000 x g for 1 min., sample was washed with 600  $\mu$ l of wash buffer type 1 and centrifuged again at 12,000 x g for 1 min. The flow-through was discarded and centrifuged again to dry the column matrix. Finally, the purified DNA was eluted with 40  $\mu$ l of sterile water. The resulting purified DNA was determined by measuring the absorbance at 260 nm.

#### **cDNA Cloning and Bacterial Culture transformation**

The  $\alpha$ -amylase purified cDNA was ligated using InsTAclone™ PCR Cloning Kit (Fermentas, USA) into pTZ57R/T plasmid vector according to the manufacture's instruction. The ligation reaction contained 3  $\mu$ l of pTZ57R/T plasmid vector, 6  $\mu$ l of 5X ligation buffer, 3  $\mu$ l of purified DNA, 1  $\mu$ l of T4 DNA ligase and 17  $\mu$ l of sterile water. The reaction mixture was incubated overnight at 4 °C. Competent *E. coli* (DH5 $\alpha$ ) cells were prepared by inoculating 2 ml of C-medium with a single bacterial colony and incubated the culture overnight at 37 °C in an incubator shaker. Subsequently, inoculated 1.5 ml of pre-warmed C-medium with 150  $\mu$ l of

the overnight bacterial culture and incubated at 37 °C for 20 min. Bacterial cells was then separated by 1 min centrifugation and discarded the supernatant. The pellet was re- suspended with 300 µl 1:1 T-solution (A) and (B), incubated 5 min on ice. The cells were centrifuged for 1 min at maximum speed and discarded the supernatant. The pellet was dissolved with 120 µl 1:1 T-solution (A) and (B), incubated 5 min on ice. Fifty µl of competent cells were added with 2.5 µl of ligation mixture, mixed and incubated 5 min on ice. Finally, the mixture was plated immediately on 100 µl/ml LB-ampicillin, 40 µg/ml X-Gal agar plates supplemented with 0.2 mM IPTG and overnight incubated at 37 °C.

### **Positive recombinant clones screening**

#### **Colony PCR screening**

White single colonies were picked by micropipette tip and mixed in PCR reaction mixture. *Tribolium castaneum*  $\alpha$ -amylase gene was amplified by the same condition as prior described.

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

Five ml of LB broth with 100 µg/ml of ampicillin was inoculated with a white colony and grown overnight at 37°C. Then, the plasmid DNA was isolated using GF-1 plasmid DNA extraction kit (Vivantis, Malaysia) following the supplier's protocols. The cells were collected by centrifugation at 6,000 x g for 2 min. The cell pellets were re-suspended in 250 µl of S1 buffer supplemented with RNase A, followed by adding 250 µl of S2 buffer. The mixture was gently mixed by inverting the tube several times. The lysate was neutralized by 400 µl of buffer NB and gently mixed by inverting the tube until a white precipitate occurred. The precipitate was separated by centrifugation at 14,000 x g for 10 min at room temperature. The supernatant was transferred to column, followed by centrifugation at 10,000 x g for 1 min. The plasmid solution was packed into column and it was washed with 600 µl of wash buffer. Plasmid was eluted by adding 80 µl of sterile water and centrifuged at 10,000 xg for 1 min. After isolation of the plasmid from recombinant clones, their nucleotide sequences were determined.

#### **Restriction analysis**

The purified plasmid DNA was confirmed by restriction enzyme digestion. The purified plasmid DNA was digested using restriction enzyme according to

the manufacture's recommendation. The reaction mixture contained 1 µg of purified plasmid DNA, 1 µl of 10X buffer, 10 U/µl of restriction enzyme (*Bam*HI and *Xho*I) and adjusted volume to 10 µl by sterile water. After incubation at 37 °C for 2 hr, the products of restriction enzyme digestion were analyzed by agarose gel electrophoresis.

### **Sequence analysis of *Tribolium castaneum* α-amylase gene**

The α-amylase nucleotide sequences from *Tribolium castaneum* were obtained with nucleotide blast in BLAST program of National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignment was examined using CLUSTALW multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). The nucleotide sequence was translated into the amino acid sequence using ExpASy translate tool (<http://web.expasy.org/translate/>).

### **3. Expression & Purification of *Tribolium castaneum* α-amylase**

#### **Construction of pET 32a(+)-*Tribolium castaneum* α-amylase gene**

The primers were designed with the corresponding restriction enzyme sites of *Bam*HI and *Xho*I at the N-terminus and C-terminus for the *Tribolium castaneum* α-amylase gene, respectively (Table 1) to clone the coding sequence into the expression vector, pET 32a (+) plasmid vector (Novagen, Germany). The expression vector (pET 32a (+)) and the recombinant plasmid (pTZ57R/T-TcasA) were digested with *Bam*HI and *Xho*I restriction enzyme. The expression vector and the DNA fragment were purified by a 1% agarose gel using illustra™ GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare). The ligation was carried out at 4 °C overnight with 1:3 ratio of pET 32a (+) plasmid vector, pTZ57R/T-TcasA recombinant plasmid, 1 µL of 10X ligation buffer and 0.5 µL 1X T4 DNA ligase (Fermentas, USA). The ligated product was transformed into BL21 (DE3) competent cells (Novagen, Germany). The correct recombinant (confirmed by restriction enzyme digestion and sequencing) was used in *Tribolium castaneum* α-amylase expression

#### **Over-expression and purification of recombinant protein**

The recombinant TcasA was over-expressed in *E. coli* BL21 (DE3) cells in the presence of isopropyl-β-thiogalactopyranoside (IPTG). Briefly, *E. coli* starter culture was grown in LB medium containing 100 µg/ml of ampicillin at the 37°C

overnight, 1% of an *E. coli* starter culture was inoculated into similar medium with starter culture. The culture was incubated at 37 °C until the cell count reached 0.4-0.8 at 600 nm optical density. The culture was induced by IPTG at 1 mM final concentration and then incubated for 1 hr at 37 °C. The cells were cooled on ice for 30 min, and harvested by centrifugation at 6000 ×g for 20 min at 4 °C, followed by removal of the medium and washed the cells pellet with PBS buffer 2 times. The cells were re-suspended in 10 ml binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.4) supplemented with 1X protease inhibitor cocktail (sigma, USA). The bacterial suspension was analyzed for expression by SDS-PAGE. The suspensions were ruptured by pulse sonication on ice at 30 sec, 5 times. The cell lysates were centrifuged at 6000 x g. Then the supernatant was transferred to a fresh tube prior to the filtration using 0.45 µm filter. The cell extract was purified with Histidine-tagged protein purification gradient elution program on an ÄKTAprime plus system (GE Healthcare, UK), the affinity chromatography using HisTrap FF column (GE Healthcare, UK). The column was eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, and 250 mM imidazole, pH 7.4). The fraction protein was collected and analyzed by SDS-PAGE. The protein band from SDS-PAGE was excised and analyzed with LC/MS by Proteomics Research Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC). The LC/MS data were submitted to database search using MASCOT (<http://www.matrixscience.com>). The data was searched against the NCBI database for protein identification.

#### **Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) for alpha-amylase**

The recombinant alpha-amylase protein from red beetle flour was determined by SDS-PAGE. Protein samples (~10 µg) were mixed with equal volumes of sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue), adding by 50 µl of 2-mercaptoethanol in each 0.95 ml of sample buffer, and boiled at 95°C for 5 min to denature the protein. The samples were analyzed on 4% stacking gel and 10% separating gel for 2 hr at 25 mA. Protein bands were visualized by staining in 0.1% (w/v) Coomassie brilliant blue R-250 solution,

50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by several washes with destain solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

#### **4. Measurement of *Tribolium castaneum* alpha-amylase activity**

##### **Protein desalting by desalting column**

The cell extract was loaded in sample tubing and ran with HiTrap Desalting program on an ÄKTApriime plus system (GE Healthcare, UK) using HiTrap™ Desalting column (GE Healthcare, UK). The column was eluted with PBS buffer. The pooled fraction protein was collected and analyzed by SDS-PAGE.

##### **Protein determination**

Protein concentration was determined by the method of Bradford using BSA as a protein standard (Bio-Rad, Germany). The concentration of BSA standard was varied at 1, 0.75, 0.5, 0.25, 0.125 and 0 mg ml<sup>-1</sup>. The protein sample (5 µl) was mixed with 1X dye reagent (250 µl) and then incubated for 15 min at room temperature. Subsequently, the mixture was measured the absorbance at 595 nm by using microplate reader (Biotek, USA).

##### **Non-denaturing polyacrylamide gel electrophoresis (Native PAGE) and zymogram analysis**

To detect the  $\alpha$ -amylase activity in a gel, native gel electrophoresis system was employed. From the standard SDS-PAGE mentioned above, the Non-denaturing polyacrylamide gel electrophoresis was performed at 4 °C by using two 10% separating gel and 4% stacking gel. The gels, sample buffer and electrophoresis solutions were prepared without SDS and reducing agent. After electrophoresis, the first gel was stained with Coomassie brilliant blue R-250 solution and destained with destain solution. The second gel was soaked in 2% triton 100 for 45 min and incubated at 37 °C for 2 hr in 1% soluble starch solution in 50 mM sodium acetate buffer pH 5.5 containing 20 mM NaCl and 2 mM CaCl<sub>2</sub>. After quick rinsing with the same buffer without starch, the gel was stained with 3% KI, 1.3% I<sub>2</sub> solution for 15 min and washed by distilled water for 5 min, then  $\alpha$ -amylase activity can be visualized as cleared bands with dark background on KI/I<sub>2</sub> solution.

##### **2-Chloro-4-nitrophenyl- $\alpha$ -D-maltotrioside substrate**

$\alpha$ -Amylase hydrolyzes the 2-chloro-p-nitrophenyl- $\alpha$ -D-maltotrioside (CNPG3) to release 2-chloro-nitrophenol and form 2-chloro-p-nitrophenyl- $\alpha$ -D-

maltoside (CNPG2), maltotriose (G3) and glucose (G). The rate of increase in absorbance was measured at 405 nm and was proportional to the  $\alpha$ -amylase activity in the sample. To measure the recombinant *Tribolium castaneum*  $\alpha$ -amylase, CNPG3 was dissolved in 20 mM sodium phosphate, 500 mM NaCl and 1mM CaCl<sub>2</sub> to make up 100 mM final concentration of stock solution. Fifty  $\mu$ l of  $\alpha$ -Amylase were added in microtiter plate and 250  $\mu$ l of 2.25 mM CNPG3 working solution. The sample was measured at 37 °C. All measurements were performed using a Hitachi 717 analyzer (Boehringer Mannheim, Germany) at 405 nm.

#### **Measurement of *Tribolium castaneum* alpha-amylase activity**

The *Tribolium castaneum* amylase activity was determined using modified of F.-Javier Gella, et al. (1996) and Klaus Lorentz, et al. (1999). Fifty micro liters of  $\alpha$ -amylase were added in microtiter plate and 250  $\mu$ l of 2.25 mM CNPG3 working solution. A blank (CNPG3 without enzyme) was run simultaneously with the reaction mixture. The sample was measured immediately at 50 °C, 30 min runtime and 1 min interval. The molar absorption coefficient of 2-chloro-4-nitrophenol used for calculations was 1549 m<sup>2</sup>/mol. The catalytic concentration is calculated by

$$A = \epsilon bc$$

#### **Effect of temperature, pH, plant inhibitors, metal ions and reagents**

Temperature dependence on  $\alpha$  amylase activity was determined at different temperatures from 20-70 °C over a 30 min incubation period. The effect of pH on  $\alpha$ -amylase was determined from pH 4-9 and performed 50 °C for 30 min. The effect of metal ions and reagents on  $\alpha$ -amylase activity were measured at pH 7 and 50 °C in present of 10 mM of each reagent in final volume such as CaCl<sub>2</sub>, NaCl, KCl, MgCl<sub>2</sub>, MnCl<sub>2</sub>, EDTA imidazole and 50  $\mu$ g of KDML 105 inhibitor separately. The thermal stability of  $\alpha$ -amylase was obtained by pre-incubating  $\alpha$ -amylase samples in 1X PBS buffer (pH 7.0) at 30, 40, 50, 60 and 70 °C for 2 h, and measuring residual activity. All experiment effect on *Tribolium castaneum*  $\alpha$ -amylase was determined using CNPG3 assay (described above). Data were compared by one-way ANOVA before the Duncan multiple range tests was used. The significant differences were at P value  $\leq$  0.05.