CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Larvae of the bamboo borer, *Omphisa fuscidentalis* were collected from bamboo forests in Amphur Maewang, Chiang Mai Province, Thailand, and kept in continuous darkness at 25°C in covered plastic containers (12 × 14 × 8 cm) lined with wet paper towels (Singtripop et al., 1999).

Larvae of the silkworm, *Bombyx mori* (Kinshu x Showa), were reared on an artificial diet (Silkmate 2M, Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12h light: 12h dark regime (Sakurai, 1983; Sakurai, 1984). The stage of larvae was determined according to Sakurai et al. (1998).

2.2 Hormones

JHA (S-methoprene; SDS Biotech, Tokyo, Japan) was dissolved in acetone at a concentration of 50 mg/ml and stored at –35°C as a stock solution. The stock solution was diluted with acetone, and a 5-µl aliquot was applied topically to the dorsal surface of individual larvae (Singtripop et al., 2002). The 20E (Sigma, St. Louis, MO, USA) was dissolved in distilled water at a concentration of 1 mg/ml and stored at –20°C until use. The stock solution was diluted with distilled water, and larvae were injected with 5-µl aliquots through the first proleg (Singtripop et al., 2000).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.3 Trehalase activity assay

Larvae were kept on ice and the midguts were excised in cold insect Ringer's solution. The midguts were cut longitudinally and washed thoroughly with cold Ringer's solution. After blotting with filter paper, the midguts were homogenized in cold 20 mM phosphate buffer (PB; pH 6.0) (Ultraturrax T25 homogenizer; IKA Laboratory Technology, Staufen, Germany), followed by sonication for 30 s. The homogenates were then centrifuged at 105,000 x *g* at 4°C for 60 min (L8-80M refrigerated ultracentrifuge; Beckman Instruments, Palo Alto, CA, USA). The resulting supernatant and precipitate were regarded as the fractions containing soluble and membrane-bound trehalase, respectively. The supernatant was subjected to a trehalase activity assay, while the precipitate was suspended in PB (pH 6.0) for measurement of trehalase activity. The amount of protein in each sample was determined prior to the trehalase assay using a protein-dye binding method (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. For the trehalase activity assay, the reaction mixture (250 µl) consisted of 62.5 µl of 40 mM trehalose (Sigma) in 20 mM PB (pH 6.0), 50 µl of the soluble or membrane-bound trehalase fraction, and 137.5 µl of PB. The mixture was incubated at 37°C for 60 min, and the reaction was stopped by heating in boiling water for 5 min. Coagulated protein was removed by centrifugation at 12,000 x *g* for 10 min at 4°C, and an aliquot of the resulting supernatant was used to measure the amount of glucose by a modified version of the hexokinase-glucose-6-phosphate dehydrogenase method (Bergmeyer et al., 1974; Knuesel et al., 1998). The reaction was performed in a total volume of 1 ml containing 50 U of hexokinase, 100 U of glucose-6-phosphate dehydrogenase, 2 mM NADP, and 2.8 mM ATP (Roche Diagnostics GmbH, Mannheim, Germany).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Trehalase activity was determined with reference to a calibration curve constructed using standard glucose (Sigma); the results are expressed as nmol glucose/μg protein/min.

2.4 In vitro incubation of the midgut

Larvae were anesthetized with diethyl ether and their midguts were exposed by cutting the epidermis along the dorsal vessel. The midguts were then ligated tightly with cotton thread at the anterior and posterior ends, and cut anterior and posterior to the ligatures. After rinsing three times with PB, the isolated midguts were incubated individually in 250 μl of PB containing 10 mM trehalose (pH 6.0) at 25°C. After incubation, the medium was heated in boiling water for 5 min, and the amounts of glucose and trehalose were determined using gas–liquid chromatography (GLC) according to Oda et al. (1997).

2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the midguts using the acid guanidinium thiocyanate–phenol–chloroform (AGPC) method (Chomczynski and Sacchi, 1987); 200 ng of the RNA was used to generate first strand cDNA. Prior to cDNA synthesis, the RNA was treated with RNase-free DNaseI (Fermentas, Burlington, Ontario, Canada) to eliminate contaminating DNA. Reverse transcription was conducted using an oligo $(dT)_{12–18}$ primer and M-MuLV-Reverse Transcriptase (Fermentas). Degenerate primers were designed based on conserved regions found in the trehalases of other insect species (*B. mori Treh-1*, GenBank accession no. **D13763** and *Treh-2*, **AB162717**; *T. molitor* **D11338**; *Drosophila melanogaster* **Q9W2M2**; *Anopheles*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

gambiae trehalase (**XP_320472**); *Aedes aegypti* trehalase (**Q16V81**); *Tribolium castaneum Treh-2* (**XM_968859**). Conserved sequences were estimated from primary sequence alignments produced using ClustalW (http://www.ebi.ac.uk/ clustalw/ index. html). The first round of PCR was performed using the following degenerate primer sets (Figs. 10A and B): for *OfTreh-1*, Ot1F (5′-TGGGAYTTYTCNCANCGNTGGT T-3′) and Ot1R (5′-NACNCCRTTNGACCANCCRAANCC-3′); for *OfTreh-2*, Ot2F (5′-GAYCTNGGNCGNAARATGAAR-3′) and Ot2R (5′-NACNCCRTTNGA CCANCCRAANCC-3′). The thermal cycling conditions were as follows: one cycle for 2 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 52°C (*OfTreh-1*) or 50°C (*OfTreh-2*), and 40 s at 72°C, and then a final extension at 72°C for 5 min.

2.6 Rapid amplification of cDNA ends (5'- and 3'-RACE)

Poly (A)-rich RNA was subjected to 5′- and 3′-RACE using a SMART^{TM} RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). For *OfTreh-1*, two reverse primers, 5-Tre1R1 (5′-AGGCGGCCAGGCGTT AGGGAAGTCC-3′) and 5-Tre1R2 (5′-GCTGCGCTAGGTAAGCCCAGTGGGAA CC-3′), were used for 5′-RACE and two forward primers, 3-Tre1F1 (5′-AGCAGTG GGACTTCCCTAACGCCTGG-3′) and 3-Tre1F2 (5′-ACGCGGAAGTTCCTGGC AAGTTCGGTG -3′), were used for 3′-RACE. For *OfTreh-2*, two reverse primers, 5-Tre2R1 (5′-TGCTCGCCTGTGTGCTCGTATGTGC-3′) and 5-Tre2R2 (5′-ACCA GACACCAACGTCTTCGTGCCAGAG-3′), were used for 5′-RACE and two forward primers, 3-Tre2F1 (5′-TCCTCTGGCACGAAGACGTTGGTGTCTG-3′) and

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

3-Tre2R2 (5′-TACCACTGTGGACAGGCTGCTACGACC-3′), were used for 3′-RACE.

2.7 Cloning

The PCR products were separated by electrophoresis on 1.2% agarose gels, and the cDNA fragments of interest were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified DNA fragments were cloned into pTZ57R/T using an InsTAclone™ PCR Cloning Kit (Fermentas), and the cloned plasmids were purified using a QIAGEN Miniprep Kit (Qiagen). The DNA was sequenced using an ABI PRISM® BigDye® Terminator (version 3.1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyzer; Applied Biosystems).

2.8 Sequence analysis

FASTA was used to search the sequence database of the European Bioinformatics Institute (EMBL-EBI) for proteins with primary sequence similarity to OfTreh-1 and OfTreh-2. A multiple sequence alignment of OfTreh-1, OfTreh-2, and other trehalases was constructed using ClustalW (http://www.ebi.ac.uk/clustalw/index.html). A phylogenetic tree was constructed by the neighbor-joining (NJ) method using the CLC free workbench (version 4.0.1) (CLC Bio; www.clcbio.com) program. *Escherichia coli* periplasmic trehalase (TreA) (Tanaka et al., 1999) was used as the outgroup, and the stability of the tree was assessed via bootstrapping with over 1000 replicates.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.9 Quantitative real-time PCR

Total RNA was isolated from midguts by the AGPC method (Chomczynski and Sacchi, 1987) then reverse transcribed (200 ng) using oligo $(dT)_{12-18}$ and M-MuLV-Reverse Transcriptase (Fermentas). The primers used were as follows: for *OfTreh-1*, OfT1-Q forward (5′-TCGACCGTCATATGCTCCAA-3′) and OfT1-Q reverse (5′-TCGTGTGGTGTCCAGTTTTCC-3′); for *OfTreh-1*, OfT2-Q forward (5′-TGGATCATCAAAGGGCTGCT-3′) and OfT2-Q reverse (5′-ATCAGGAGCGGTG GTTGAGA-3); and for *OfRpL3* (*O. fuscidentalis* ribosomal protein L3, GenBank accession no. **EF453378**), OfRpL3-Q forward (5′-TCTACCCCAAGAAGAGGTCTC G-3′) and OfRpL3-Q reverse (5′-ACGACAGTCCTCAGACATGTGC-3′). *OfRpL3* was used as an endogenous control. Recombinant plasmid DNAs containing individual RT-PCR products were purified for preparation of the absolute standards; the concentrations of the plasmids were determined spectrophotometrically at 260 nm. Serial dilutions were prepared at a final concentration of 10^2 to 10^8 copies for *OfTreh-1* and *OfTreh-2* or 10^2 to 10^6 copies for *OfRpL3* per 25 μl of reaction mixture to generate external standard curves. Quantitative real-time PCR was conducted using a QuantiTect SYBR Green PCR Kit (Qiagen) and a Chromo4™Real-Time Detector (Bio-Rad). Each reaction was run in triplicate and contained 2 μl of cDNA template and 0.3 μM primers in a final volume of 25 μl. The cycling parameters were 95°C for 15 min to activate the polymerase, followed by 45 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed to ensure that only a single product was amplified. Transcription of the trehalase genes was normalized to

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

the mRNA level of *OfRpL3*. The data were analyzed using Opticon Monitor™ software version 3.1 (Bio-Rad).

2.10 Changes in concentration of the inhibitor in *O. fuscidentalis* hemolymph

Diapausing larvae were treated with 0.1µg JHA or 1µg 20E, hemolymph samples were collected every 2 days after JHA application and every day after 20E injection for trehalase inhibitor assay. Hemolymph was collected into chilled 1.5-ml plastic tubes containing small crystals of phenylthiourea, and centrifuged at 10,000 x *g* for 5 min at 4°C to remove hemocytes. Hemolymph (137.5 µl) was incubated with 50 µl of the midgut homogenates at 37°C for 5 min. The mixture was incubated with 62.5 µl of 40 mM trehalose at 37°C for 60 min, and the reaction was stopped by heating in boiling water for 5 min. The mixture was centrifuged at 12,000 x *g* for 10 min at 4°C, and an aliquot of the resulting supernatant was used for assessing glucose amounts as described in Materials and Methods. The amounts of glucose in the hemolymph samples were also determined in the same manner. The amount of the released glucose in each determination was then calculated by subtracting the amount of glucose in the hemolymph sample from that in the mixture after the reaction.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.11 Trehalase inhibitor extraction from *B. mori* hemolymph

Hemolymph was collected into a chilled 1.5 ml plastic tube containing a small amount of phenylthiourea powder, and centrifuged at 10,000 x *g* for 5 min at 4°C to remove hemocytes. For trehalase inhibitor-M, a methanol-extractable inhibitor, hemolymph (200 µl) was diluted with distilled water (400 µl), boiled for 10 min, chilled on ice and centrifuged at 15,000 x *g* for 10 min. The resulting supernatant was

mixed with 4 volumes of ice-cold methanol, kept at 4°C for 2 h and centrifuged at 15,000 x *g* for 10 min to remove precipitates. The supernatant was transferred to a new tube to which distilled water was added so as to give 80% methanol concentration, and then the same volume of hexane was added. After the tube was mixed vigorously and centrifuged briefly to separate the aqueous methanol and hexane layers, and the lower layer was transferred to a new tube. The solvent was evaporated using centrifugal evaporator. The resulting residue was dissolved with PB of the same volume as the volume of hemolymph, and used for inhibitor assay. For trehalase inhibitor-P, a proteinaceous inhibitor, hemolymph was dialyzed against 500 volumes of distilled water at 4°C for 24 h using dialysis tube with 14 kDa cut off (Nacalai tesque, Kyoto, Japan) with changes of the water two times during the dialysis. The turbid dialysate was centrifuged at 10,000 x *g* to yield the clear supernatant and used for inhibitor assay. The dialysate was concentrated for further experiments using ultrafiltration apparatus with 10 kDa cut off (Centricon YM10, Millipore, Bedford, MA, USA)

2.12 Trehalase inhibitor assay

Trehalase inhibitor solution (137.5 µl) was mixed with 10 µl of soluble or membrane-bound trehalase fraction and incubated at 37°C for 10 min. Then, 62.5 µl of 40 mM trehalose was added, adjusted the volume to 250 µl with PB and incubated at 37°C for 60 min. When hemolymph was used directly for assessing the inhibitory activity, 137.5 µl hemolymph was added to the reaction mixture for trehalase activity instead of trehalase inhibitor fraction. The reaction was stopped by heating in boiling

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

water for 5 min. The mixture was centrifuged at 12,000 x *g* for 10 min at 4°C, and an aliquot of the resulting supernatant was used for assessing glucose amounts as described in Materials and Methods. The amounts of glucose in the inhibitor extracts or hemolymph were determined in the same manner. The amount of the released glucose in each assay was then calculated by subtracting the amount of glucose in the inhibitor sample from that in the mixture after the reaction.

2.13 Treatment with trypsin

Inhibitor preparations (150 µl) were added with 10 µl trypsin in PB (10 mg/ml, Nacalai tesque). The solution was incubated for 5 h at 37°C and then the reaction was stopped by an addition of 10 µl of trypsin inhibitor (10 mg/ml, soybean trypsin inhibitor, Sigma). The trypsin-treated solution was centrifuged at 10,000 x *g* at 4°C for 5 min, and the resulting supernatant was used immediately for trehalase inhibitor assay.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved