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Characterization of putative soluble and membrane-bound trehalases in a hemipteran insect, *Nilaparvata lugens*

Jianhua Gu [a,1], Ying Shao [a,1], Chengwei Zhang [b], Zewen Liu [a,*], Yongjun Zhang [c,**]

[a] Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China
[b] The Service Center of Technology, Changzhou Entry-Exit Inspection and Quarantine Bureau, Changzhou 213022, China
[c] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

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ABSTRACT

Trehalose is the main blood sugar of insects, and the enzyme trehalase is involved in energy metabolism and controlling trehalose levels in cells. Two forms (soluble and membrane-bound) of trehalase and the corresponding genes (NlTre-1 and NlTre-2) were identified from the brown planthopper, *Nilaparvata lugens*. Both NlTre-1 and NlTre-2 contain trehalase signature motifs, and NlTre-2 contains a putative transmembrane domain. Comparison of trehalase activity and gene mRNA level at different developmental stages, or following application of 20-hydroxyecdysone (20E), suggests that NlTre-1 and NlTre-2 encode a soluble trehalase and a membrane-bound trehalase respectively. Soluble trehalase activity accounted for the majority of total trehalase activity in *N. lugens*. Only soluble trehalase activity and NlTre-1 mRNA level could be induced by 20E. Additionally, only soluble trehalase activity was significantly higher in macropterous individuals than in brachypterous morphs. These results indicate that only soluble trehalase is differentially expressed between macropterous and brachypterous individuals and is more responsive to hormone stimulus.

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1. Introduction

Trehalose is a non-reducing disaccharide consisting of two α-glycosidically linked glucose units. This disaccharide is found in many organisms as diverse as bacteria, yeast, fungi, nematodes, plants, insects and some other invertebrates, but is absent in mammals (Elbein et al., 2003; Tang et al., 2008). In these organisms, trehalose may serve as a source of energy, a carbohydrate store, or an agent for protecting proteins and cellular membranes from inactivation or denaturation caused by a variety of environmental stress conditions, including desiccation, dehydration, heat, cold and oxidation (Crowe et al., 1984; Garg et al., 2002; Elbein et al., 2003). In yeast and plants, it may also serve as a signaling molecule to direct or control certain metabolic pathways or even to affect growth (Elbein et al., 2003).

In insects, trehalose is the main blood sugar and is present in high concentration in the hemolymph (Wyatt, 1967; Becker et al., 1996; Thompson, 2003). Trehalose is the main reserve sugar in the hemolymph of flying insects and is also indispensable for thermotolerance in larvae (Wyatt, 1967; Thompson, 2003). Trehalose is synthesized mainly in the insect fat body and is rapidly released into the hemolymph (Becker et al., 1996; Thompson, 2003). In order to utilize blood trehalose, insect tissues possess an enzyme, trehalase (EC 3.2.1.28) that catalyses 1 mol of trehalose into 2 mol of glucose. Trehalase is believed to be located on the plasma membrane on the blood side, because the cell has to incorporate glucose from extracellular trehalose (Azuma and Yamashita, 1985a,b; Valaitis and Bowers, 1993; Yaginuma et al., 1996; Mitsumasu et al., 2005). Insects are believed to have two types of trehalase, soluble trehalase (Tre-1; Takiguchi et al., 1992) and membrane-bound trehalase (Tre-2; Mitsumasu et al., 2005). At present, two forms of trehalase have been cloned and characterized in several insect species, such as *Apis mellifera*, *Bombyx mori*, *Omphisa fuscidentalis*, *Spodoptera exigua* and *Tribolium castaneum* (Mitsumasu et al., 2005; Lee et al., 2007; Tang et al., 2008; Tatun et al., 2008). However, no information about trehalase genes or proteins from hemipteran insects has been obtained.

The brown planthopper (*Nilaparvata lugens*), a hemipteran insect, is a major rice pest in many parts of Asia. Wing polymorphism is known to be a common and ecologically important trait in this species. The long winged (macropterous) adults possess the ability of migrate over long-distances. Because trehalose is the main reserve sugar in the hemolymph of flying insects and trehalase is one of main

* Corresponding author. Tel.: +86 25 84399051; fax: +86 25 84399051.
** Corresponding author. Tel.: +86 10 62815929; fax: +86 10 62815929.
E-mail addresses: jemunson@njau.edu.cn (Z. Liu), yjzhang@ippcaas.cn (Y. Zhang).
[1] Both the authors contributed equally to this work.

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factors regulating trehalose levels in insect hemolymph (Tang et al., 2008), it is interesting to make a study on trehalases in *N. lugens* and to relate this to different morphs of this insect. In this report, we show the presence of two forms of trehalase, soluble and membrane-bound, in *N. lugens*, and we identify the genes that may encode each type. Developmental changes in two morphs of trehalase activity and developmental expression of two genes were compared. The regulation of trehalase activity and mRNA level by 20-hydroxyecdysone (20E), and differences in trehalase activity between macropterous and brachypterous insects have also been studied.

2. Material and methods

2.1. Insect culture and treatment with 20-hydroxyecdysone

Nilaparvata lugens was a laboratory strain obtained from China National Rice Research Institute in September 2001. Insects were kept indoors at 25 (±1) °C, 70–80% relative humidity under a 16/8 h light-dark cycle. Under laboratory conditions, the duration of *N. lugens* life cycle is about 30–35 days, in which the duration of last-larval (5th instar) stage is 3–4 days. 20-Hydroxyecdysone (20E) (Sigma, St. Louis, MO, USA) was dissolved in methanol at a concentration of 1 mg/ml and stored at −20 °C until use. The treatment method followed the micro-topical application technique reported by Nagata (1982) with some modification. Under carbon dioxide anesthesia, a droplet (0.1 μL) of 20E methanol solution was applied topically to the prothorax notum of 5th instar macropterous female nymphs or macropterous female adults (soon after ecdysis) with a hand microapplicator (Burkard Manufacturing Co. Ltd., Rickmansworth, UK). Controls used methanol alone.

2.2. Trehalase activity assay

The separation of two trehalase proteins was carried out as described by Tatun et al. (2008). Thirty milligrams of insect whole bodies were homogenized in 1 ml of 100 mM phosphate buffer (PB, pH 6.0) at 0 °C followed by sonication for 30 s. The cuticle debris was removed by centrifugation at 1000 × *g* at 4 °C for 10 min. The homogenates were then centrifuged at 105,000 × *g* at 4 °C for 60 min. The resulting supernatant and precipitate were regarded as the fractions containing soluble and membrane-bound trehalase, respectively. The supernatant was subjected directly to a trehalase activity assay, while the precipitate was resuspended in PBS for measurement of trehalase activity. Protein content was determined 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 (200 μl) consisted of 50 μl of 40 mM trehalose (Sigma, St. Louis, MO, USA) in 100 mM PBS, 40 μl of the soluble or membrane-bound trehalase fraction, and 110 μl of PBS. 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 × *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 (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). Trehalase activity was determined with reference to a calibration curve constructed using standard glucose (Sigma, St. Louis, MO, USA).

2.3. Amplification of two trehalase genes

Total RNA was isolated from 10 individuals of the 5th instar female using a Trizol kit (Invitrogen, Foster, CA, USA). Synthesis of first-strand cDNAs was carried out according to the reverse transcriptase XL (AMV) (Takara, Dalian, Liaoning, China) protocol with oligo dT_{18}. The first-strand cDNA (1 μl) was used as a template for PCR. The degenerate primers were designed (Fig. 1), based on the conserved residues among insect trehalases. The degenerate primers were TreF (GCNGCNGARWSNGGNATGGAYTT) and TreR (TTNGGRTARTCCCAYTGYTCNCC). The components of PCR were PCR reaction buffer containing 0.1 mM dNTP, 5 μM each primer, and 1.0 U of Go-Taq DNA polymerase (Promega, Madison, Wisconsin, USA) in a total volume of 20 μl. Thermal cycling conditions were 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min. The last cycle was followed by final extension at 72 °C for 10 min. The amplified product was separated onto agarose gel and purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, Wisconsin, USA). Purified DNA was ligated into the pGEM-T easy vector (Promega, Madison, Wisconsin, USA) and several independent subclones were sequenced from both directions to identify a fragment different from Clone NLSG4316 from the BPH EST Database. The full-length cDNAs for two fragments were obtained by the rapid amplification of cDNA ends (RACE) according to Generacer™ Kit (Invitrogen, Foster, CA, USA) with gene-specific primers (GSPs). The RACE products were purified and sequenced as described above.

2.4. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

mRNA levels were measured by qRT-PCR using the One Step SYBR PrimeScript RT-PCR Kit (Takara, Dalian, Liaoning, China). Total RNA from *N. lugens* was treated with DNase I (Sigma, St. Louis, MO, USA) and then used as template. qRT-PCR was performed in a 25 μl total reaction volume containing 5 ng of total RNA, 0.5 μl primer mix containing 10 μM each of forward and reverse gene-specific primers, 0.5 μl of Ex TaqTM HS (5 U/μl), 0.5 μl of PrimeScript RT Enzyme Mix, 12.5 μl of 2× One Step SYBR RT-PCR Buffer and 8.5 μl of H_2O. Two kinds of negative controls were set up: non-template reactions (replacing total RNA by H_2O) and minus reverse transcriptase controls (replacing PrimeScript RT Enzyme Mix by H_2O). qRT-PCR was done with the following cycling regime: initial incubation of 42 °C for 5 min and 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s. Standard curves were obtained using a 10-fold serial dilution of pooled total RNAs from 20 individuals. mRNA levels were quantified in relation to the expression of β-actin (EU179846; Liu et al., 2008). The primer pair for each gene was designed to amplify a 200–300 bp PCR product, which was in each case verified by nucleotide sequencing. Only data that showed good efficiency (≥85%) and correlation coefficient (≥95%) were included in the analysis. Means and standard errors for each time point were obtained from the average of three independent sample sets. Gene-specific primers for NlTre-1, NlTre-2 and β-actin were listed as: NlTre-1-F: GTC TCA CTG GTC AAG CGG TTC G; NlTre-1-R: CTG TAT GAT TCG GGT CTC GGA C; NlTre-2-F: TCG TGC CAG GTG GAC GGT TTA G; NlTre-2-R: CAG AAC TCG AAC TCT TTC TCC AG; β-F: TGG ACT TCG AGC AGG AAA TGG; β-R: ACG TCG CAC TTC ATG ATC GAG.

3. Results

3.1. Detection of the two types of trehalase

Ultracentrifugation at 105,000 × *g* was used to divide the whole body homogenates from the macropterous female adult *N. lugens* (soon after ecdysis) into two fractions, supernatant and precipitate. Trehalase activity was measured in these two fractions; activity was 39.4 ± 4.2 μmol mg protein^{-1} min^{-1} for the soluble fraction and

Fig. 1. The alignment of amino acid sequences of two trehalases *N. lugens* with the sequences of other insect trehalases: numbers on the right side of the alignment indicate the position of residues in the sequence of each protein. Identical amino acid residues are indicated by the letters below the sequences. Trehalase signature regions and the glycine-rich region are indicated by the underline and double underline. The degenerate primers are indicated by the dashed line. Putative transmembrane regions are boxed. AmTre-1 and AmTre-2, *Apis mellifera* (XM_393963 and NM_001112671); BmTre-1 and BmTre-2, *Bombyx mori* (NM_001043993 and NM_001043445); OfTre-1 and OfTre-2, *Omphisa fuscidentalis* (EF426724 and EF426723); SeTre-1 and SeTre-2, *Spodoptera exigua* (EU427311 and EU106080); NlTre-1 and NlTre-2, *Nilaparvata lugens* (FJ790319 and FJ790320).

12.2 ± 1.9 μmol mg protein^{-1} min^{-1} for the insoluble fraction, indicating that whole body samples from macropterous female adults contain both soluble and membrane-bound forms of trehalase. Whole body samples from the 5th instar macropterous female nymph also showed trehalase activity in both soluble (32.4 ± 2.3 μmol mg protein^{-1} min^{-1}) and insoluble (11.9 ± 0.7 μmol mg protein^{-1} min^{-1}) fractions. In each case, the majority of the trehalase activity was present in the soluble fraction, which accounted for about three-quarters of the total activity.

3.2. Isolation of cDNAs encoding two trehalases

Searching the BPH EST Database http://bphest.dna.affrc.go.jp/; Noda et al., 2008) with the amino acid sequences of two *A. mellifera* trehalases gave a homolog fragment (Clone ID: NLSG4316) of insect trehalase with 54% identity at the amino acid level to *A. mellifera* trehalase 1 (AmTre-1). Because two trehalase genes (soluble and membrane-bound) had been identified from several insect species (Mitsumasu et al., 2005; Lee et al., 2007; Tang et al., 2008; Tatun et al., 2008), we designed a pair of degenerate primers so that we could amplify another trehalase gene from *N. lugens*. We then used the RACE technique to clone too full-length cDNAs encoding *N. lugens* trehalases. The first cDNA (NlTre-1; FJ790319) has an open reading frame (ORF) of 1641 bp and 546 deduced amino acids, while the second cDNA (NlTre-2; FJ790320) has an ORF of 1851 bp and 616 deduced amino acids. In the deduced amino acid sequence of each of these two trehalases, there are two trehalase signatures and a highly conserved glycine-rich region. In NlTre-2, a putative transmembrane region was found at the C-terminal of the deduced amino acid sequence (Fig. 1). Although NlTre-1 showed approximately the same degree of sequence identity to both soluble and membrane-bound forms of trehalase (42–46%), NlTre-2 showed a greater degree of sequence identity to membrane-bound trehalases (54–63%) than soluble trehalases (40–47%) from other insect species (Fig. 1). The sequence information indicated that NlTre-1 was a putative soluble trehalase and NlTre-2 was a putative membrane-bound one, which need further confirmation.

3.3. Developmental expression patterns and the regulation by 20E of two trehalase genes

The developmental changes in enzyme activity for the two different forms of trehalases, and the developmental expression of the two trehalase genes were compared, with or without 20E application (Fig. 2). From the ecdysis of the 5th instar macropterous female nymph, the activities of both the soluble and the membrane-bound trehalase kept stable, although a slight increase in the soluble enzyme's activity was observed from the 4th day after ecdysis into the fifth instar onward (Fig. 2A). An increase was also found in the level of Nltre-1 mRNA levels from the 4th day after ecdysis onward (Fig. 2B). In all samples, the activity of the soluble trehalase was much higher than the activity of the membrane-bound enzyme, and the level of NlTre-1 mRNA level was much higher than NlTre-2, which is consistent with (but does not prove) that NlTre-1 encodes a soluble trehalase while NlTre-2 encodes a membrane-bound trehalase.

When 8 ng 20E/insect was applied to the 5th instar macropterous female nymph (soon after ecdysis), a large increase in both soluble trehalase activity and the level of NlTre-1 mRNA was observed at the 5th day, which decreased gradually after the 6th day (Fig. 2). In contrast, no increase in membrane-bound trehalase activity and NlTre-2 mRNA level was observed under the same conditions. These results provide additional circumstantial evidence that NlTre-1 encodes a soluble trehalase and NlTre-2 encodes a membrane-bound trehalase. These results also indicate

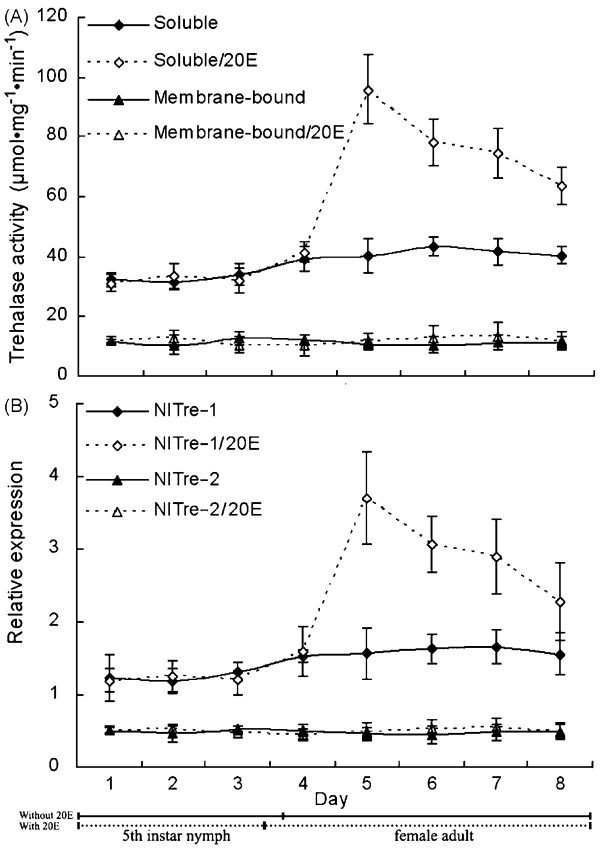

Fig. 2. Developmental changes of trehalase activities and gene mRNA levels and their regulation by 20-hydroxyecdysone (20E). (A) Activities of soluble and membrane-bound trehalases. (B) Levels of two *N. lugens* trehalase mRNAs detected by the quantitative real-time RT-PCR. The dose of 20E was 8 ng/insect, applied to 5th instar macropterous female larvae (soon after ecdysis). Under the *X*-axis, the durations of 5th instar nymph and adult stages are indicated by single line for control (without 20E) and dotted line for 20E treatment (with 20E). The data represent mean values ± SE of at least three repeats. The mRNA level is normalized relative to β-actin transcript levels.

that the regulation of the soluble trehalase activity occurs mainly at the level of gene transcription. It was found that the 20E-treated nymphs had a slightly shorter duration of 5th instar (3.41 ± 0.25 days) than in the untreated nymphs (3.69 ± 0.28 days), although the difference was not significant at the $P < 0.05$ level.

Different doses of 20E were applied to 5th instar macropterous female nymphs and soluble and membrane-bound trehalase activities were measured on the 5th day after application. Between 2 ng 20E/insect and 8 ng 20E/insect, 20E increased the soluble trehalase activity in a dose-dependent manner. A dose of 1 ng 20E/insect did not effectively increase the level of soluble trehalase activity, and the induction reached its peak at the dose of 8 ng 20E/insect. Doses greater than 8 ng did not increase soluble trehalase activity any further (Fig. 3). Membrane-bound trehalase activity was unaffected by 20E at any of the doses examined.

3.4. Difference of trehalase activity between macropterous and brachypterous insects

The soluble and membrane-bound trehalase activities were compared between macropterous and brachypterous females on the 8th days after ecdysis into the 5th instar. In macropterous females, a small increase in soluble trehalase activity and NlTre-1 mRNA level was observed from the 4th day of the ecdysis into the fifth instar onwards (Figs. 2 and 4). However, no such increase occurred in brachypterous females (Fig. 4). The soluble trehalase

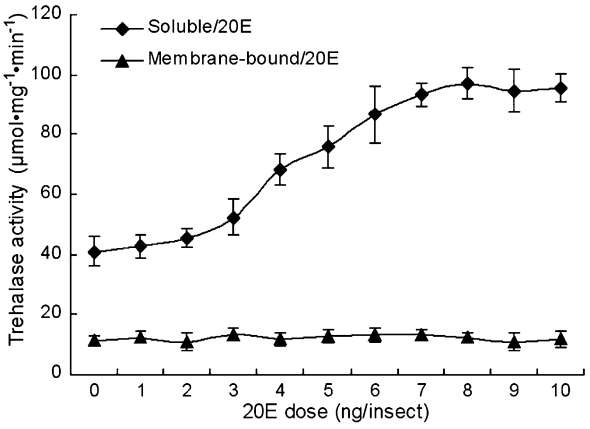

Fig. 3. Dose–response of soluble and membrane-bound trehalase activity to 20E. The hormone was topically applied 5 days before measuring enzyme activity. The data represent mean values ± SE of at least three repeats.

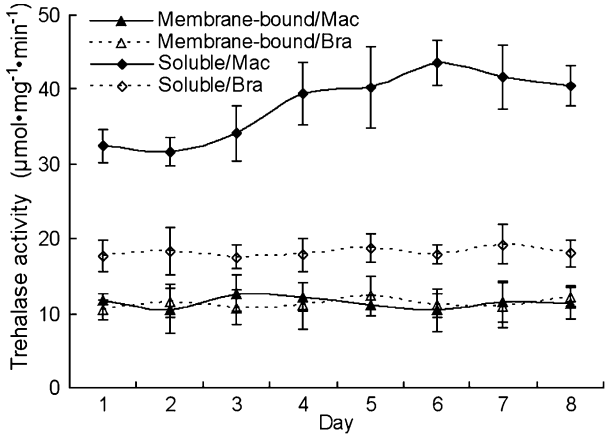

Fig. 4. Comparison of soluble and membrane-bound trehalase activity between macropterous and brachypterous females. The data represent mean values ± SE of at least three repeats.

activity of macropterous females was much higher than that of brachypterous females at all times examined (Fig. 4). The difference in the soluble trehalase activity between two types of adults increased after the emergence of female adults (at 4th day). At the same time, NlTre-1 mRNA level of macropterous females was also much higher than that of brachypterous females (data not shown). In contrast, no difference was observed in the membrane-bound trehalase activity between macropterous and brachypterous females.

4. Discussion

The brown planthopper, *N. lugens*, belongs to the Order Hemiptera, in which the biochemistry and molecular biology are poorly studied. This pest undergoes incomplete metamorphosis and does not possess a pupal stage. Because this insect is small, and because it is difficult to dissect the tissues, such as larval midgut, ovary and fat body, from the nymph or adults of this pest, we used whole body samples to clone the cDNAs and to determine trehalase activity and mRNA levels. We used ultracentrifugation to separate two forms of trehalase, soluble and membrane-bound, finding that the soluble form of the enzyme accounted for the majority of the total activity in both nymphs and adults. We were able to clone two cDNAs, NlTre-1 and NlTre-2, encoding insect trehalases from *N. lugens*. Analysis of the deduced amino acid sequences of NlTre-1 and NlTre-2 indicated the presence of two trehalase signatures and a highly conserved glycine-rich (GGGGEY) region. These signatures are specific to trehalase and are not present in any other protein (Tatun et al., 2008). In addition, NlTre-2 has a putative transmembrane helical region, which is absent from NlTre-1. These sequence characteristics indicated that NlTre-1 and NlTre-2 might encode a soluble trehalase and a membrane-bound trehalase, respectively. Our finding that soluble trehalase activity and NlTre-1 mRNA level are induced to a similar extent by topical application of 20E, but that membrane-bound trehalase activity and NlTre-2 mRNA level are not induced at all, gave the same indication that NlTre-1 might encode a soluble trehalase, while NlTre-2 might correspond to a membrane-bound trehalase.

Soluble and membrane-bound trehalase activities were compared between macropterous and brachypterous females (5th instar nymphs and adults). In brachypterous females, soluble trehalase activity accounted for two-third of the total activity, which is significantly lower than the three-quarters that was found in macropterous females. We found no obvious difference between macropterous and brachypterous females in the activity of membrane-bound trehalase activity, nor in the level of in NlTre-2 mRNA (data not shown). In contrast, soluble trehalase activity was much higher in macropterous females than that in brachypterous females. The macropterous (long winged) morph of adult *N. lugens* possesses long-distance migration ability. Because trehalose, the target of trehalase, serves as a source of energy in insects (Elbein et al., 2003), it is an interesting question whether the high (soluble) trehalase activity in macropterous females plays an important role in the long-distance migration of *N. lugens*. This is currently being investigated in our laboratory.

Trehalase activity in various insects is at least in part under hormonal regulation, its activity being regulated by hormones, such as juvenile hormone (JH), 20-hydroxyecdysone (20E) and diapause hormone (DH) (Yamashita et al., 1972; Ogiso and Takahashi, 1984; Yaginuma and Happ, 1989). Exogenous 20-hydroxyecdysone (20E) has been shown to lower the hemolymph trehalose concentration in the lepidopterous *B. mori* and *O. fuscidentalis*, indicating that 20E could elevate trehalase activity (Oda et al., 2000; Singtripop et al., 2002). In *O. fuscidentalis*, the genes (OfTre-1 and OfTre-2) encoding two forms of trehalase have been cloned, but only soluble trehalase activity and mRNA level of the corresponding gene OfTre-1 could be induced by the exogenous 20E (Tatun et al., 2008). Here we also showed that only the soluble trehalase activity and NlTre-1 mRNA level could be induced by 20E, which occurred in a dose-dependent manner. An increase in soluble trehalase activity was observed at the 4th day of the ecdysis into the fifth instar of macropterous females, but not brachypterous females. This increase in soluble trehalase activity corresponded to an estimated increase in hemolymph ecdysteroid concentration for adult eclosion of macropterous females (data not shown). In *Drosophila melanogaster*, 20E was found to indirectly influence alkaline phosphatase activity via regulating dopamine (DA) level (Rauschenbach et al., 2007). Because, in the present study, the effects of the 20E-treatment were only observed at the 5th day after hormone application, it is possible that the influence of 20E or internal ecdysteroid on soluble trehalase activity might also be indirect, perhaps being mediated by another hormone or substance. Whether the effect is direct or indirect, our data suggest that endogenous ecdysteroid may be an important factor in regulating soluble trehalase activity.

In conclusion, here we have identified two forms of trehalase and the corresponding genes from *N. lugens*, which is as far as we know the first identification of a trehalase gene in a hemipteran species. Soluble trehalase activity accounted for the majority (about three-quarters) of the total trehalase activity. Only soluble trehalase activity and the corresponding gene NlTre-1 mRNA were

responsive to 20E induction and this form of trehalase was significantly different between macropterous and brachypterous females.

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