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Regulation of hemolymph trehalose level by an insulin-like peptide through diel feeding rhythm of the beet armyworm, *Spodoptera exigua*

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ABSTRACT

Like vertebrate insulins, some insect insulin-like peptides (ILPs) play crucial roles in controlling immature growth, adult lifespan, and hemolymph sugar level. An ILP gene (SeILP1) was predicted from a transcriptome database of *Spodoptera exigua*. SeILP1 encodes 95 amino acid sequence and shares sequence homologies (33–83%) with other insect ILPs, in which six conserved cysteine residues are found in the predicted B–A chains. SeILP1 was expressed in all developmental stages of *S. exigua*. However, SeILP1 expression was tissue-specific because the transcript was detected in fat body and epidermis, but not in hemocytes and gut. Its expression increased with feeding activity. Hemolymph trehalose levels of the fifth instar larvae maintained a relatively constant level at 2.31 ± 0.62 mM. However, starvation induced a significant increase of the hemolymph trehalose level by more than twofold in 48 h, at which few SeILP1 was transcribed. RNA interference of SeILP1 using its specific double-stranded RNA induced a significant increase of hemolymph trehalose level. Interestingly, a bovine insulin decreased hemolymph trehalose level in a dose-dependent manner. These results indicate that SeILP1 plays a role in suppressing hemolymph trehalose level in *S. exigua*.

Introduction

Insulins are peptide hormones and consist of insulin, insulin-like growth factor (IGF), and relaxin. They mediate metabolism, growth, reproduction, and life span in vertebrates. In invertebrates, insulin-like peptides (ILPs) are identified. More than 30 putative ILP genes are encoded in the genome of the nematode, *Caenorhabditis elegans* [42]. For mollusks, seven ILP genes in *Lymnea stagnalis* [49] and one in *Aplysia californica* [16] have been characterized. In crustaceans, an isopod, *Armadillium vulgare*, produces an ILP from the androgenic gland [40]. The blue crab, *Callinectes sapidus*, produces two isoforms of ILPs from the androgenic gland and the hepatopancreas [11]. In insects, a number of ILPs have been identified in different orders [57].

The first insect ILP, bombyxin, was found in the silkworm, *Bombyx mori*, during the process to identify prothoracicotropic hormone [35,36]. Bombyxins regulate insect growth [37], carbohydrate metabolism [46], and ovarian development [17]. Insulin-like peptides in insects are homologous in molecular form to mammalian insulins as well as being functionally equivalent [8]. Insect genomes contain multiple genes encoding insulin-like molecules; eight in *Drosophila melanogaster* [13,18,24], seven in the mosquito *Anopheles gambiae* [32], and 39 in *B. mori* [2,28].

Bombyxins are expressed predominantly in the brain [28,29] in contrast to the vertebrate insulin, which is expressed in the pancreas. However, several insect ILPs have been known to be expressed in other tissues including fat body, suggesting the presence of insect IGFs [2,38,39,47]. The synthesized ILPs are released into hemolymph and bound to high-affinity binding proteins to be transported to target cells [48]. At the target cells, ILPs specifically interact with insulin receptor (InR) and trigger insulin signal pathway to regulate growth, metabolism, reproduction, and longevity [12,20].

Insulin in vertebrates induces anabolic metabolism by activating glucose uptake and conversion into glycogen while it inhibits glycogen breakdown. The primary circulating sugar in insects is trehalose, the non-reducing disaccharide of glucose. Insulin signaling in some insects is involved in the regulation of circulating trehalose levels [9,46,54,57]. Trehalose regulation by ILPs is explained by controlling expression of trehalase in *B. mori* [28,46] and *Tribolium castaneum* [57], by a direct molecular interaction with trehalase in *Tenebrio molitor* [7] or by regulating the trehalose biosynthesis in *C. elegans* [27]. In addition, a control of trehalose transporter activity by ILPs has been also suggested because glucose transporter 4 is

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translocated to the plasma membrane in response to insulin signal in vertebrates [22,25].

The beet armyworm, *Spodoptera exigua*, is a major insect pest in vegetables with its broad host range [60]. Especially, its massive feeding activity during last instar allows the larvae to grow more than four times in body weight and gives serious economic damage to crops [30]. Its major sugar in hemolymph (= insect blood) is trehalose [41]. Like other insects to keep a constant level of hemolymph trehalose, *S. exigua* needs to control the fluctuating trehalose levels along with nutrient uptake from the gut to the hemolymph under a circadian feeding rhythm. In this control of hemolymph trehalose, ILP may play a crucial role as an endocrine signal. However, no ILP was known in *S. exigua*. This study reports the first ILP gene of *S. exigua* and its physiological function as a down-regulator of hemolymph trehalose level.

Materials and methods

Insect rearing and test sample preparation

Larvae of *S. exigua* were reared on an artificial diet [21] at 25 °C, 16:8 h (L:D) photoperiod, and 60% relative humidity. Adults were supplied with 10% sucrose solution for diet and kept at the larval rearing conditions for mating and oviposition. Different developmental stage samples consisted of newly laid eggs, first to fifth instar larvae (L1–L5), 2–3 days old pupae, and both sex adults. For different tissues, three days old L5 larvae were used to collect hemocytes, fat body, epidermis, and gut. After collection in 100 mM phosphate buffered saline (PBS, pH 7.4), all tissues were washed three times with PBS. Gut contents were removed from the gut tissues.

Feeding diel rhythm assay

Two days old L5 larvae were used for assessment of feeding amount in different times in a day. Each larva was kept individually in a 9 cm diameter Petri dish with cabbage diet. Feeding activity was measured by weighing cabbage. Thus, a feeding amount at every 3-h time zone was calculated by the reduction of cabbage weights. Light and dark cycle used 16:8 h photoperiod, in which light-on was at 8 am and light-off was at 12 pm. Each time treatment was replicated three times.

Starvation experiment

One day old L5 larvae were used for a long term (48 h) starvation experiment. Control larvae were fed cabbage ad libitum. The larvae under starvation were individually kept in the 9 cm Petri dish with a moisturized filter paper to prevent desiccation and avoid any cannibalism. Under these conditions, no larvae under starvation were died. The treated larvae were used for RT-PCR or hemolymph trehalose measurement as described below.

RNA extraction and cDNA preparation

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To prepare different developmental insect samples, about 5000 eggs for egg stage, 20 larvae for L1–L3 stages, 5 larvae for L4 stage, 3 larvae for L5 stage, 3 pupae for pupal stage, 5 adults for each male and female adult sample were used for RNA extraction. To prepare different tissue samples of L5 larvae, about 50 larvae were sacrificed for collecting hemocytes and 5 larvae were used for collecting fat body, epidermis, and gut in each RNA extraction. One microgram of total RNA was incubated at 70 °C for 3 min and cDNA was synthesized by reverse transcriptase using RT-premix (Intron Biotechnology, Seoul, Korea) containing an oligo dT primer according to the manufacturer's instruction and subsequently treated with RNase H (Promega, Madison, WI, USA).

Identification of a putative ILP gene from S. exigua *('SeILP1') by bioinformatics*

An ILP gene sequence was obtained from a congeneric species, *S. littoralis* with a GenBank accession number of HQ451072. This sequence was used as a query to search homologous gene(s) from transcriptome database of *S. exigua* using NCBI-BlastN tool. Two transcript sequences were obtained. One transcript was highly homologous to other known ILPs and contained a full open reading frame. This gene was named as SeILP1. The other transcript was partial and showed poor sequence homology with other known ILPs.

RT-PCR and quantitative real time RT-PCR (qRT-PCR) of SeILP1

To screen the expression of SeILP1 in different developmental stages and tissues, RT-PCR was used. The synthesized single-stranded cDNA was used as a template for PCR amplification with gene-specific primers (5′-CTTTCTGTGTTCTTGTGGGAGA-3′ and 5′-CTTTACTAGAAGAGAAGGTAGGTAGGTAG-3′) with 35 cycles under conditions of 1 min at 94 °C for denaturation, 1 min at 50 °C for annealing, and 1 min at 72 °C for extension. A ribosomal gene, RL32, was used for a positive control of RT-PCR using its gene-specific primers (5′-ATGCCCAACATTGGTTACGG-3′ and 5′-TTCGTTCTCCTGGCTGCGGA-3′).

To quantify the decrease of SeILP1 after RNA interference treatment, qRT-PCR was performed on a Bioneer Exicycle™ using SYBR green chemistry and real time fluorescence measurements with the gene-specific primers of SeILP1. Template cDNAs were constructed as described above. The reaction mixture (20 μl) consisted of 10 μl enzyme/substrate complex, each 5 pmol of forward and reverse primer (each 2 μl), and 50 ng of cDNA (6 μl). The PCR condition began with an initial activation of Hot-start Taq DNA polymerase by heat treatment at 95 °C for 10 min and was followed by 35 cycles of 15 s at 95 °C, 30 s at 50 °C, and 40 s at 72 °C with a final extension for 10 min at 72 °C. RL32 was used as an internal control in each sample using the same cDNA template and was amplifies with RL32 primer as mentioned above. Fluorescence values were measured and amplification plots were generated in real time by an Exicycler TM program. Melting curve in each reaction was assessed to confirm a single PCR product. Quantitative analysis followed a comparative C_T ($\Delta\Delta C_T$) method [33]. Each qRT-PCR was replicated with three independent biological samples.

Hemolymph trehalose measurement using a high performance liquid chromatography (HPLC)

Hemoylmph from L5 larvae was collected into 1.5 ml tubes containing a few granules of phenylthiocarbamide (Sigma–Aldrich Korea, Seoul, Korea), and diluted with distilled and deionized water. After centrifugation at 400 × *g* for 5 min, the supernatant plasma was cleaned with Sep-Pak C18 cartridge (Walters Associates, Milford, MA, USA), which was prewashed with distilled and deionized water. The plasma samples were further cleaned by passing through a 0.22 μm syringe filter (Pall Corporation, Ann Arbor, MI, USA) to prevent the HPLC column from any bacterial contamination. Analysis of the composition and amount of polyols was carried out using an ion exchange HPLC (BioLC, Dionex, Sunnyvale, CA, USA) equipped with a guard column (CarboPac MA1, 4 × 50 mm, Dionex) and a main column (CarboPac MA1, 4 × 250 mm, Dionex). A sample was injected with 5 μl volume. Elution buffer was 400 mM NaOH at a constant rate of 0.4 ml min^{-1}. The separated samples