Analysis of β-furanosidase Activity and Gene Expression in the Midgut of Fifth-instar Silkworm (*Bombyx mori*) Larvae

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Abstract [**Objectives**] The present study was conducted to investigate the change rule of β-fructofuranosidase gene expression and its enzyme activity in the midgut of 5th instar silkworm (*Bombyx mori*), in order to provide a reference for illustrating the enzymatic mechanism of using β-fructofuranosidase to absorb sucrose nutrition from mulberry leaves. [**Methods**] Real-time fluorescent quantitative PCR was applied to analyze the expression of *BmSuc1* and *BmSuc2* in midgut of 5th-instar silkworm larvae, meanwhile the activities of β-fructofuranosidase was determined. [**Results**] *BmSuc1* was expressed in the midgut of 5th-instar silkworm larvae at different developmental stages. Its expression was upregulated at the beginning of the 5th instar and during the peak feeding period, whereas *BmSuc2* expression remained very low throughout the entire 5th instar. The activity of β-fructofuranosidase was relatively high during the peak feeding period of 5th-instar larvae, showing a trend of increasing first and then decreasing. [**Conclusions**] The expression pattern of the *BmSuc1* gene and the changes in β-fructofuranosidase activity were generally consistent with the physiological process of sugar nutrient absorption and utilization from mulberry leaves in 5th-instar silkworms. It suggests that *BmSuc1*, as a sucrose hydrolase gene, plays a major role in the digestion and absorption of sucrose nutrients from mulberry leaves in the midgut tissue.
Key words *Bombyx mori*; β-Fructofuranosidase; *BmSuc1*; *BmSuc2*; Gene expression
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1-Deoxynojirimycin (DNJ) can inhibit the activity of α-glucosidase, but has no inhibitory effect on β-fructofuranosidase[1-2]. By inhibiting the activity of α-glucosidase in the gut of insects, DNJ prevents the decomposition and absorption of sucrose nutrients, thereby disrupting normal growth and development, and even causing death[3-4]. Mulberry leaves, branches and roots are rich in DNJ. The oligophagous silkworm (*Bombyx mori*), which feeds exclusively on mulberry leaves throughout its life cycle, overcomes the inhibitory effect of DNJ on α-glucosidase and mitigates its toxicity primarily by relying on β-fructofuranosidase to decompose and absorb sucrose nutrients from mulberry leaves[3-6]. The 5th instar stage of silkworms represents the period of highest mulberry leaf consumption. Investigating the expression patterns of the β-fructofuranosidase gene in the midgut during this stage and analyzing the functional activity and enzymatic characteristics of sucrose hydrolases in the silkworm midgut holds significant scientific importance for elucidating the enzymatic adaptation mechanism by which silkworms circumvent the toxic effects of the mulberry alkaloid DNJ. Sucrose is a preferred primary sugar nutrient for animals, including insects. There are two kinds of hydrolases responsible for sucrose decomposition, one of which is α-glucosidase that catalyzes the glucose side group, and the other is β-fructofuranosidase that catalyzes the fructose side group[1]. α-Glucosidase is widely present in plants, animals, and microorganisms. Although β-fructofuranosidase has been extensively reported in microorganisms and plants, the long-standing consensus has been that animals lack β-fructofuranosidase and that sucrose digestion and absorption in animals primarily rely on the hydrolytic activity of α-glucosidase[1,7]. Early research reported the presence of β-fructofuranosidase in the intestinal fluids of a few insect species[8-10], but no related gene had been cloned or identified. It was not until 2008 that Daimon *et al.*[5] first discovered two genes in the silkworm genome with high homology to bacterial β-fructofuranosidase genes, designated *BmSuc1* and *BmSuc2*, and confirmed that the proteins they encode exhibit characteristic β-fructofuranosidase activity in silkworms' midguts. Furthermore, studies have shown that homologous genes of β-fructofuranosidase also exist in non-mulberry-feeding insects such as the eri-silkworm and the Chinese oak silkworm. The eri-silkworm possesses two such genes (*ScSuc1* and *ScSuc2*), while the Chinese oak silkworm has three (*ApSuc1a*, *ApSuc1b*, and *ApSuc2*). However, none of these genes exhibit the enzymatic activity characteristic of β-fructofuranosidase, suggesting a close association between β-fructofuranosidase function and the dietary preferences of mulberry-feeding/non-mulberry-feeding insects[11]. The mulberry-feeding insect silkworm primarily relies on β-fructofuranosidase rather than α-glucosidase to decompose sucrose in mulberry leaves, which is why the high concentration of DNJ in mulberry leaves has no toxic effect on it. To date, no reports have been published on the activity of β-fructofuranosidase in the silkworm midgut or the expression patterns of the *BmSuc1* and *BmSuc2* genes. In this study, the expression levels of β-fructofuranosidase genes in the midgut of silkworm larvae were detected throughout the entire 5th instar stage

using real-time quantitative PCR, and the activity of β-fructofuranosidase during this period was measured. This study preliminarily clarified the variation patterns of *BmSuc1*, *BmSuc2* and β-fructofuranosidase in the midgut of 5th-instar silkworms, providing insights into the adaptive mechanism by which silkworms circumvent the toxic effects of the mulberry alkaloid DNJ.

Materials and Methods
Experimental materials

The experimental silkworms were of the Jingsong × Haoyue strain, artificially hatched and reared at 25.5 – 28.0 ℃ with 60% – 70% humidity, and fed with mulberry leaves. Midgut tissues were collected from the beginning of the 5th instar to the mature larval stage. The midgut of each silkworm was longitudinally divided into two portions: one for RNA extraction and the other for enzyme activity assays. Three replicates were set for each sampling, and five silkworms were taken from each replicate. The samples were collected and stored at −80 ℃ for subsequent use. RNAiso Plus, the reverse transcription kit PrimeScript^{TM} II 1st Strand cDNA Synthesis Kit, PrimeScript^{TM} RT reagent Kit with gDNA Eraser, Taq DNA Polymerase and the fluorescent quantitative reagent SYBR$^{®}$ Premix Ex Taq^{TM} II (Tli RNaseH Plus) were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The protein quantification kit and sucrase assay kit were obtained from Nanjing Jiancheng Bioengineering Institute.

RNA extraction and reverse transcription

Total RNA was extracted from the midgut tissues following the instructions of the RNAiso Plus reagent. The obtained total RNA was then treated with the PrimeScript^{TM} RT reagent Kit with gDNA Eraser to remove genomic DNA. The RNA prepared using the above method was diluted by adding 2 μl of total RNA to 100 μl of DEPC water. The OD_{260}, OD_{280} and OD_{260}/OD_{280} ratios were measured using a nucleic acid/protein analyzer to calculate the RNA concentration. According to the instructions of the PrimeScript^{TM} II 1st Strand cDNA Synthesis Kit, the extracted RNA was reverse transcribed into cDNA. Samples with an OD_{260}/OD_{280} ratio between 1.80 and 2.0 were selected for real-time quantitative PCR experiments.

Primer design and synthesis

Primers were designed using Primer Premier 5.0 software in accordance with real-time PCR requirements. The primer sequences are listed in Table 1.

Table 1　Primers used in real-time fluorescent quantitative PCR

Gene name	Primer sequence
BmSuc1	F: 5′-AATCCAGTCCTCTCCTACGTGC-3′
	R: 5′-TCCGGTCTGATACGTGTTCTTG-3′
BmSuc2	F: 5′-ACGTGCAACTGTGACTCTCCTG-3
	R: 5′-CTGATGCCTCCTGTTAGGGAAG-3
Actin3 (internal reference)	F: 5′-CGGGAAATCGTTCGTGAT-3′
	R: 5′- ACGAGGGTTGGAAGAGGG-3′

PCR amplification of target genes

Prior to fluorescent quantitative PCR detection, conventional PCR was first performed using the designed primers. This step served two purposes, the first of which was to verify primer specificity and check for primer-dimer formation, and the second was to preliminarily assess the approximate transcription levels of the target genes in the silkworm midgut, providing qualitative reference data for subsequent real-time fluorescent quantitative PCR analysis.

Using the primers listed in Table 1, PCR amplification was performed with the reverse-transcribed cDNA from the silkworm midgut as the template. The procedure was carried out in accordance with the instructions provided with the TaKaRa Taq^{TM} reagent. The amplification program was as follows: pre-denaturation at 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 30 s, and a final extension at 72 ℃ for 10 min. PCR products were detected using 1% agarose gel electrophoresis. Electrophoresis was conducted at 5 V/cm, and the results were observed and recorded after 25 – 30 min using a UVP gel imaging system.

Real-time fluorescent quantitative PCR detection

Real-time fluorescent quantitative PCR was performed according to the instructions of the SYBR$^{®}$ Premix Ex Taq^{TM} II (Tli RNaseH Plus) kit. The reaction system had a total volume of 20 μl, and the reaction parameters were as follows: denaturation at 95 ℃ for 1 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 30 s. The results were recorded using a StepOne real-time PCR amplifier from ABI company, with each sample tested in triplicate. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

β-Fructofuranosidase activity assay

Preparation of enzyme solution: Silkworm midgut samples were taken and homogenized in 1.0 ml of ice-cold potassium phosphate buffer (0.1 mol/L, pH 7.0) on ice. The homogenate was centrifuged at 8 000 r/min for 5 – 8 min at 4 ℃, and the supernatant was collected. Protein content was determined following the instructions of the protein quantification kit, and enzyme activity was measured according to the sucrase assay kit protocol.

Statistical Analysis

Data processing and graphing were performed using Excel 2007, while statistical analysis was conducted with SPSS 13.0.

Results and Analysis
PCR amplification and detection of target genes

The results in Fig. 1 indicate that transcripts of both the *BmSuc1* and *BmSuc2* genes were detected in the midgut cDNA of 5th-instar silkworm larvae. No significant primer-dimer bands were observed, confirming that the designed primers were suitable for real-time quantitative PCR experiments. Furthermore, by comparing the width and intensity of the amplified bands, it was observed that the band for *BmSuc1* was brighter, while that for *BmSuc2* was very faint. It suggested, to some extent, a difference in transcription level between *BmSuc1* and *BmSuc2*. However, a more accurate analysis of these differences requires further validation through real-time quantitative PCR detection.
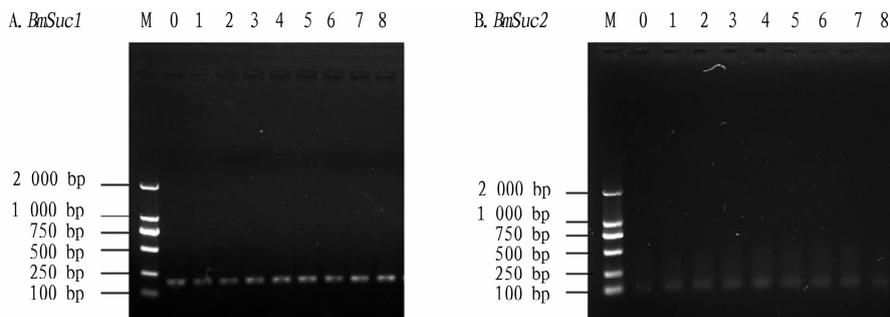
M: DL2000 Marker; 0: newly moulted 5th-instar larvae; 1 – 8: larvae from day 1 to day 8 of the 5th instar.

Fig .1　**PCR amplification and detection of *BmSuc1* and *BmSuc2***

Analysis of β-fructofuranosidase gene expression in the midgut of 5th-instar silkworms

Using real-time quantitative PCR, the expression levels of the β-fructofuranosidase genes *BmSuc1* and *BmSuc2* in the midgut from the beginning to the end of the 5th instar were quantitatively analyzed. The results are shown in Fig. 2. The figure indicates that the transcriptional expression of *BmSuc2* fluctuated slightly, remaining very low from the early to the late larval stage. In contrast, the expression level of *BmSuc1* varied significantly throughout the instar stage. Starting relatively high at the beginning of the 5th instar, it showed a slight drop on day 1, followed by a steady rise from day 2 to a peak on day 4. A decline began on day 5, reaching its lowest point by days 7 – 8. The expression of *BmSuc1* throughout the instar stage showed an initial decrease, followed by a gradual increase to a peak, and then a subsequent decline. This pattern indicates that the transcriptional expression dynamics of *BmSuc1* and *BmSuc2* in the midgut of 5th-instar silkworms are inconsistent.
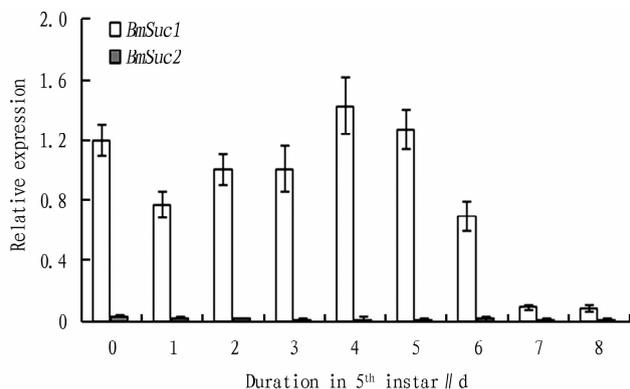

Fig. 2　**Transcription levels of *BmSuc1* and *BmSuc2* in the midgut of 5th-instar silkworm larvae**

Changes in β-fructofuranosidase activity in the midgut of 5th-instar silkworms

The variation in β-fructofuranosidase activity in the midgut of 5th-instar silkworm larvae is shown in Fig. 3. As illustrated in Fig. 3, the enzyme activity exhibited an initial increase followed by a decrease from the beginning to the end of the 5th instar. From the beginning of the 5th instar to the 2nd day of feeding, β-fructofuranosidase activity showed no significant change, remaining at approximately 80 U/mg prot. Starting from the 3rd day, the enzyme activity

gradually increased, peaking on the 5th day (158.82 U/mg prot). Subsequently, the enzyme activity declined steadily, reaching its lowest level by the end of the larval stage.
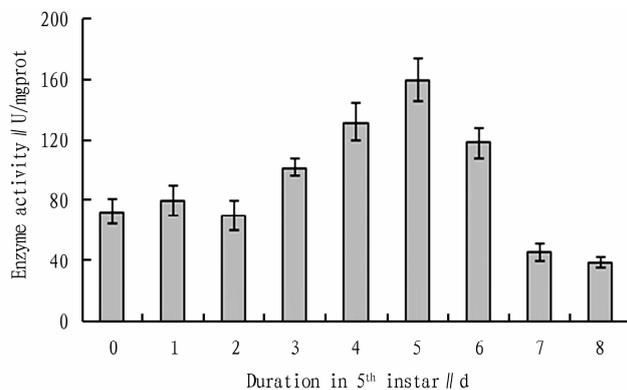

Fig. 3　**The changes of β-fructofuranosidase activity in 5th instar silkworm larvae**

Discussion

The silkworm genome contains two β-fructofuranosidase genes (*BmSuc1* and *BmSuc2*). Among them, *BmSuc1* is specifically expressed in the midgut tissue of silkworms and possesses full enzymatic function. In contrast, *BmSuc2* shows extremely low expression levels in the midgut, and sequence alignment analysis reveals that *BmSuc2* lacks enzyme active sites, suggesting that its product likely does not exhibit β-fructofuranosidase activity[5]. In this study, real-time quantitative PCR detected a certain level of *BmSuc1* expression in the midgut tissues of 5th-instar silkworms, with relatively higher expression observed at the beginning of the 5th instar and during the peak feeding period. In contrast, *BmSuc2* expression remained very low throughout the entire 5th instar, almost undetectable. Based on these findings, it is hypothesized that *BmSuc1*, as a sucrose hydrolase, plays a major role in the digestion and absorption of sucrose nutrients in the midgut.

The midgut of the silkworm is primarily responsible for the digestion and absorption of nutrients. Macromolecules in mulberry leaves, such as carbohydrates, proteins, and lipids, are first decomposed into small molecular compounds by the digestive fluids in the midgut. These compounds are then absorbed by the midgut epithelial cells and transported via the bloodstream to other tissues, providing energy for life activities such as growth and

development[12]. Sucrose is a primary nutritional sugar source preferred by many insects. The β-fructofuranosidase in the insect midgut tissue mainly decomposes sucrose into glucose and fructose, providing a sugar source for the organism[1,13]. Alkaloids in mulberry leaves, such as D-AB1 and DNJ, are potent inhibitors of α-glucosidase and exhibit high toxicity to non-mulberry-feeding insects like the cabbage moth and the castor silkworm[14-15]. Yet, the silkworm relies solely on mulberry leaves as its food source. Studies have shown that the silkworm utilizes β-fructofuranosidase to hydrolyze sucrose in mulberry leaves into utilizable monosaccharides, which are then absorbed and used by the silkworm body[15]. The results of this study indicated that β-fructofuranosidase activity showed an initial increase followed by a decrease during the entire 5th-instar stage of silkworms, a pattern generally consistent with the physiological process of sugar nutrient absorption and utilization from mulberry leaves in the larvae at this stage. The silkworms reached their peak feeding period around days 4 and 5 of the 5th instar, during which β-fructofuranosidase activity was relatively high, and the expression level of the *BmSuc1* gene also peaked on day 4. During the peak feeding stage, silkworms have a high demand for mulberry leaves and sugar nutrients. The larvae ingest large amounts of mulberry leaves, requiring more sucrose hydrolases to digest and absorb sugar nutrients. At this stage, sucrose hydrolase genes are highly expressed, and enzyme activity remains at a relatively high level.

Conclusions

The expression pattern of the *BmSuc1* gene and the changes in β-fructofuranosidase activity were generally consistent with the physiological process of sugar nutrient absorption and utilization from mulberry leaves in 5th-instar silkworms. It suggests that *BmSuc1*, as a sucrose hydrolase gene, plays a major role in the digestion and absorption of sucrose nutrients from mulberry leaves in the midgut tissue.

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