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A novel sucrose hydrolase from the bombycoid silkworms , , and with a substrate specificity for sucrose

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Although membrane-associated sucrase activity has been detected in the midgut of various lepidopteran species, it has not yet been identified and characterized at the molecular level. In the present study, we identified a novel sucrose hydrolase (SUH) gene from the following three bombycoid silkworms: , , and and named them , , and , respectively. The EST dataset showed that is one of the major glycoside hydrolase genes in the larval midgut of . . These genes were almost exclusively expressed in the larval midgut in all three species, mainly at the feeding stage. SUHs are classified into the glycoside hydrolase family 13 and show significant homology to insect maltases. Enzymatic assays revealed that recombinant SUHs were distinct from conventional maltases and exhibited substrate specificity for sucrose. The recombinant BmSUH was less sensitive to sugar-mimic alkaloids than TvSUH and ScSUH, which may explain the reason why the sucrase activity in the . midgut was less affected by the sugar-mimic alkaloids derived from mulberry.

1. Introduction

Sucrose is a major disaccharide in the carbohydrate translocation and storage in plants. Herbivorous insects digest dietary sucrose into its constituent monosaccharides using midgut sucrase (Mittler and Meikle, 1991; Febvay et al., 1995; Ashford et al., 2000). Insect midgut sucrase has been identified as either a soluble enzyme or being associated with the membrane. It is generally thought that insect sucrase activity depends mainly on α-glucosidases (Terra and Ferreira, 1994; Carneiro et al., 2004). On the other hand, several studies have reported the occurrence of β-fructofuranosidase in lepidopteran insects (Santos and Terra, 1986; Sumida et al., 1994a; Carneiro et al., 2004; Daimon et al., 2008). Although a large variety of α-glucosidase genes have been recently identified in Diptera (Ferreira et al., 2010; Gabrisko and Janecek, 2011; Gabrisko, 2013; Zhang et al., 2013), there are only a few studies on α-glucosidase genes of Lepidoptera. All digestive enzymes in the Lepidoptera except those required for initial digestion are localized in the membrane fraction of midgut cell homogenates (Sumida et al., 1990; Ferreira et al., 1994; Terra and Ferreira, 1994, 2012).

Previous studies have described the presence of membrane-associated sucrase in the larval midgut of (Lepidoptera: Crambidae) (Carneiro et al., 2004). This sucrase displayed substrate specificity to sucrose (Carneiro et al., 2004). Although sucrose-specific sucrase has been reported only from . in the Lepidoptera, we speculated that similar enzymes were also distributed in other lepidopteran species. Sucrase activity in the membrane fraction of the midgut homogenate was also detected in (Sumida et al., 1994b; Hirayama et al., 2007). However, the gene encoding the membrane-associated sucrase has not yet been identified at the molecular level. In the present study, we analyzed the RNA-seq data of larval midguts of the three species, . , , (Bombycidae), and (Saturniidae), all of which belonged to superfamily Bombycoidae, Lepidoptera (http://silkbase.ab.a.u-tokyo.ac.jp/), and identified novel genes encoding sucrose-specific hydrolases (SUHs), which were almost exclusively expressed in the midgut of each insect species.

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2. Materials and methods

2.1.

Larvae of . . (p50T strain) were reared at 25 °C on fresh mulberry ( ) leaves under a 12 h light–12 h dark photoperiod. Larvae of . , a strain originating from a population collected on Ishigaki Island, Okinawa, Japan, were reared at 25 °C on Chinese banyan ( ) leaves (Daimon et al., 2012). Larvae of . . were reared at 25 °C on the tree of heaven ( ) leaves. To precisely stage the larvae, we used the onset of head capsule slippage, which is the separation of an old head capsule from a new head capsule, as a developmental marker.

2.2.

The protein sequences were analyzed using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/, Petersen et al., 2011), the SOSUI system (http://harrier.nagahama-i-bio.ac.jp/sosui/) in conjunction with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Glycosylphosphatidylinositol (GPI) lipid-anchoring was judged using the PredGPI system (http://gpcr.biocomp.unibo.it/predgpi/). The domains and functional sites in the proteins were identified using the InterPro (http://www.ebi.ac.uk/interpro/) and the Carbohydrate-Active enZYmes Database (http://www.cazy.org/Glycoside-Hydrolases.html). The amino acid sequences were searched in the NCBI protein database (http://www.ncbi.nlm.nih.gov/), SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp), and butterfly genome databases (http://www.butterflygenome.org/and http://monarchbase.umassmed.edu/). The amino acid sequences of alpha amylase catalytic domain from proteins showing homology to SUHs were aligned using ClustalW, and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates.

2.3. *d* -

Tissues of last instar larvae were collected, washed three times in ice cold phosphate-buffered saline (PBS) and stored at −80 °C. The tissues used for analysis were as follows: silk gland, midgut, hindgut, testis, ovary, Malpighian tubule, fat body, wing disc, and epidermis. Total RNA was isolated using TRIzol (Invitrogen). The contaminating genomic DNA was digested with RNase-free DNase1 (Takara, Japan). First-strand cDNA was synthesized using the TaKaRa RNA PCR Kit (Takara, Japan). PCR was performed under the following conditions: 94 °C for 2 min followed by 30 cycles (27 cycles for 49:) of 94 °C for 30 s; 59 °C for 30 s; and 72 °C for 1 min. The primers used for PCR are listed in Supplementary Table 1.

2.4. *d* *fi*

Recombinant SUHs were produced with a baculovirus expression system using the Bac-to-Bac system (Invitrogen). The

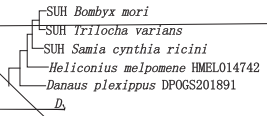
3. Results

3.1. d fi d . ,
. d . .

To identify the novel sucrose hydrolase gene, we searched the RNA-seq database for the larval midguts of three bombycoids, . , . , and . (http://silkbase.ab.a.u-tokyo.ac.jp). Except β-fructofuranosidase, lepidopteran sucrases are α-glucosidases (EC 3.2.1.20). In , 27 genes have been categorized as "α-glucosidase" (GO:0004558) in the FlyBase (http://flybase.org/). Therefore, we searched the RNA-seq datasets using these 27 α-glucosidases as queries. As a result, we found 13 contigs homologous to them in the . RNA-seq assembly. Among them, we found a novel maltase-like gene with an N-terminus hydrophobic amino acid sequence and a high expression level in each transcriptome of the three species, though its biochemical function is unknown. We named this gene , , and , for . , . , and . , respectively. The nucleotide sequences have been deposited in the DDBJ database (accession nos. AB905205–AB905207). Each of , and contained an open reading frame encoding 606 amino acid residues. Their residues of 7–29 were hydrophobic and potentially formed a transmembrane domain based on TMHMM and SOSUI though we could not exclude the possibility that they were a part of the signal peptides (Fig. S1). We also checked for GPI-anchoring motifs in SUHs using the web-based software, PredGPI. The result suggested that SUHs were not GPI-anchored. The amino acid sequence analysis using the InterPro indicated that the SUHs belong to glycosyl hydrolase family 13. In addition, the SUHs possessed three conserved catalytic residues, which are also observed in many α-glucosidases (Fig. S1).

BmSUH, TvSUH, and ScSUH showed 49%, 49%, and 48% identity to . maltase A1 (NP_476627), respectively. In the genome of . , there are three paralogues of , BGIBMGA003055, BGIBMGA003056, and BGIBMGA003057, showing 48%–51% amino acid identity to BmSUH. They encoded putative maltases, which carry putative signal peptides at their N-termini but do not possess transmembrane domains as predicted by the TMHMM program. There was only a single ortholog in the genomes of the butterfly, , whereas there was one ortholog and one closely-related paralog in the genome of the Monarch butterfly, .

A phylogenetic analysis was performed using the neighbor-joining method to investigate the evolutionary relationships among SUHs, maltases, and other insect α-glucosidases (Fig. 1). The phylogenetic tree indicated that BmSUH, TvSUH, and ScSUH belonged to α-glucosidases (EC 3.2.1.20) including maltases (Gabrisko, 2013) and the putative sucrase of (Price et al., 2007). However, the SUHs in these bombycoid silkworms and papilionoid butterflies, . and . , formed a monophyletic clade and were clearly distinct from maltases and other α-glucosidases in insects, suggesting that SUHs have



diverged from other α-glucosidases during the evolution of lepidopteran insects.

3.2. . . . . fi .

The nucleotide sequence of . hit 46 EST tags derived from the larval midgut using SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp), indicating that it is more highly expressed than another sucrase gene, *1*, whose EST count was only 4. It suggests that BmSUH is a major sucrose hydrolase in the larval midgut, at least in . . . . Subsequently, we examined the expression profile of the . genes with RT-PCR using total RNA prepared from day 3 (. . . and . . . . .) and day 2 (. . . .) last instar larvae. The results indicated that the . genes were expressed mainly in the larval midgut (Fig. 2A). In . . . , . was highly detected in the anterior and posterior parts of the midgut, and a weak signal was detected in the middle part (Fig. 2B). Expression of . and . was observed in all parts of the midgut. Furthermore, the . mRNAs were abundantly detected during the feeding stage but drastically decreased at the wandering stage of the three insect species (Fig. 2C).

3.3. . . . . d . fi . . . . . .

To examine the biochemical properties of the SUHs, we generated recombinant baculoviruses expressing His-tagged SUHs. Western blot analysis using anti-His antibody showed that the recombinant SUHs were successfully produced and accumulated in High Five cells and were not secreted into the medium (Fig. 3A). We subsequently examined the distribution of recombinant SUHs in High Five cells. As shown in Fig. 3B, we observed that all three

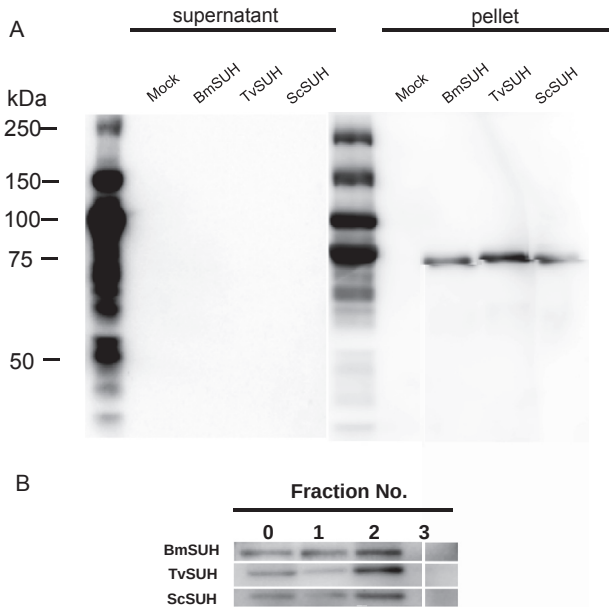


Fig. 3. Expression of recombinant SUHs using a baculovirus expression system. (A) The baculovirus-infected High Five cell medium (supernatant) and cell lysate (pellet) were separated using SDS-PAGE and were analyzed using Western blot with anti-His antibody. (B) Subcellular distribution of recombinant SUHs in High Five cells. The fractions used for analysis were as follows: the supernatant of the cell homogenate (fraction 0), fractions rich in nuclei (fraction 1), microsomes, membrane-associate protein (fraction 2), and cytosol (fraction 3). The molecular weights of the protein standards are shown on the left.

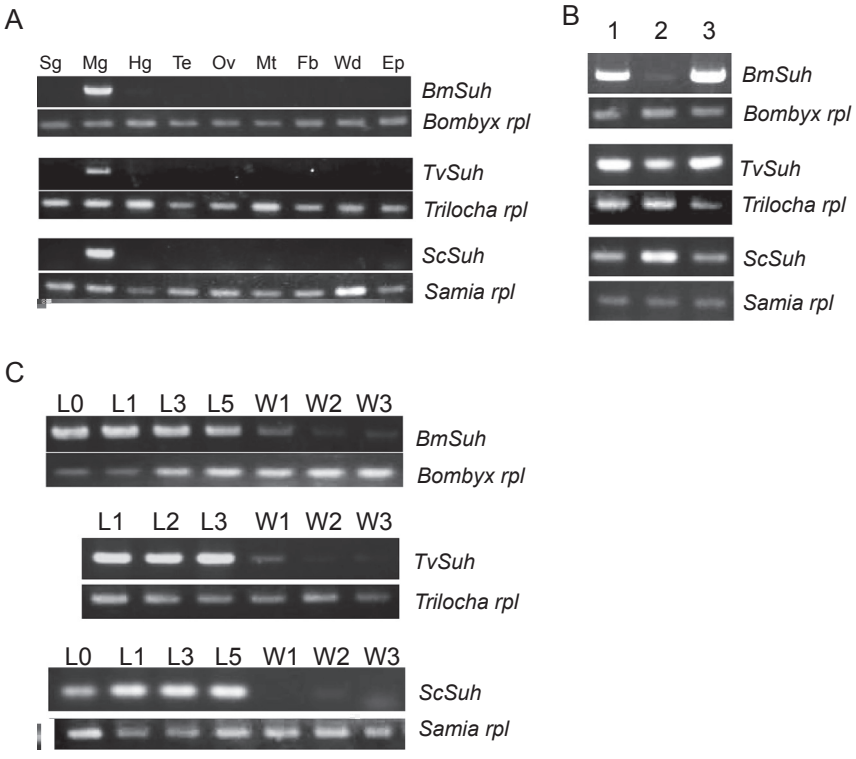


Fig. 2. Expression profiles of the . mRNAs in three bombycoids. (A) Expression profiles of the . mRNAs in three insect species. RT-PCR analyses were performed using total RNA from day 3 (. . . . .) or day 2 (. . . .) last instar larvae. Tissues used for analysis were as follows: silk gland (Sg), midgut (Mg), hindgut (Hg), testis (Te), ovary (Ov), Malpighian tubule (Mt), fat body (Fb), wing disc (Wd), and epidermis (Ep). (B) Different parts of the midgut used for analysis were as follows: lane 1, anterior part of the midgut; lane 2, middle part of the midgut; and lane 3, posterior part of the midgut. (C) Developmental expression of the . mRNAs in the midgut. Total RNA extracted from the midgut was analyzed using RT-PCR. Ln: day n of the last instar larvae; Wn: n days after the beginning of wandering. . . . . ( ) was used as an internal control.

recombinant SUHs abundantly existed in the fraction 2 compared with that in the other fractions. This finding suggests that the SUH proteins were mainly associated with the membrane.

We then purified the recombinant SUHs from baculovirus-infected cells using nickel-chelating chromatography. SDS-PAGE (Fig. 4A) and immunoblot analyses (Fig. 4B) revealed that the molecular weights of the purified proteins were approximately 70 kDa, which was consistent with the predicted size.

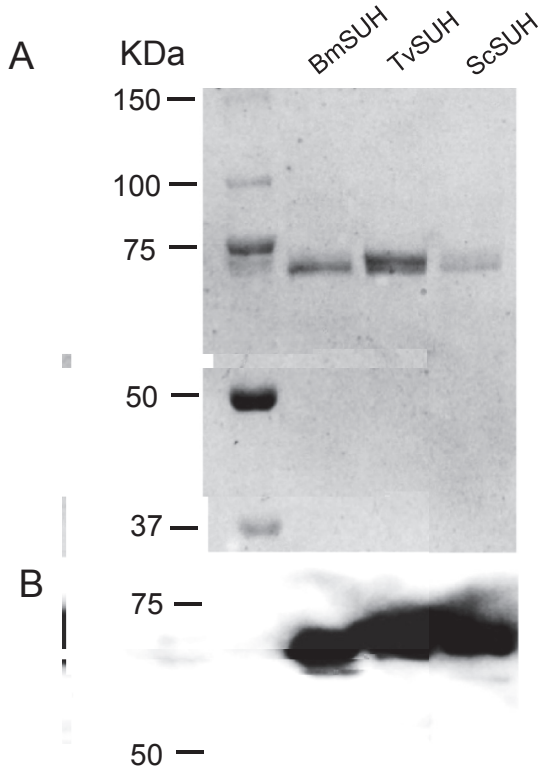


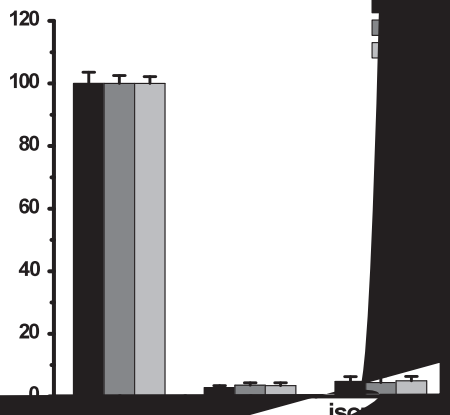
Fig. 4. Purification of recombinant SUHs. (A) Purification was performed using nickel chromatography. Purified protein was electrophoresed, and the gel was stained with Coomassie Brilliant Blue. The molecular weights of the protein standards are shown on the left. (B) The purified proteins were analyzed by immunoblot with the anti-His antibody. The molecular masses of the protein standards are shown on the left.

3.4. *fi d*

We further analyzed the enzymatic f of th
nant SUHs. Substrate specificity of th ant
examined using several sugar substrates in
recombinant SUHs were active on sucro on
stachyose, which lacks the α-glucosyl e re
SUHs showed measurable activity onl ucros
maltose, isomaltose, or trehalose (Fig. resul
strate that the recombinant SUHs hav spe
sucrose and are distinctive from maltas ofura

The pH stability of the SUHs was de sing
the substrate. The recombinant SUHs we d at
values (pH 4.0–11.0). The optimum pH ombi
was 8.0–9.0, but they remained highly n a p
6.0–10.0 (Fig. 6).

Subsequently, we calculated the kine ers o
An analysis of the recombinant SUH act erfor
presence of increasing sucrose concen e
values of BmSUH, TvSUH, and ScSUH we , 12.



11.8 ± 0.6 nmol/min/μg and 3.5 ± 0.4, 3.9 ± 0.5, and 3.7 ± 0.7 mM, respectively (Fig. S2).

3.5. - 1 d 1-

Mulberry latex contains extremely high amounts of alkaloid sugar-mimic glycosidase inhibitors such as D-AB1 and 1-DNJ, which are known as competitive inhibitors of α-glycosidase (Asano et al., 2001; Yao et al., 2003; Konno et al., 2006; Watanabe et al., 2013). To examine the effect of these inhibitors on the SUH hydrolytic activity, we analyzed SUH activity using sucrose as the substrate in the presence of D-AB1 and 1-DNJ. D-AB1 showed full inhibition at a low concentration. The IC_{50} value of D-AB1 for BmSUH was 44.2 μM, and was 3.9-fold and 3.4-fold higher than those for TvSUH and ScSUH, respectively (Fig. 7A). 1-DNJ slightly affected the activity of BmSUH, and its IC_{50} value was more than 1000 μM (Fig. 7B). The IC_{50} values of 1-DNJ for TvSUH and ScSUH were 236 and 359 μM, respectively, indicating that they are more sensitive to 1-DNJ than BmSUH.

4. Discussion

Previous studies have shown that the digestive enzymes of lepidopteran insects, except those required for initial digestion, are immobilized on the surface of midgut cells (Terra and Ferreira, 2012). However, the precise properties of membrane-associated sucrases are unknown. In this study, we identified a novel gene encoding a sucrose hydrolase (SUH) from each of the bombycoid silkworms. Biochemical studies showed that these SUHs have enzymatic activities as sucrose-specific hydrolases. All of the recombinant SUHs were recovered in the membrane fraction when expressed in High Five cells, suggesting that they are associated with membrane. These findings identified a key element underpinning the membrane-associated sucrases in lepidopteran insects.

Our analysis revealed that mRNAs were most exclusively expressed in the larval midgut. In addition, the EST database showed that was transcribed abundantly in the larval midgut. Moreover, the genome database search revealed that the genomes of two papilionoid butterflies contained the genes orthologous to them, whereas SUH gene-like sequences were not found in other genomes of insects and metazoans. These findings indicates that SUHs are novel, major sucrose hydrolases in the larval midgut of lepidopteran insects.

The sequence comparison revealed that the SUHs have several conserved residues presumably involved in the catalytic mechanism and showed 48%–49% identity to . maltase A1, which was the most intensively transcribed, and is probably involved in sugar digestion (Gabrisko, 2013). In addition, the SUHs showed 43% overall sequence identity to . (Hemiptera) gut sucrase, which is membrane bound, and probably the sole gut sucrase (Price et al., 2007). Like the . α-glucosidase, SUHs also have a hydrophobic C-terminal region, which could potentially function as a membrane association region. To find signal peptides, we used SignalP 4.1, which could discriminate between signal peptides and transmembrane regions (Petersen et al., 2011). It predicted that none of the three SUHs had signal peptides cleaved by the signal peptidase. On the other hand, when we used PrediSi and Signal-CF, the results showed that each SUH had a signal peptide constituting of 22 residues. Further studies are required to distinguish whether the signal peptides are cleaved or not and whether SUHs are trapped in the cell glycocalyx or integrated into the membrane.

Unlike typical α-glucosidases, the recombinant SUHs showed measurable activity only against sucrose. A sucrose hydrolase with specificity to sucrose has been previously isolated and characterized from d (Kim et al., 2004, 2008). Although this enzyme has also been categorized into glycosyl hydrolase family 13, it shows low sequence similarity to the bombycoid SUHs. β-fructofuranosidase shows little sequence similarity to SUH and belongs to glycoside hydrolase family 32. Despite the high sequence similarity between lepidopteran SUH and maltase, SUH is a peculiar hydrolase with substrate specificity for substrate. Further studies are required to determine the crystal structures of SUHs and active sites optimized for sucrose hydrolysis.

The recombinant SUHs displayed a very broad range of optimal pH, whereas the . β-fructofuranosidase showed an optimum pH of approximately 7 (Sumida et al., 1994a; Carneiro et al., 2004; Daimon et al., 2008). The SUH activity with a wide pH range may be an adaptation to the higher pH milieu of the digestive juice of lepidopteran larvae (Sumida et al., 1994b; Pytelkovám et al., 2009; Terra and Ferreira, 2012). These enzymatic properties of SUHs are similar to those of the . sucrase, which displays specificity for sucrose and shows a very broad range of optimal pH of approximately 6–11 (Carneiro et al., 2004). Because the papilionoid butterflies possess the genes orthologous to the bombycoids genes in our database search, SUH may be widely distributed among lepidopteran insects. This finding highlights an evolutionarily conserved role of SUH in their digestion system.

Sugar-mimic alkaloids are observed in mulberry latex and occur up to 18% of the dry mass in the latex (Konno et al., 2006).

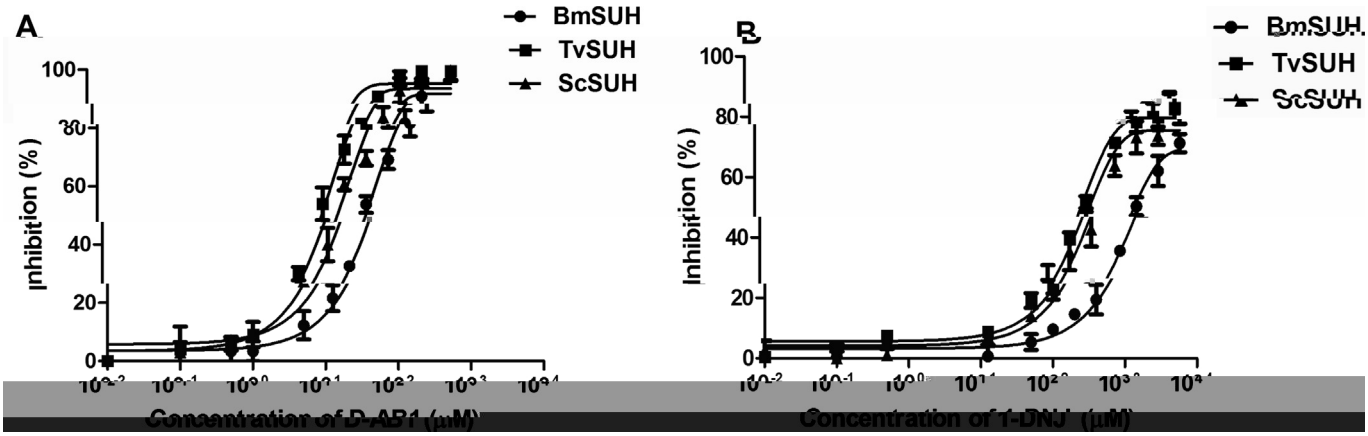


Fig. 7. Effect of sugar-mimic alkaloids on the hydrolysis of sucrose by recombinant SUHs. (A) Inhibitory effects of D-AB1. (B) Inhibitory effects of 1-DNJ. Recombinant SUHs (200 ng) were incubated for 20 min at 30 °C with increasing concentrations of the sugar-mimic alkaloid. Glucose released during the reaction was measured at 505 nm using the Glucose C II-Test Wako. Error bars are standard deviations of the average of three independent experiments.

These compounds prevent the growth of nonmulberry-feeding herbivores by inhibiting midgut sucrase (soluble and membrane-associated fractions) and digestion of sucrose (Sumida et al., 1990; Hirayama et al., 2007). It has been unclear why . membrane-associated sucrase activities are less affected by the alkaloids. In this study, we showed that BmSUH was less sensitive to sugar-mimic alkaloids than SUHs from nonmulberry feeders, in particular to D-AB1, which may partially explain the reason why the midgut membrane-associated sucrase activity of . was not inhibited by high concentrations of sugar-mimic alkaloids. Further analysis of structural changes associated with the adaptation of BmSUH to sugar-mimic alkaloids will be of great interest.

Previous studies showed that the digestive system in lepidopterans is more complex than those in other insects and very different from them (Terra and Ferreira, 2012). The present study elucidated that SUH is a major sucrase hydrolase, which appears to have diverged from other α-glucosidases at an early stage of lepidopteran evolution. The presence of SUH together with β-fructofuranosidase strongly supports that the Lepidoptera have evolved a specific mechanism that enables the larval midgut to utilize sucrose as a nutrient efficiently. Further insight into the diversity and catalytic properties of α-glucosidases will further improve our understanding of lepidopteran digestive system.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.04.005.

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