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A novel β-fructofuranosidase in Coleoptera: Characterization of a β-fructofuranosidase from the sugarcane weevil, *Sphenophorus levis*

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ABSTRACT

β-fructofuranosidases or invertases (EC 3.2.1.26) catalyze the hydrolysis of sucrose into fructose and glucose. β-fructofuranosidases have been widely described in microorganisms, but were not known in the animal kingdom until very recently. There are studies reporting lepidopteran β-fructofuranosidases, but no β-fructofuranosidase gene sequence or encoding transcript has previously been identified in beetles. Considering the scarcity of functional studies on insect β-fructofuranosidases and their apparent non-occurrence among coleopterans, the aim of the present study was to investigate the occurrence and characterize a β-fructofuranosidase transcript identified in a cDNA library from the sugarcane weevil, *Sphenophorus levis* (Curculionidae). To validate that the β-fructofuranosidase sequence (herein denominated *Sl-β-fruct*) is indeed encoded by the *S. levis* genome, PCRs were performed using genomic DNA extracted from the larval fat body as well as DNA from the midgut with microbial content. Amplification of *Sl-β-fruct* gene using larval fat body DNA indicated its presence in the insect's genomic DNA. The *Sl-β-fruct* gene was cloned in *Pichia pastoris* to produce the recombinant enzyme (r*Sl-β-fruct*). Molecular weight of the recombinant protein was about 64 kDa, indicating possible glycosylation, since the theoretical weight was 54.8 kDa. The substrate specificity test revealed that r*Sl-β-fruct* hydrolyzes sucrose and raffinose, but not melibiose or maltose, thereby confirming invertase activity. The pH curve revealed greatest activity at pH 5.0, demonstrating r*Sl-β-fruct* to be an acidic β-fructofuranosidase. Quantitative PCR (qRT-PCR) analyses indicated that the production of mRNA only occurs in the midgut and reaches the greatest expression level in 30-day-old larvae, which is the expected pattern for digestive enzymes. Chromatography of glycosidases from *S. levis* midguts showed two enzymes acting as β-fructofuranosidase, indicating the presence of a *Sl-β-fruct* isoform or a β-fructofuranosidase from insect intestinal microbiota. Moreover, it was found that α-glucosidases do not act on sucrose hydrolysis. Phylogenetic analyses indicated this enzyme to be similar to enzymes found in other coleopteran and lepidopteran β-fructofuranosidases, but also closely similar to bacterial enzymes, suggesting potential horizontal gene transfer. Despite this, the enzyme seems to be restricted to different groups of bacteria, which suggests distinct origin events. The present study expands the concept of the occurrence of β-fructofuranosidase in insects. Despite the few descriptions of this gene in the animal kingdom, it is possible to state that β-fructofuranosidase is crucial to the establishment of some insects throughout their evolutionary history, especially members of the Lepidoptera and Coleoptera clades.

1. Introduction

Beta-fructofuranosidases or invertases (EC 3.2.1.26) catalyze the hydrolysis of sucrose into fructose and glucose and were first purified from yeast ferment in the second half of the 19th century (O'Sullivan and Tompson, 1890). Alpha-glucosidases (EC 3.2.1.20) can also catalyze the hydrolysis of sucrose and are commonly found in all animals, including in the midgut of insects. However, β-

Abbreviations: rSl-β-fruct, recombinant β-fructofuranosidase from Sphenophorus levis; DNS, dinitrosalicylic acid.

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fructofuranosidases are specific to β-fructosyl, whereas α-glucosidases are specific to the α-glucosyl moiety of the substrate (Terra and Ferreira, 1994).

Beta-fructofuranosidases have been widely described in microorganisms, but were not known in the animal kingdom until very recently, when found in the order Lepidoptera (Santos and Terra, 1986; Sumida et al., 1994; Carneiro et al., 2004). The first reports on the occurrence of transcripts encoding β-fructofuranosidase in animals were in a midgut cDNA library of *Helicoverpa armigera* larvae (Pauchet et al., 2008) as well as *Bombyx mori*, in which the enzyme was characterized and immunolocalized in the midgut and silk gland of larvae (Daimon et al., 2008). Subsequently, β-fructofuranosidase was found in the transcriptome of *Manduca sexta* larvae (Pauchet et al., 2010). The combined data indicate that β-fructofuranosidases are common among species of Lepidoptera and are encoded by the genomes of these insects.

Studies report invertase activity in the midgut of coleopteran species (Hirashima et al., 1989; Devi and Singh, 2011), but no such enzyme activity has previously been described for beetles or their microbiota. Furthermore, no specific β-fructofuranosidase activity assay has been described, which suggests that the detected sucrose hydrolysis may result from common α-glucosidase activity. No β-fructofuranosidase encoding genes or likely conserved motifs were found in the *Tribolium castaneum* genome (Richards et al., 2008).

Considering the scarcity of functional studies on insect β-fructofuranosidases and their apparent non-occurrence among Coleoptera, the aim of the present study was to investigate the occurrence and characterize a β-fructofuranosidase transcript identified in a cDNA library from the sugarcane weevil, *Sphenophorus levis* (Curculionidae). The *S. levis* β-fructofuranosidase (denominated *Sl-β-fruct*) was also produced in a recombinant way (r*Sl-β-fruct*) to verify its substrate specificity for β-fructofuranosidase and the gene was amplified from larval fat body genomic DNA. The present study reports a novel β-fructofuranosidase identified from a coleopteran source, which is expressed in the midgut of *S. levis* larvae as a digestive enzyme. This finding offers further information on the evolutionary origin of β-fructofuranosidase, thereby paving the path for theoretical hypotheses regarding this enzyme in insects.

2. Material and methods

2.1. Clone sequencing and characterization

The clone *MBL_S._levis_libr_022F06* isolated from the *S. levis* larval cDNA library constructed at our laboratory (CloneMiner™ cDNA Library Construction Kit) was completely sequenced in the MegaBACE 1000 Flex using the DYEnamic ET Terminator sequencing kit (GE Healthcare) and deposited in the GenBank under accession number KJ934794. The BioEdit program v7.0.5.3 (Hall, 1999) was used to assemble the clone sequences and obtain the full-length β-fructofuranosidase sequence. A signal peptide prediction was performed using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP) and the open reading frame (ORF) was compared to homologous sequences selected by similarity from the GenBank using BlastX and BlastN (http://www.ncbi.nlm.nih.gov/blast). An amino acid alignment was performed using ClustalW (Thompson et al., 1994) in BioEdit.

2.2. β-fructofuranosidase origin

To prove that the β-fructofuranosidase sequence (*Sl-β-fruct*) found in the cDNA library is indeed an enzyme encoded by the *S. levis* genome, polymerase chain reactions (PCRs) were performed using genomic DNA extracted from the fat body as well as DNA from the midgut with microbial content.

Approximately 200 mg of fat body and whole intestine were treated with 0.32 mg/mL of proteinase K and the DNA extraction was performed with UltraPure™ phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (Invitrogen) following the manufacturer's instructions. DNA extraction from the fat body was performed under aseptic conditions to avoid bacterial contamination.

Both DNA preparations were tested for bacterial DNA contamination by PCR using specific primers for the bacterial 16S rRNA gene (338F-ACTCCTACGGGAGGCAGCAG and 1492R-GGTTACCTTGTTACGACTT) (Lane, 1991). This reaction was conducted with recombinant *Taq* DNA polymerase (Fermentas) with initial denaturation at 94 °C for 5 min, followed by 34 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 10 min. This test is important to prove that the DNA extracted from the fat body is free of microbial DNA contamination.

The specific primers described in the next section (*Sl-β-fruct*_F and *Sl-β-fruct*_R) were used to amplify the complete genomic sequence of the β-fructofuranosidase gene. This reaction was conducted with Elongase® enzyme (Invitrogen) with initial denaturation at 94 °C for 4 min, followed by 34 cycles of 94 °C for 40 s, 52 °C for 45 s, 72 °C for 3 min and final extension at 72 °C for 10 min.

2.3. Sl-β-fruct heterologous expression

2.3.1. Construction of expression vector

The *Sl-β-fruct* ORF without the signal peptide (Fig. 1) was amplified by PCR using the primers *Sl-β-fruct*_F (AAACTCGAGAAAAGAGAAAACATTAGCTGGTATCC) and *Sl-β-fruct*_R (TTTTCTAGATCAATGATGATGATGATGATGTTCACAAATTCTCCAGATTTTGTACG). The reverse primer contained a His-tag coding sequence (underlined) and a stop codon (TGA). The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and digested with *Xho* I and *Xba* I restriction enzymes. After further purification, the fragment was cloned into the expression vector pPICZαA (INVITROGEN) previously digested with the same enzymes. Chemically competent *E. coli* DH5α cells were transformed with the construct denominated pPICZαA-*Sl-β-fruct*, which was confirmed by sequencing.

2.3.2. Transforming, screening and protein expression

The plasmid pPICZαA-*Sl-β-fruct* was linearized with the enzyme *Pme* I and 1 μg was used for transformation in the *Pichia pastoris* strain KM71H. The electroporation parameters were 1.5 kV, 25 μF and 200 Ω in a 0.2-cm cuvette. The transformants were selected by plating in YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol and 1.5% bacteriological agar) containing either 100 mg/mL or 500 mg/mL of zeocin. Several transformants were verified by colony PCR, as described elsewhere (Akada et al., 2000). Screening for recombinant protein expressing clones was performed in 24-well plates using the EasySelect™ *Pichia* Expression Kit (Invitrogen). Culture supernatants were verified by SDS-PAGE and DNS enzymatic assays (explained in greater detail below). A scale-up expression was performed with the highest protein-expressing colony identified in the screening. Inoculation was performed in 10 mL of BMGY (1% yeast extract, 2% peptone, 100 mM of potassium phosphate buffer – pH 7.0, 1.34% yeast nitrogen base, 1.64 μM of biotin and 1% glycerol) and incubation was performed at 250 rpm and 30 °C for 24 h. The culture was transferred to 500 mL of BMGY and incubated at 250 rpm and 30 °C for 24 h. The medium was changed by centrifuging cells at 1500 g for 5 min and solubilizing in 100 mL BMMY (same as BMGY, except that glycerol was replaced with 0.5% methanol). Methanol was added daily for a final