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Identification of membrane proteins of the midgut of *Zabrotes subfasciatus* larvae associated with the insecticidal mechanism of PF2 lectin

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ABSTRACT

The gut of insects, as well as other animals, is rich in glycoconjugates that have important biological functions required for growth and development. Interaction of lectins (carbohydrate binding proteins) with these conjugates can result in anti-physiological and insecticidal effects. PF2 lectin from *Olneya tesota* seeds shows insecticidal activity against *Zabrotes subfasciatus* larvae (bean pest). In order to evaluate the impact of plant lectins on pests, it is important to understand the composition, distribution, and physiological role of glycoconjugates present in the gut. In this study, the binding of PF2 to midgut membrane glycoproteins from 16-day-old larvae was evaluated using 2-DE (two-dimensional electrophoresis) and lectin blotting assay performed with biotinylated PF2. LC–MS/MS analysis identified several glycoproteins that could act as targets for PF2 recognition and that are involved in energy metabolism, cell motility and division, and vesicle movement among other processes. Each of these proteins is physiologically important in such a way that interference by PF2 could result in insecticidal activity.

Introduction

Coleopteran pests often cause serious damage to stored grains. In Latin America, the major pest of this class is the Mexican bean weevil, *Zabrotes subfasciatus*. This insect attacks legumes such as *Phaseolus vulgaris* causing great economic losses (Cárdenas-Morales et al., 2008). Some plant defense proteins (lectins, α-amylase and protease inhibitors, arcelins and vicilins) are highly selective for specific pests and their use as insecticidal agents could reduce the use of contaminating chemical agents as well as their impact on non-target species including mammals (Daoust et al., 1985).

The gut of insects, as well as other animals, is rich in glycoconjugates that are important in maintaining the normal function of the gut (Peumans and van Damme, 1995). Some plant defense proteins, such as carbohydrate-binding proteins or lectins, interact with gut glycoconjugates resulting in anti-physiological or toxic effects (Chrispeels and Raikhel, 1991). To be effective as an insecticidal agent, lectins must resist enzymatic proteolysis within the insect gut and operate in a relatively hostile environment (Peumans and van Damme, 1995; Zhu-Salzman and Salzman, 2001; Brunelle et al., 2004). Insecticidal activity of plant lectins has been shown for a large array of insect orders such as Coleoptera, Diptera, Homoptera, and Lepidoptera (Vandenborre et al., 2011b). We reported that PF2 lectin from *Olneya tesota* seeds produced 100% mortality to *Z. subfasciatus* when incorporated into an artificial diet at a level of 0.5% *w*/w. In addition, we demonstrated that PF2 lectin is resistant to protease digestion, while histochemical analysis showed PF2 binding to epithelial cells of the midgut of *Z. subfasciatus* (Lagarda-Diaz et al., 2009).

Recent studies using immobilized PF2 affinity chromatography of the soluble midgut fraction showed that PF2 recognized several glycoproteins of *Z. subfasciatus* that included tubulin, cytochrome C oxidase, lysozyme and alpha amylase (Lagarda-Diaz et al., 2012). Moreover, PF2 interacted with the glycosylated form of amylase and reduced the enzyme activity (Lagarda-Diaz et al., 2014). As non-glycosylated forms of amylase can ease the inactivation, other targets could be expected as part of PF2 insecticidal mechanism. Therefore, the aim of this study is to understand the interaction of PF2 lectin with membrane proteins that are expressed during its early larval development in *Z. subfasciatus* midgut.

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Materials and methods

Insect rearing

Colonies of *Z. subfasciatus* were reared for several generations on *P. vulgaris* cv. Peruano and kindly donated by the Entomology Laboratory of the Universidad of Sonora. Insects were maintained at 27 °C with 65–75% relative humidity and light for 12 h daily (Rodriguez-Quiroz et al., 2000).

Sample preparation

Two hundred 16-day-old larvae were selected as previously reported (Rodriguez-Quiroz et al., 2000) and cold-immobilized. The midguts were dissected into cold 40 mM Tris Base solution. Larval midguts were surgically separated using tweezers and gut portions taken were posterior to proventriculus and anterior to the Malpighian tubules segments. Only actively feeding larvae with food filling the gut tract were chosen. The midgut proteins were extracted step by step with ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA). Midgut tissues were homogenized in a cold 40 mM Tris Base solution containing a cocktail of complete protease inhibitors (Roche Applied Science, Mannheim, GER) and subsequently centrifuged at 434,902*g* for 20 min at 4 °C. The pellet was suspended in 8 M urea, 4% CHAPS, 2 mM TBP, 40 mM Tris, 0.2% ampholytes (pH 3–10), vortexed for 5 min and; centrifuged at 434,902*g* for 20 min at 4 °C. The supernatant containing the membrane proteins (of intermediate solubility) was kept at −80 °C. After of diluted of samples to a concentration of 1% CHAPS, the protein concentration was estimated using DC Protein Assay (Bio-Rad).

Separation of proteins by 2-DE and lectin blotting assay

Membrane-bound proteins (150 μg) were mixed with rehydration buffer (8 M urea, 4% CHAPS, 2 mM TBP, 40 mM Tris, 0.2% ampholytes) and applied onto 7 cm IPG strips pH 4–7 (Bio-Rad, Hercules, CA) for 16 h rehydration at room temperature. Isoelectric focusing (IEF) was performed on a Protean IEF Cell (Bio-Rad) for 10,000 V-hr at 50 μA per strip.

After IEF, the strips were equilibrated for 30 min in a solution containing 0.375 M Tris pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT and traces of bromophenol blue, followed by 30 min in the same solution, but containing 2.5% (w/v) iodoacetamide instead of DTT. For the second-dimension resolution, the strips were placed on a 12% polyacrylamide-SDS gel and covered with 0.5% agarose. The separation was conducted under constant voltage (200 V). Analytical gels were used for lectin blotting assay whereas preparative gels were subjected to silver staining (Wray et al., 1981).

Lectin blotting assay was performed as described by Towbin et al. (Towbin et al., 1979); 2-DE separated proteins were transferred onto a nitrocellulose membrane at 0.8 mA/cm^2 for 2.5 h, using a semi-dry blotter (LABCONCO, Kansas City, MO). Membranes were blocked overnight with PBST (0.02 M KH2PO4/K2HPO4, 0.9% NaCl, 0.1% (v/v) Tween-20, pH 7.2) containing 2.5% (w/v) bovine serum albumin (BSA). The membrane was then washed with PBST and further incubated for 3 h with biotinylated-PF2 lectin (8 μg/ml) followed by streptavidin–peroxidase for 1.5 h. The color reaction was developed at room temperature by addition of peroxidase substrate, 0.075% of 3, 3′-diaminobenzidine 4 HCl (DAB).

Nanoscale liquid chromatography–tandem mass spectrometry (nanoLC-MS/MS) and protein identification using MS/MS data sets and database search

Selected protein spots were excised from the preparative gels and sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona, Tucson, Arizona) for nanoLC-MS/MS analysis as previously described (Lagarda-Diaz et al., 2012). The protein spots were manually excised from the gel, destained, and in-gel digested with commercial Proteomax. After digestion, tryptic peptides were extracted from the gel pieces with 5% formic acid/5% acetonitrile. Microbore HPLC system (TSP4000, Thermo) was modified to operate at capillary flow rates using a simple T-piece flow splitter. Columns (inner diameter: 8 cm by 100 mm) were prepared by packing 100 A°, 5-mm Zorbax C18 resin at 500-psi pressure into columns with integrated electrospray tips made from fused silica, pulled to a 5-mm tip using a laser puller (Sutter Instrument Co.). Peptides were eluted in a gradient using buffer A (90% H_2O, 10% methanol, 0.5% formic acid, and 0.01% trifluoroacetic acid) and buffer B (98% methanol, 2% H_2O, 0.5% FA, and 0.01% trifluoroacetic acid). After an initial wash with buffer A for 1 min, peptides were eluted with a linear gradient from 0 to 70% buffer B over 35 min, followed by a 70–90% buffer B gradient over 2 min and a 3-min wash at 90% buffer B. Samples were introduced onto the analytical column using a Surveyor auto sampler (Surveyor, Thermo Finnigan). MS was scanned, followed by three MS/MS scans of the highest peak within the initial MS scan. Other instrument parameters included the following: precursor ion at 2.0 Da, MS/MS normalized collision energy at 30%, default charge state of precursor at 2, minimum MS scan signal threshold at 500, activation (Q) at 0.250, activation time at 30 ms, and exclusion mass width at around 61.5 Da. MS/MS spectra datasets were used to generate. DTA extension files and proteins were identified using .DTA extension files and the PEAKS Studio v7.0 (Bioinformatics Solutions Inc. Ontario, CAN) search engine. The PEAKS in chorus function was used to combine PEAKS and XTandem database search engines analyses. Searches were conducted against an in-house database containing the Cucujiformia subset of the NCBInr protein database (114,176 sequences, September 2014) and the Contaminants collection from The Max Plank Institute of Biochemistry. Martinsried, Germany (247 sequences, September 2014). Searches were also conducted against an in-house database containing the Cucujiformia subset of the NCBInr nucleotide database after removing of too short, too long, and dirty sequences (114,047 and 103,341 ESTs and GenBank sequences, respectively. September 2014) and the contaminants collection was also included. Trypsin was selected as the specific protease, and one missed cleavage was allowed. The mass tolerance for precursor and fragment ions was set to 10 ppm and 0.8 Da, respectively. Carbamidomethyl cysteine was set as fixed modification and oxidation of methionine was specified as variable modification. However, not other post-translational modifications were set to be identified. False discovery rate was estimated using a decoy database and the peptide target in chorus false discovery rate was set to 1% and an in chorus score >50 and the presence of at least two peptides were considered necessary for confident identification.

Bioinformatics analysis

The identified proteins were submitted to a Gene Ontology (GO) analysis using Blast2GO v3.0.10 (www.blast2go.org) (Conesa et al., 2005). The analysis was performed by searching (BLAST-P) the protein sequences of the most likely *Z. subfasciatus* orthologous (previously obtained after MS/MS data-based protein identification) against the NCBInr protein database. The input parameters used were as follows: number of BLAST-hits requested for each query, 20; BLAST expect value (i.e., e Value), 1e-25. Then, GO mapping was performed to obtain GOs for hits retrieved by the BLAST-P step and annotation was obtained (annotation parameters were as follows: e-value hit filter; 1e-25; anotation cutoff, 55; and GO weight 5), combined graphs of biological processes, molecular functions and cellular components of the proteins were obtained with the Make Combined Graph Tool.

The sequences of the most likely orthologous proteins obtained after MS/MS-based protein identification (against organisms listed in the Cucujiformia subset of the NCBInr protein database) were obtained