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Binding of PF2 Lectin from *Olneya tesota* to Gut Proteins of *Zabrotes subfasciatus* Larvae Associated with the Insecticidal Mechanism

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ABSTRACT: *Zabrotes subfasciatus* (Boheman) is the main pest of common beans (*Phaselous vulgaris*). Wild legume seeds from *Olneya tesota* contain a lectin, PF2, that shows insecticidal activities against this insect. The binding of PF2 to midgut glycoproteins of 20-day-old larvae was evaluated using PF2 affinity chromatography. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of the proteins retained on the gel revealed several putative glycoproteins, ranging in mass from 17 to 97 kDa. Subsequent protein digestion and analysis by liquid chromatography−tandem mass spectrometry (LC−MS/MS) provided amino acid fragments that identified an α-tubulin, cytochrome *c* oxidase subunit I, an odorant receptor, and a lysozyme from available insect sequence databases. The potential of these proteins to serve as part of the mechanisms involved in the insecticidal activity of PF2 to *Z. subfasciatus* is discussed.

KEYWORDS: *PF2 lectin, Olneya tesota, wild legume, Zabrotes subfasciatus, insect midgut glycoproteins, affinity chromatography*

INTRODUCTION

Legumes are one of the world's most important sources of food because they are a rich storage of carbohydrates, lipids, and proteins.[1] Both in the field and during storage, legumes are vulnerable to insect attacks. Coleopterans of the Bruchidae family are the main pests of the seeds of legumes; the association between bruchids and legumes is highly specific. In Mexico, the bruchid *Zabrotes subfasciatus* (Boheman; Mexican bean weevil) is the main postharvest pest of the common bean, a major food source.[2,3] Although common beans are endowed with several defense proteins, including lectins and α-amylase and protease inhibitors that protect the seeds against widely different herbivores, cultivated legume seeds are generally nonresistant to *Z. subfasciatus*.

Some lectins present in wild legume seeds are effective insecticidal agents against insect pests.[4] Lectins are ubiquitous proteins or glycoproteins that reversibly bind to specific mono- or oligosaccharides.[5] Lectins have also been associated with defense mechanisms of plants against insect predators.[6,7] Some are highly selective to specific pests, and their development and use as insecticidal agents could reduce the environmental impact of such agents on nontarget species, including mammals. Lectins bind to glycoconjugates present in the midguts of insects and can cause local or systemic deleterious effects in the development of the insects or, in some cases, death.[8] To be effective, these proteins must resist enzymatic proteolysis within the insect gut and operate in this relatively hostile environment.[9]

Affinity chromatography with immobilized lectins has been used to isolate glycoprotein ligands for further characterization. In insect studies, this approach has been applied successfully to identify lectin receptors.[8,10] Recently, we reported 100% mortality for *Z. subfasciatus* larvae fed an artificial diet that contained 0.5% (w/w) PF2, a lectin isolated from the seeds of a desert wild legume, *Olneya tesota*. We found that PF2 is resistant to protease digestion, and our histochemical studies showed that this lectin bound epithelial cells of the midgut of *Z. subfasciatus*.[11] We further characterized the complex carbohydrate recognized by PF2 as a tetrasialylated triantennary oligosaccharide.[12] The aim of the current study was to use PF2 immobilized on agarose to obtain midgut-soluble glycoproteins from 20-day-old *Z. subfasciatus* larvae and to evaluate the potential of those proteins as putative participants in the mechanisms responsible for the insecticidal activity of PF2. It is important to emphasize that this study is the first step of an exploratory analysis of potential receptors and ligands of the midgut tissues of *Z. subfasciatus* that recognize PF2, which will eventually lead to the elucidation of its insecticidal mechanism.

MATERIALS AND METHODS

Materials. Agarose activated with divinyl sulfone (Mini-LeaKR) was from Kem-En-Tec (Hellerup, Denmark). Broad-range sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) molecular-weight standards were from BioRad (Hercules, CA).

Insects. Colonies of *Z. subfasciatus* were reared for several generations on *Proteus vulgaris* cv. Peruano and kindly donated by the Entomology Laboratory of University of Sonora. Insects were kept under controlled conditions (27 °C, 65−75% relative humidity, and light for 12 h daily).[13]

Plant Material. Seeds of *O. tesota* were collected from mature trees located in the Sonora Desert of Hermosillo, Mexico. Mature pods containing two to six dry seeds were collected and transported to the laboratory. Seeds were removed from pods and stored at 4 °C.

Lectin Purification. PF2 lectin was purified according to ref 12. *O. tesota* seeds were grounded, and meals were defatted by hexane extraction. Hexane was removed by aeration under a chemical hood.

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The flour was suspended in a 0.9% NaCl solution (1:10, p/v) containing 0.02% sodium azide and 0.2 mM phenylmethanesulfonyl fluoride, stirred for 2 h at 4 °C, and then centrifuged at 800*g* for 15 min. The extract was clarified by glass fiber filtration and kept at 4 °C until use. For PF2 purification, fetuin was coupled to activated agarose (Mini-Leak) following the procedure developed by Mini-Leak Kem-En-Tec, as reported by ref 12. Briefly, the crude extract (15 mL) was injected into an agarose−fetuin chromatography column (10 × 100 mm), previously equilibrated with phosphate-buffered saline (PBS) [0.02 M KH_2PO_4/K_2HPO_4, 0.9% NaCl, and 0.02% sodium azide (pH 7.2)]. Unbound protein was washed off with 10 column volumes of equilibrium buffer, and PF2 eluted with 2 column volumes of 0.05 M glycine−HCl buffer (pH 2.5). Lectin-containing fractions were pooled, dialyzed against water at 4 °C, freeze-dried, and stored at −20 °C until use.

Gut Tissue Preparation. The midguts of 20-day-old larvae were cold-immobilized and dissected into cold 40 mM Tris Base solution containing a complete cocktail of protease inhibitors (Roche). Larval midguts were surgically separated using tweezers, and midgut portions were collected (posterior to proventriculus and anterior to the Malpighian tubule segments). Only actively feeding larvae with food filling the gut tract were chosen. The midguts were rinsed in a cold 40 mM Tris Base solution containing protease inhibitors, homogenized using an Ultra-Turrax T25 homogenizer at 8000 rpm for 1 min, with the sample immersed in ice and centrifuged at 434902*g* for 20 min at 4 °C in an ultracentrifuge (Beckman). The supernatant containing the gut-soluble proteins was kept at −80 °C, and the protein concentration was estimated as described by ref 14 using bovine serum albumin as a standard.

PF2-Sepharose Affinity Chromatography. Purified PF2 was cross-linked to glycidol-activated Sepharose 4BCl (PF2-Sepharose).[15] The supernatant prepared from the gut tissues was adjusted to pH 7.0 for optimal binding to PF2 and applied onto a 2 mL PF2-Sepharose column equilibrated with PBS. The column was washed with PBS containing a complete cocktail of protease inhibitors. Proteins were followed by absorbance at 280 nm. Glycoproteins retained on PF2-Sepharose were eluted with 0.02 M glycine−HCl buffer (pH 2.5) until the absorbance at 280 nm was baseline.

PAGE. Fractions obtained from affinity chromatography were analyzed by 12% SDS−PAGE.[16] Gels were silver-stained.

Nanoscale Liquid Chromatography−Tandem Mass Spectrometry (NanoLC−MS/MS). For spectrometry analysis, samples were sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona, Tucson, Arizona). The protein bands were manually excised from gel, destained, and in-gel-digested with trypsin. After digestion, tryptic peptides were extracted from the gel pieces with 5% formic acid (FA)/5% acetonitrile (ACN). Microbore HPLC system (TSP4000, Thermo, San Jose, CA) was modified to operate at capillary flow rates using a simple T-piece flow splitter. Columns (8 cm × 100 μm inner diameter) were prepared by packing 100 Å, 5 μm Zorbax C18 resin at 500 psi pressure into columns with integrated electrospray tips made from fused silica, pulled to a 5 μm tip using a laser puller (Sutter Instrument Co., Novato, CA). 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 (TFA)]. 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, following by 70−90% buffer B over 2 and 3 min wash at 90% buffer B. Samples were introduced onto the analytical column using a Surveyor autosampler (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 ±1.5 Da. All matched peptides were confirmed by visual examination of the spectra. All spectra were analyzed using nonredundant insect protein. The results were also validated using XTandem, another search engine, and with Scaffold, a program that relies on various search engine results (i.e., Sequest, XTandem, and MASCOT), which uses Bayesian statistics to reliably identify more spectra.[17]

RESULTS AND DISCUSSION

Affinity Chromatography. Some plant lectins have shown toxicity toward different insect pests.[6,7] Under normal circumstances, insects consume plant lectins through the diet, making it likely that first candidate recognition factors would be located in the digestive tract. Because gut cells of insects express glycoproteins that are important in maintaining the normal function of the tissue, we thought it plausible that these glycoproteins might also serve as potential ligands for PF2 recognition. This notion was also supported by work by others in our group who demonstrated binding of biotinylated PF2 to *Z. subfasciatus* midgut tissues using immunohistochemistry.[11] Thus, we evaluated the binding of midgut-soluble glycoproteins isolated from 20-day-old *Z. subfasciatus* larvae to PF2-Sepharose using affinity chromatography. Two fractions were obtained from this separation, one corresponding to unbound proteins (PBS at pH 7.2) and a second fraction eluted with 0.02 M glycine−HCl at pH 2.5 (Figure 1). The proteins eluted in the second fraction (F2-20) were resolved by 12% SDS−PAGE. We assumed these to be glycoproteins or glycoprotein subunits, and SDS−PAGE suggested the relative molecular mass of these proteins as 97, 67, 60, 47, 30, 22, and 17 kDa (inset of Figure 1). Alternatively, we sought to detect the glycoproteins by Western blotting using biotin-labeled PF2 and found that PF2 recognized the glycoproteins in the elution (data not shown).

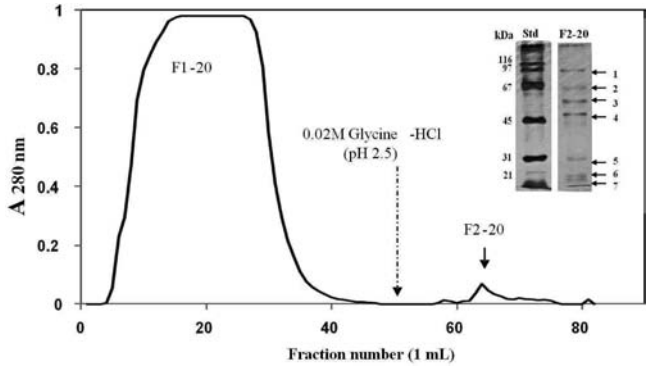

Figure 1. Affinity chromatography using PF2-Sepharose of *Z. subfasciatus* midgut protein extracts from 20-day-old larvae. PF2-Sepharose was prepared and packed, animals were dissected, and proteins were extracted as described in the Materials and Methods. The gel was equilibrated with PBS (pH 7.2) and eluted with 0.02 M glycine−HCl (pH 2.5). (Inset) SDS−PAGE of fractions F2-20. The gel was silver-stained. Std, molecular mass markers; F1-20, ununbound proteins; and F2-20, eluted proteins.

NanoLC−MS/MS Analysis of Putative Midgut Proteins. To identify the proteins that recognized PF2, they were resolved by SDS−PAGE and the band was cut out and subjected to protease digestion. The peptides were analyzed using LC−MS/MS. Spectral analysis provided a peptide sequence corresponding to each of the original bands identified by SDS−PAGE. Initial analyses of the peptide fragments using peptide mass fingerprinting identified four potential proteins from the databases of related Coleopterans, *Ptomaphagus tenuicornis*, *Megacephala chilensis* and *Tribolium castaneum*, including cytochrome *c* oxidase subunit 1 (CCO 1), an

Table 1. Putative Midgut Proteins from *Z. subfasciatus* Recognized by PF2-Sepharose Affinity Chromatography[a]

band	accesion number GenBank ID	description	organism	molecular mass[b] (kDa) predicted	molecular mass[b] (kDa) SDS−PAGE	peptide(s)	coverage (%)	glycosylation sites
3	EEZ99331.1	α-tubulin	*Tribolium casteum*	50	60	(K)DVNAAIATIK(T) (K)VGINYQPPTVVPGGDLAK(V)	6	1
4	EFA01255.1	odorant receptor	*Tribolium casteum*	44	45	(K)ILLTFMTNCTNNDSIALA(G)	5	2
6 and 7	ADG45723.1	CCO 1	*Ptomaphagus tenuicornis*	30	20	(K)SINSLNMVTSLEWLQNMP(P)	7	2
6 and 7	ADK44629.1	CCO 1 Coleoptera	unclassified Coleoptera	24	20	(S)LSLLLMSSMIDK(G)	5	1

[a]Proteins were obtained by affinity chromatography on PF2-Sepharose, resolved by SDS−PAGE, and analyzed by protease digest and nanoLC−MS/MS. Peptide mass fingerprinting was used to identify potential proteins from the databases of related Coleopterans, *P. tenuicornis*, *M. chilensis*, and *T. castaneum*. [b]Predicted, the mass of the protein identified by comparative sequence analysis; SDS−PAGE, estimated mass of the protein eluted from PF2-Sepharose (Figure 1).

Figure 2. Comparison of the CCO 1 amino acid sequences among the different species of insects. The N-glycosylation site in boxed, and the identified peptide is shaded.

odorant receptor, and α-tubulin. Table 1 shows the peptides and the mass of the protein identified from the predicted database sequence, as well as the mass suggested from the SDS−PAGE for the protein isolated from midgut using PF2-Sepharose. In all cases, the deduced amino acid sequences for the proteins identified by spectral analysis were a nearly exact match for the respective peptide sequence. Identity comparison using BLAST analysis confirmed the presence of the peptide in CCO 1 and revealed linkages with CCO 1 from other organisms, including deduced amino acid sequences from *Z. subfasciatus* and *Acanthoscelides obtectus* (GenBank accession numbers DQ459035 and AY676639.1, respectively). These insects belong to the Bruchidae (Coleoptera) family and are closely related to *Z. subfasciatus*. The deduced amino acid sequences of these two CCO 1 are shown with that for *P. tenuicornis* in Figure 2. CCO 1 is highly conserved (>80% identity) among these insects. Two potential N-glycosylation sites are present in the predicted CCO 1 from *Z. subfasciatus* (N107 and N169) and *P. tenuicornis* (N121 and N183), while a third site has been reported in *A. obtectus* (N108, N170, and N235) (Figure 2). Another putative glycosylation site also has been detected in mammalian CCO 1 (not shown), and the glycosylation of cytochrome and mitochondrial localized proteins has also been documented, particularly for cytochromes *b* and P450.[18]

CCO 1 enzymes are highly conserved proteins and are involved in the terminal catalysis of the mitochondrial respiratory chain, electron transport, and proton translocation across the mitochondrial membrane.[19] Studies of the ultrastructure of midgut cells show numerous small mitochondria that occupy a zone just behind the microvilliar border.[20] Although we would not expect CCO 1 to serve as an extracellular receptor for PF2, it could interact with PF2, if PF2 is internalized by the midgut epithelial cells.

An odorant receptor (OR) from *T. casteum* was also identified by peptide analysis. These receptors from *Drosophila* have glycosylation sites.[21] In mammals, OR belongs to the large superfamily of G-protein-coupled receptors.[22] Studies in humans show that the function of the tongue bitter taste receptors, members of the TAS2R family, is influenced by the respective glycosylation pattern.[23] It has been hypothesized that lectins could interfere with these glycosylated receptors and thereby hamper proper function or even send false signals to the nervous system. However, no interaction of lectins with any

Table 2. Identification of Midgut Proteins from *Z. subfasciatus* Obtained by Searching in Insect Databases[a]

band	accesion number GenBank ID	description	organism	molecular mass[b] (kDa) predicted	molecular mass[b] (kDa) observed on SDS−PAGE	peptide(s)	coverage (%)
3	AAL69327.1	egg white lysozyme	*Gallus gallus*	16	60	(K)FESNFNTQATN(R) (N)TDGSTDYGILQINS(R)	22

[a]Proteins were obtained by affinity chromatography on PF2-Sepharose, resolved by SDS−PAGE, and analyzed by protease digest and nanoLC−MS/MS. Peptide sequences were analyzed using nonredundant databases. [b]Predicted, the mass of the protein identified by comparative sequence analysis; SDS−PAGE, estimated mass of the protein eluted from PF2-Sepharose (Figure 1).

taste- or odor-related insect protein has yet been reported.[4] Whether this type of protein is expressed in the midgut of insects also is unknown. However, recognition of an OR by PF2 is interesting and will be addressed in further studies.

Our analyses identified as well an α-tubulin. α-Tubulin is a known structural protein that is part of microtubules that are fundamental components of the spindle in cell division and involved in dynein/kinesin-based cell trafficking. The tubulins are essential for the correct functioning of the insect midgut for growth and nutrient uptake.[24] Although it is generally accepted that, with the exception of O-GlcNAcylation, cytosolic proteins are not glycosylated, there are several reports of tubulin glycosylation. In mammals, work by others indicates that α- and β-tubulins contain N-linked oligosaccharides.[25] More recently, it was reported that the lectin *Galantus nivalis* (GNA) recognized α-tubulins from *Bombix mori*, *Apis mellifera*, *Drosophila melanogaster*, and *Acyrthosiphon pisum*; each of these α-tubulins had putative glycosylation sites.[26]

As expected, comparative spectral analyses also indicted the presence of proteins, such as phaseolin, PHA lectin, and amylase inhibitors. These proteins were present in the food source of the insect. The identification of insect gut proteins can be complicated by contamination with dietary proteins.[27] Nonetheless, we chose to dissect feeding insects to obtain proteins expressed under feeding conditions. Another important factor that influenced our analyses is the quality of the available insect databases. Proteins from the Coleopterans are not yet well-represented among the available databases, and annotation of those available is ongoing. Databases from insects of this order have lagged behind that of insects from other orders, such as Diptera.

Our analysis of nonredundant databases also found two identical peptides for a lysozyme from chicken (Table 2). Several types of lysozymes have been described, including those from chicken (lysozyme type c), goose (type g), phage, bacteria, plant, and invertebrates (type i).[28] The type c lysozyme has been identified in insects from the orders of Lepidoptera, Diptera, Orthoptera, Hymenoptera, Isoptera, and Hemiptera.[29,30] In particular, *D. melanogaster* have both types c and i lysozymes.[31] Lysozymes are defined by their enzymatic hydrolysis of the β-1,4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan layer in bacterial cell walls. In insects, the innate defense response is mediated in part by pattern recognition proteins that stimulate expression of effectors, including the antibacterial protein lysozyme.[28] Lysozymes are expressed in various insect tissues, including hemocytes, fat body, and tissues from the digestive tract and gut throughout growth from larva to adult.[32,33] In Dipteran, such as *Drosophila*, it is hypothosized that lysozyme secreted into the midgut, has functions not as a self-defense enzyme but as a digestive enzyme by acting on bacteria incorporated as food.[33] Typically, the molecular mass for these lysozymes is about 14 kDa. Our peptide originated from a band suggested by SDS−PAGE to be approximately 60 kDa (Table 2). Recently, a lysozyme from the Coleopteran *T. casteum* was predicted from genomic sequencing. A search for lysozymes from insects revealed a lysozyme from *Culex quinquefasciatus* with a molecular mass of 96 kDa, while that from *T. casteum* is 66 kDa. These findings suggest that the 60 kDa protein eluted from PF2-Sepharose could correspond to a lysozyme from *Z. subfasciatus*. It is important to point out that lysozymes from *T. casteum* and *C. quinquefasciatus* each have one putative glycosylation site (N294 and N624, respectively). There have also been reports of glycosylated forms of the hen egg white lysozyme.[34] Interestingly, studies by others indicate that human lysozyme mutated to introduce a N-glycosylation site resulted in a glycosylated enzyme with increased stability.[34,35] A lysozyme from *Z. subfasciatus* has not been reported to our knowledge. However, we have isolated a protein by lectin affinity chromatography that identifies a lysozyme. It is therefore tempting to speculate that the lysozyme of *Z. subfasciatus* is a glycoprotein and that a component of insecticidal action of PF2 could be interference with insect immunity or with its digestive function, breaking down ingested bacteria in the gut.

In conclusion, PF2 recognizes several proteins in the midgut of *Z. subfasciatus*, some of which might be potential factors related to its insecticidal mechanism. However, further studies are necessary to support this conclusion.

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Notes

The authors declare no competing financial interest.

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