Centro de Investigación en Alimentación y Desarrollo, A.C.

GLICOPROTEOMA DEL INTESTINO DE *Zabrotes subfasciatus* ASOCIADO AL MECANISMO INSECTICIDA DE LA LECTINA PF2 DE *Olneya tesota*

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Resumen

Las pérdidas en el frijol almacenado provocadas por insectos continúan siendo un grave problema en México y en América Latina. Asociado a esto, el control químico de las larvas resulta en la contaminación del grano y posibles daños al consumidor. Bajo este escenario, se planteó el presente estudio enfocado en la glicoproteómica del intestino de *Zabrotes subfasciatus* (plaga del frijol) y la identificación de glicoproteínas involucradas en el mecanismo insecticida de la lectina PF2. El intestino de insectos, como el de otros animales, es rico en glicoconjugados. Éstos tienen funciones biológicas muy importantes en el crecimiento y desarrollo de los organismos. Las lectinas que reconocen estos glicoconjugados pueden interferir con sus funciones, teniendo efectos insecticidas. En insectos-plaga es muy importante conocer la composición, distribución y el rol fisiológico de estos glicoconjugados a fin de evaluar el efecto insecticida de lectinas. La lectina PF2 de *Olneya tesota* ha demostrado tener actividad insecticida contra *Zabrotes subfasciatus* (plaga del frijol). En este estudio se lograron identificar potenciales receptores para PF2 en el intestino del insecto. Mediante el uso de técnicas cromatográficas (afinidad e intercambio iónico) y electroforéticas (1D y 2D PAGE) se aislaron y purificaron las glicoproteínas solubles y de membrana reconocidas por PF2 y con el uso de la espectrometría de masas y búsquedas en bancos de secuencias, se obtuvo la identidad de las glicoproteínas. Los resultados mostraron proteínas relacionadas con la estructura celular (tubulina y actina), proteínas sensoriales, del sistema de defensa (lisozima), proteínas mitocondriales (ATPasa, citocromo oxidasa, porina), involucradas en los procesos de digestión (α-amilasa) y a una glicoproteína tipo factor de crecimiento (*imaginal disc growth factor*, IDGF). Además, algunas de estas glicoproteínas solo están presentes en determinado estado larval del insecto. La identificación de los receptores de PF2 en *Z.*

subfasciatus no sólo contribuye a ampliar el conocimiento de su mecanismo de acción insecticida, sino también proporcionará nueva información sobre las glicoproteínas del intestino del insecto.

Palabras clave: lectina PF2, glicoproteínas del intestino de insectos, LC-MS/MS.

Abstract

Losses caused by insects to stored beans remain a serious problem in Mexico and Latin America. Chemical control of larvae results in contamination of grains and possible toxicity to consumers. Under this scenario, this study focused on the glycoproteomic of the zabrotes midgut and the identification of glycoproteins involved in the insecticide mechanism of PF2 lectin. The gut of insects, as well as other animals, is rich in glycoconjugates that have important biological functions required for growth and development. Lectins (carbohydrate binding proteins) can interact with these glycoconjugates resulting in insecticidal effects. In order to evaluate the effect of insecticidal lectins in pests, it is important to know the composition, distribution and physiological role of the glycoconjugates present in the gut. PF2 lectin from *Olneya tesota* has insecticidal activity against *Zabrotes subfasciatus* larvae (bean pest). In this work, we evaluated the binding of PF2 to midgut glycoproteins using chromatography (ion exchange and affinity) and electrophoretic techniques (1D and 2D-gel electrophoresis) followed by lectin blotting developed with biotinylated PF2. After protease treatment and analysis by LC-MS/MS (of peptides) the identified glycoproteins were related with cellular structure (tubulin and actin), an odorant receptor, defense system (lysozyme), energy metabolism (ATPase, oxidase cytochrome, porin), digestive system (amylase) and an imaginal disc growth factor. In addition, some of these glycoproteins were present only during certain larval stages of the insect. The potential of these glycoproteins to serve as part of the mechanism involved in the insecticidal activity of PF2 to *Z. subfasciatus* is discussed. Also, these results provide new information on insect glycoproteins.

Keywords: PF2 lectin, insect midgut glicoproteins, LC-MS/MS

Sinopsis

Zabrotes subfasciatus (Boheman) (Coleoptera, Bruchidae), conocido comúnmente como el gorgojo del frijol mexicano, es la principal plaga que ataca al frijol almacenado en América Latina. El control de esta plaga es mediante el uso de insecticidas químicos, pero debido a que su residualidad provoca daños al medio ambiente y a la salud, su empleo está cada vez más restringido (Vasconcelos y Oliveira, 2004). En las últimas décadas diversas investigaciones han sido enfocadas a la búsqueda y aplicación de moléculas con actividad insecticida que se encuentren de forma natural en las plantas, tales como las lectinas (Vandenborre *et al.*, 2011b). Estas son definidas como proteínas o glicoproteínas que se unen a carbohidratos simples o complejos de manera específica y reversible (Sharon, 2007).

A la fecha se sabe que las lectinas pueden tener diferentes modos de acción insecticida y estos pueden estar en función del receptor (glicoproteína) a la cual se unan. Disciplinas como la proteómica y la glicoproteómica han sido piezas clave para identificar receptores de lectinas insecticidas (Capitulo I).

En nuestro grupo de trabajo se han estudiado a las lectinas de leguminosas silvestres del desierto de Sonora y se ha probado que la lectina PF2 purificada de las semillas de *Olneya tesota* (Palo fierro), es tóxica para larvas de *Z. subfasciatus* (Lagarda-Diaz *et al.*, 2009).

PF2 es una lectina que reconoce carbohidratos complejos y mediante estudios de histoquímica se observó que reconoce estructuras en el intestino de *Z. subfasciatus*. Lo anterior, aunado a que PF2 es resistente a la proteólisis *in vitro*

por enzimas digestivas de *Z. subfasciatus* forman parte de su mecanismo insecticida (Lagarda-Diaz *et al.*, 2009). El resistir la proteólisis en el intestino, les permite a las lectinas no ser degradadas y mantenerse biológicamente activas permitiéndoles interactuar con estructuras glicosiladas en el intestino del insecto (ChrispeelsyRaikhel, 1991). La función biológica de estructuras glicosiladas, tales como las glicoproteínas, puede ser afectada por la unión de lectinas.

De acuerdo a lo anterior, la hipótesis planteada para este trabajo fue que las glicoproteínas presentes en el intestino medio de *Z. subfasciatus* reconocidas por la lectina PF2 son importantes para la sobrevivencia del insecto. Estas glicoproteínas pueden encontrarse ya sea de forma soluble dentro o fuera de las células, ó incorporadas en las membranas celulares.

Con la finalidad de ampliar el conocimiento acerca del mecanismo insecticida de PF2, el objetivo planteado para este trabajo fue caracterizar el glicoproteoma del intestino de *Z. subfasciatus* asociado al mecanismo insecticida de la lectina PF2. Para lo anterior, fue indispensable identificar las glicoproteínas del intestino de *Z. subfasciatus* que son reconocidas por la lectina PF2. El esquema general de este estudio está representado en la figura 1.

Utilizando cromatografía de afinidad con PF2 acoplada, se purificaron las glicoproteínas solubles que son reconocidas por PF2, a partir de larvas de 20 días de edad. Mediante espectrometría de masas y búsquedas en bancos de secuencias se lograron identificar proteínas relacionadas con la estructura celular, proteínas sensoriales, sistema de defensa y proteínas involucradas en la cadena de transporte de electrones. Algunas de estas glicoproteínas no habían sido reportadas en *Z. Subfasciatus* , por lo que este estudio proporciona nueva información acerca de las glicoproteínas del insecto (Capitulo II).

Por otra parte, trabajos anteriores han sugerido que PF2 podría interferir con enzimas digestivas del insecto tales como la α-amilasa, por lo que en este

trabajo se decidió abordar el efecto de PF2 sobre la amilasa de *Z. subfasaciatus*. Los resultados mostraron que PF2 reduce la actividad de amilasas en un 40%. Además, tres isoformas con actividad de α-amilasa fueron detectadas (α-1, α-2, α-3), de las cuales sólo una de ellas (α-3) presenta el carbohidrato reconocido por PF2. La identidad de amilasa reconocida por PF2 fue confirmada utilizando un anticuerpo anti-amilasa y por espectrometría de masas. Estos resultados sugieren que la disminución en la actividad de α-amilasa podría ser atribuida a la unión de PF2 a α-3. Si esta forma glicosilada de amilasa (α-3) es la principal en las etapas tempranas del desarrollo larval del insecto, la unión de PF2 a la enzima representaría su principal mecanismo insecticida; de lo contrario constituiría sólo una parte del mecanismo insecticida de PF2 (Capítulo III).

Se ha reportado que lectinas insecticidas pueden unirse a proteínas unidas a las membranas celulares. Haciendo uso de las condiciones adecuadas de extracción de proteínas de membrana, en este estudio, se obtuvieron dichas proteínas del intestino de *Z. subfasciatus*. La unión de PF2 a las proteínas de membrana del intestino fue evaluada mediante electroforesis en dos dimensiones, seguida por una lectino-detección con PF2 biotinilada. Las glicoproteínas detectadas fueron analizadas mediante espectrometría de masas y los resultados apuntaron a proteínas mitocondriales, estructurales y a una glicoproteína tipo factor de crecimiento (Capítulo IV). Esta última es de particular importancia para el insecto debido a que se expresa en determinada etapa del desarrollo larval.

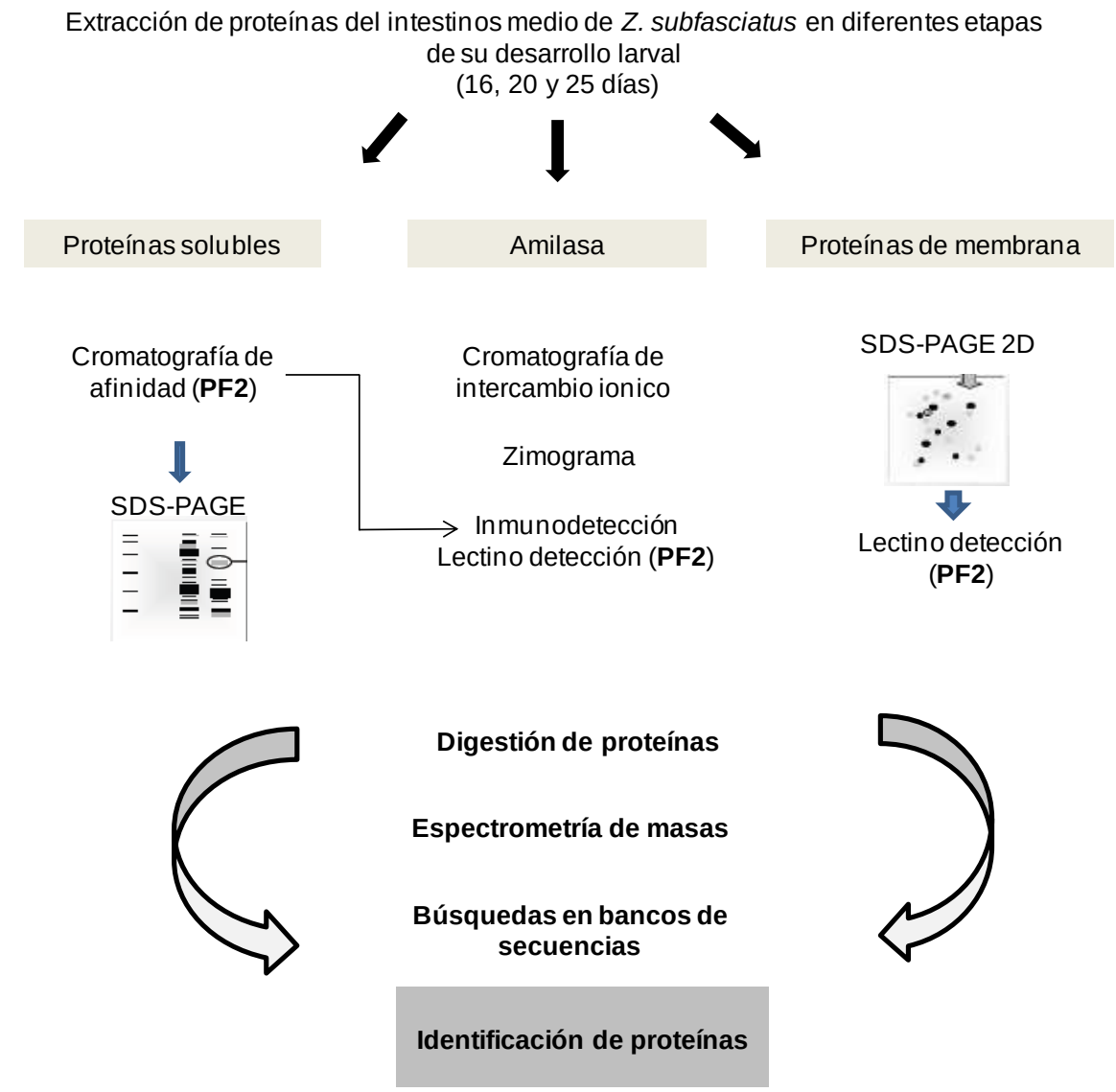

Figura 1. Esquema general de trabajo.

Conclusión

El presente estudio demuestra que el mecanismo insecticida de la lectina PF2 en *Z. subfasciatus* involucra la interacción con glicoproteínas solubles y de membrana del intestino del insecto, las cuales poseen funciones muy importantes en el metabolismo energético, estructura celular, como promotoras del crecimiento y en los procesos digestivos.

En un primer contacto PF2 podría reconocer receptores del olfato del insecto y alterar su funcionamiento. Posteriormente, PF2 puede unirse a una de las principales enzimas digestivas del insecto como la α-amilasa glicosilada, la cual se puede encontrar de forma soluble o adherida a la membrana (como lo se mostró en este trabajo) e inhibir su actividad. Además, PF2 puede reconocer la actina presente en los filamentos de las células epiteliales, la cual ha sido relacionada con el modo de acción insecticida de las tóxinas Cry de *B. thuringiensis.* Por último la internalización de en las células epiteliales podría resultar en la unión de la lectina a proteínas mitocondriales, las cuales son piezas clave en el metabolismo energético.

Este estudio muestra que las lectinas tienen mecanismos insecticidas multifactoriales, dependientes de la etapa de desarrollo larvario del insecto y de la expresión de sus glicoconjugados.

CAPITULO I

ANTECEDENTES

DAÑOS E IMPORTANCIA ECONÓMICA *DE ZABROTES SUBFASCIATUS* COMO PRINCIPAL PLAGA DEL FRIJOL

El frijol es uno de los productos agrícolas con mayor importancia socioeconómica en México, su producción anual alcanza los 8 millones de toneladas y genera ganancias con un valor de 10 millones de pesos (SAGARPA, 2010). Además, el frijol es considerado un alimento básico en la población ya que es una fuente accesible de proteínas, carbohidratos, minerales, vitaminas del complejo B y fibra (Hellendoorn, 2007).

Durante su almacenamiento, la estabilidad del frijol se ve afectada por el ataque de diversas plagas, las cuales provocan pérdidas no sólo en la cantidad, sino también en la calidad de la semilla (Sandoval, 1984). En el almacenamiento, la ineficiencia en el control de plagas es tal, que las perdidas en el frijol almacenado pueden llegar hasta el 35% (Permul *et al.*, 2008).

Z. subfasciatus figura dentro de las principales plagas que ataca al frijol almacenado. Las hembras depositan los huevos sobre la semilla y posteriormente las larvas perforan el grano para desarrollarse dentro de él (Southgate, 1979). El daño causado favorece la entrada de hongos y bacterias y por tanto, la aparición de pudriciones secundarias provocadas principalmente por algunas especies de *Penicillium* sp y *Aspergillum*. Algunas especies de *Aspergillus* que atacan a los granos como *A. flavus* y *A parasiticus* producen micotoxinas (Schoonhoven *et al.*, 1988). Debido a lo anterior, éstos insectos no sólo pueden provocar pérdidas del alimento, sino también un posible daño toxicológico debido al consumo de micotoxinas (Robinson, 2005).

MECANISMOS INSECTICIDAS DE LECTINAS DE PLANTAS

A la fecha, existe escasa información acerca del mecanismo de acción insecticida de las lectinas. Sin embargo, un prerrequisito importante es que la lectina resista los ambientes proteolíticos del intestino del insecto. Una vez en él, las lectinas pueden: (a) unirse a glicoconjugados expuestos en las células epiteliales a lo largo del intestino, provocando alteraciones en la estructura y metabolismo de estas células (Chrispeels y Raikhel, 1991;Peumans y Van Damme, 1995); (b) unirse a la membrana peritrófica (presente en la mayoría de los insectos y que separa el lumen de las células epiteliales), impidiendo la absorción de nutrientes (Eisemann y Binnington, 1994); (Zhu-Salzman *et al.*, 2003); (c) unirse a enzimas glicosiladas, inhibiendo los procesos digestivos (Macedo *et al.*, 2007) (Figura 1).

Estudios de inmunohistoquímica han demostrado que las lectinas pueden llegar a los cuerpos de grasos, tubos de malfigio, ovarios y hemolinfa de los insectos e inducir una variedad de efectos sistémicos y actuar directa o indirectamente sobre otros órganos (Fitches *et al.*, 2001). La presencia de lectinas en ovarios ha sido relacionada con efectos en la fecundidad del insecto (Gatehouse *et al.*, 1998). También se piensa que las lectinas se unen al aparato quimiosensor de los herbívoros, bloqueando las señales químicas de los alimentos e interfiriendo así con la absorción de nutrientes y provocando cambios en los patrones de expresión de las células intestinales.

La unión de las lectinas a glicopoteínas, puede provocar diferentes efectos insecticidas tales como el bloqueo de receptores o transportadores, activación o inactivación de enzimas ó provocar efectos miméticos (Sadeghi *et al.*, 2008b;

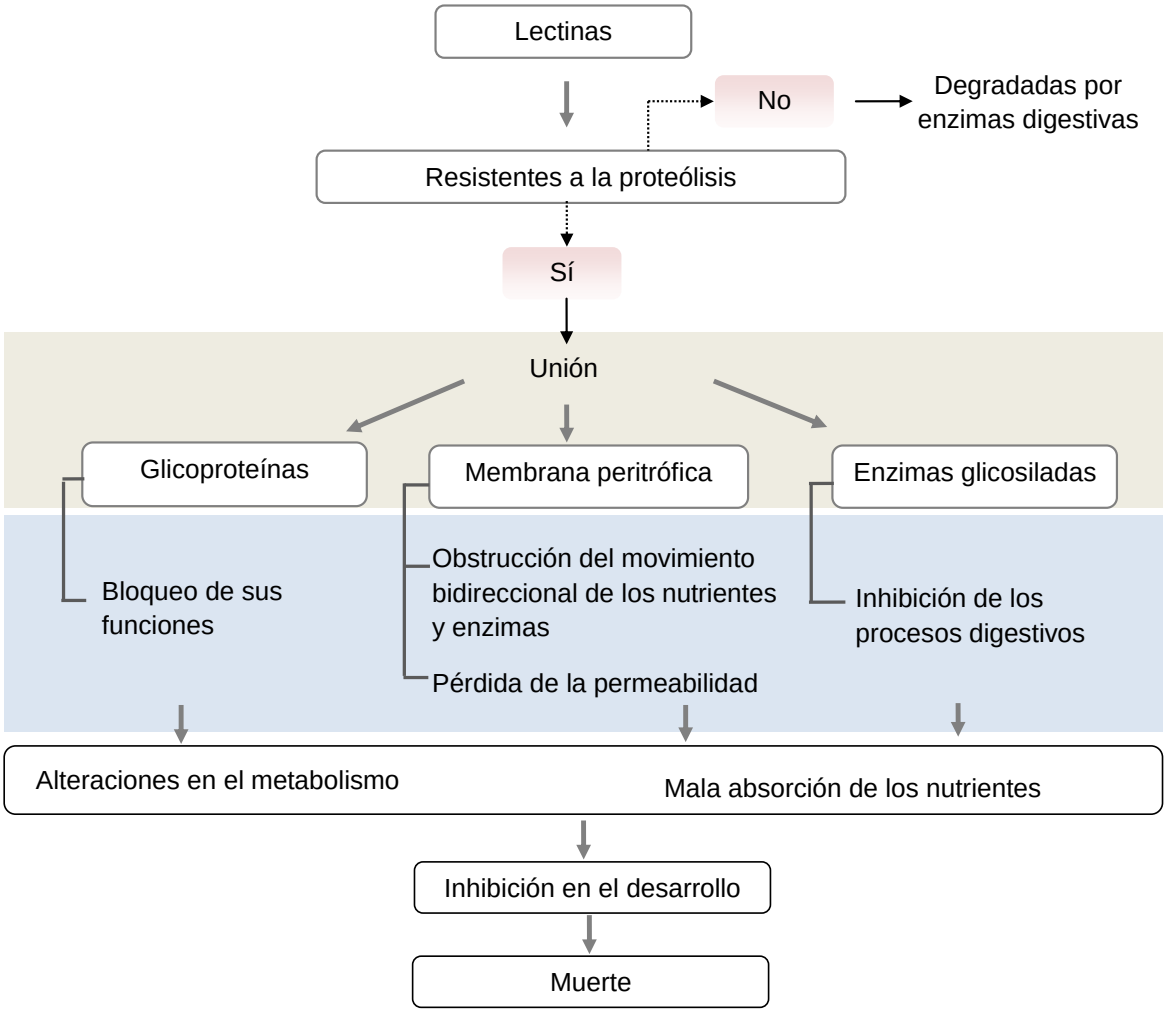

Figura 2. Componentes del mecanismo insecticida de lectinas

Shahidi-Noghabi *et al.*, 2009). Por ejemplo se ha reportado que la lectina *Galantus nivalis* (GNA) reconoce a una subunidad de ferritina (glicosilada con manosa) en el intestino de larvas de *Nilaparvata lunges*. Dicha unión sugiere que el mecanismo insecticida de GNA involucra la interferencia de la lectina en el metabolismo de hierro del insecto (Du *et al.*, 2000). Otro mecanismo insecticida para GNA ha sido reportado en el mosquito *Acyrthosiphon pisum*, GNA al igual que *Canavalia ensiformis* (Con A) reconocen una aminopeptidasa (altamente glicosilada con manosas) unida a las membranas microviliares del intestino del insecto, la unión de las lectinas a la enzima inhibe su actividad provocando un decremento en la absorción de aminoácidos (Cristofoletti *et al.*, 2006).

Los receptores de lectinas en el intestino de insectos también pueden ser carbohidratos que se encuentren formando polisacáridos tales como la quitina (residuos de N-acetil-glucosamina) (Cohen, 1993). Este polisacárido es el principal componente de la membrana peritrófica (MP) presente en la mayoría de los insectos, a excepción de los homópteros (Tellam *et al.*, 1999). El mecanismo insecticida de lectinas como la aglutinina de germen de trigo (WGA) involucra su unión a los residuos de N-acetil-glucosamina de la quitina, dicha unión obstruye los poros de la MP, reduce su permeabilidad y daña su integridad (Cohen, 1993) .

Por otra parte, la sensibilidad de diferentes especies de insectos a los efectos insecticidas por la ingestión de lectinas puede variar. Así, existen lectinas que sólo retrasan el desarrollo del insecto y otras que tienen efectos letales. El efecto insecticida va depender: a) de la resistencia de la lectina a la proteólisis por enzimas digestivas del insecto; b) de su especificidad a los glicoconjugados del intestino, la importancia fisiológica de estos glicoconjugados y la región del intestino a la que se une la lectina (Bandyopadhyay *et al.*, 2001); c) del historial dietario del insecto, la composición de la dieta, la concentración de lectina

administrada, así como de la frecuencia del consumo (Macedo *et al.*, 2002; Sadeghi *et al.*, 2008a).

De esta manera, el mecanismo insecticida de una lectina en particular va estar en función del tipo de glicoproteína a la cual se una, que a su vez va ser dependiente del patrón de glicosilación del insecto, el cual puede cambiar según la etapa de desarrollo, por la infección por patógenos, variaciones en la dieta u otros factores.

USO DE LECTINAS EN EL CONTROL DE PLAGAS DE INSECTOS

Se han introducido los genes que codifican para lectinas en plantas que son susceptibles al ataque de insectos. Lo anterior con la finalidad de volver a las plantas resistentes al ataque de plagas. Lectinas como Con A*,* ACA (*Amaranthus caudatus),* GSII (*Griffonia simplicifolia*), WGA, ASA (*Allium sativum*) y GNA han incrementando la resistencia de la planta al ataque de insectos. La lectina de GNA, específica para manosa, se ha expresado genéticamente en chícharo, arroz, caña de azúcar y tomate, protegiendo efectivamente a la planta del ataque de plagas (Vasconcelos y Oliveira, 2004). Plantas transgénicas de arroz que expresaban a la lectina GNA demostraron resistencia a *Nephotettix virescens* y *N. lugens,* disminuyendo la sobrevivencia del insecto hasta en un 50% (Foissac *et al.*, 2000; Nagadhara *et al.*, 2003). La lectina *Pisium sativum* expresada en semillas de oleaginosas reduce el crecimiento de larvas del gorgojo *Meligethes aeneus*. De manera similar, la lectina *Amaranthus caudatus* (ACA) expresada en plantas de algodón transgénicas inhibe el desarrollo de *Aphis gossypii* (Wu *et al.*, 2008). La lectina de la hoja de *Allium sativum* (ASAL) ha sido expresada en plantas de arroz y ha demostrado tener un efecto negativo en la sobrevivencia y en la fecundidad de *N. virescens* y *N. lunges* (Yarasi *et al.*, 2008).

Además de alimentarse de los cultivos, algunos insectos plaga sirven como vectores de enfermedades virales para las plantas. Las lectinas, además de brindar resistencia a las plantas contra el ataque de insectos, pueden ayudar a disminuir la transmisión de enfermedades virales. Plantas transgénicas de arroz que expresaban ASAL tuvieron una menor incidencia a la infección por el virus del tungro, después del ataque de *N. virescens* (Saha *et al.*, 2006).

Por otra parte, los niveles de expresión de lectinas en plantas pueden ser variables, incluso diferentes niveles de expresión se han observado en replicas de clones. Algunas de las variaciones pueden estar en función de las condiciones de cultivo. Alternativamente, las variaciones entre las replicas de clonas puede ser consecuencia del grado de metilación, el cual va aumentando conforme el desarrollo de la planta y como consecuencia, pueden hacer la expresión del transgen mas receptiva a estímulos ambientales (Meyer, 1995). Se ha observado que la expresión de GNA en papas transgénicas va aumentando durante el desarrollo de la planta, siendo las plantas maduras los que expresan los niveles más altos, y estos están correlacionados con la resistencia de la planta al ataque de *Lacanobia oleracea* (Down *et al.*, 2001). Esto es muy importante ya que el grado de toxicidad de algunas lectinas está relacionado con su concentración en la dieta del insecto.

Por otra parte, la toxicidad de algunas lectinas restringe su uso como factores de resistencia para plantas. Mientras unas lectinas son moderadamente tóxicas, otras pueden ser altamente tóxicas, tales como la ricina y abrina (Endo *et al.*, 1987; Wu *et al.*, 2001) . La lectina GNA es considerada no toxica para mamíferos porque su unión al yeyuno es muy baja (Pusztai *et al.*, 1990). No obstante, estudios realizados en ratas alimentadas con papas transgénicas que expresaban a la lectina GNA, demostraron lesiones estomacales, cambios en el revestimiento del colon y un efecto como factor de crecimiento. Sin embargo, el daño no fue atribuido a la lectina sino al proceso de ingeniería genética utilizado para expresarla, específicamente a un promotor llamado CaMV. No obstante,

en otros estudios no se ha observado un efecto negativo del promotor CaMV. Estudios posteriores demostraron que la introducción de un transgen en las plantas podría provocar cambios importantes en los niveles de expresión de los genes (Down *et al.*, 2001). Además, las proteínas pueden tener modificaciones pos-traduccionales, tales como la glicosilación. Estas modificaciones tienen efectos significativos tanto estructurales como en la función biológica de las proteínas. El patrón de glicosilación en los organismos va depender del paquete de glicosiltransferasas que posea y éste puede ser diferente entre especies. De esta forma el éxito en la expresión de una glicoproteína con función biológica puede depender de su correcta glicosilación que es proporcionada por el sistema de expresión.

La información anterior indica que es necesario generar conocimiento no solo a nivel de proteínas de defensa de plantas, sino también a nivel de composición de carbohidratos y glicoproteínas de insectos. Esto permitiría conocer que proteínas de defensa son especificas sólo para insectos plaga y reduciría el riesgo de afectar a insectos benéficos.

La mayoría de las plantas en las que el humano basa su alimentación contienen cantidades apreciables de proteínas de defensa en sus semillas (lectinas, arcelinas, inhibidores de α.amilasas, entre otras) (Hellendoorn, 2007). En general, estas moléculas son muy resistentes a la desnaturalización por calor. A pesar de que se ha observado que algunas proteínas de defensa pueden tener efectos deletéreos para el humano cuando se encuentran activas (Pusztai, 1989), éstas pueden ser inactivadas mediante exposiciones prolongadas de cocción, por acidez o por degradación proteolítica en el intestino, por ejemplo, la lectina del frijol PHA-E tiene efectos anti-nutricionales o anti-fisiológicos para el humano, no obstante la lectina es inactivada cuando el frijol se somete a cocción por el tiempo necesario.

Por lo tanto, la utilización de proteínas insecticidas es una alternativa para el control de plagas, no sólo en su utilización convencional (insecticidas en polvo), sino también como su uso en plantas modificadas genéticamente que las puedan expresar.

PROTEÓMICA

El término proteoma fue usado por primera vez en 1994 para describir a todo el conjunto de proteínas codificadas en un genoma (Wilkins *et al.*, 1996). Más tarde el término fue ampliado e involucró no sólo a todas las proteínas codificadas, sino también a sus modificaciones e isoformas.

Diversos autores señalan que la proteómica puede dividirse en dos ramas: de expresión y funcional (ChoudharyyGrant, 2004; Kocks *et al.*, 2003). La proteómica de expresión abarca la descripción total de las proteínas expresadas en un organismo ó en un determinado tejido, así como los niveles de expresión diferencial en muestras sometidas a diferentes condiciones. Por su parte la proteómica funcional, involucra el estudio de la función de las proteínas dentro de un sistema, su interacción proteína-proteína, proteína-ADN/ARN, modificaciones postraduccionales y la regulación de su expresión. La expresión de proteínas puede variar de acuerdo al tipo celular y estado de desarrollo, pero también en respuesta a factores externos tales como situaciones de estrés, alimentación ó enfermedades (Blackstock y Mann, 2000).

Herramientas de la Proteómica

La proteómica parte del uso de técnicas de cromatografía y electroforesis para la resolución de proteínas intactas. Posteriormente las proteínas pueden ser digeridas proteolíticamente y analizadas por técnicas de espectrometría de masas (MS) (Yates *et al.*, 2009).

Debido a su alta capacidad de resolución, la electroforesis en dos dimensiones (2D PAGE) es la técnica más utilizada para el estudio del proteoma a partir de muestras complejas de proteínas. La 2D PAGE separa a las proteínas en una primera dimensión de acuerdo a su punto isoeléctrico utilizando tiras con gradientes de pH inmovilizados (IPG). Posteriormente, las proteínas son separadas de nuevo en una segunda dimensión en base a su masa molecular en geles de poliacrilamida (Gorg *et al.*, 2004). De esta manera una gran cantidad de proteínas (3000 a 10,000) pueden ser visualizadas simultáneamente en un gel (Shi y Paskewitz, 2006). La ausencia o presencia puntos (spots) permite hacer comparaciones sobre la expresión diferencial de proteínas provenientes de muestras sometidas a diferentes tratamientos.

El estudio del proteoma también puede ser abordado mediante la combinación de dos o más técnicas de cromatografía o electroforesis. Por ejemplo, las proteínas pueden separarse por cromatografía de afinidad en base a ligandos específicos, seguido por cromatografía de intercambio iónico en base a su carga, para posteriormente ser resueltas mediante 2D PAGE ó digeridas y analizadas por MS para su identificación. Alternativamente, las proteínas también pueden ser identificadas mediante secuenciación del extremo N-terminal, detección con anticuerpos específicos, composición de aminoácidos, co-migración con proteínas conocidas, y sobre-expresión y deleción de genes (Gevaert y Vandekerckhove, 2000).

Debido a su rapidez y elevada sensibilidad, la MS se ha convertido en una pieza clave para la identificación de proteínas (Yates *et al.*, 2009). Gran parte del éxito de la proteómica depende de la capacidad de la MS para lograr identificar cantidades de proteínas cada vez más pequeñas a partir de muestras complejas.

En la MS, los péptidos trípticos obtenidos a partir de la digestión proteolítica de proteínas son convertidos en iones en fase gaseosa mediante técnicas de ionización suave. Los dispositivos de MS no sólo son capaces de medir la masa, sino también de fragmentar los péptidos trípticos. Los espectros de fragmentación son comparados con las masas peptídicas teóricas de proteínas presentes en bases de datos para revelar la identidad de la proteína (Yates *et al.*, 2009)

Por lo tanto, el desarrollo de técnicas de MS, así como la secuenciación de genomas a gran escala y el desarrollo de bases de datos de proteínas, son esenciales para el estudio del proteoma.

GLICOPROTEÓMICA

Las glicoproteínas tienen funciones muy importantes en el reconocimiento biológico, transmitiendo mensajes entre las células o dentro de ella. Por ello, diversos grupos de investigación se han enfocado en su estudio. La glicoproteómica es la disciplina encargada estudiar el perfil de proteínas glicosiladas en un sistema biológico. La glicoproteómica parte del enriquecimiento de glicoproteínas y de las tecnologías analíticas y computacionales de la proteómica para la identificación sistemática y la cuantificación de glicoproteínas en una muestra compleja (Kim y Misek, 2011).

Es bien conocido que una gran cantidad de las proteínas solubles y de membrana producidas por la célula eucariota, tienen unidos covalentemente diversas estructuras de glicanos (RuddyDwek, 1997). Dicha glicosilación es altamente específica y puede variar de acuerdo al organismo, etapa de desarrollo y estado fisiológico. Los tipos de glicosilación más comúnes y ampliamente estudiados son la N- y O- glicosilación. La primera se basa en la unión de una N-acetilglucosamina a la amida de una asparagina en una

secuencia consenso de Asn-X-Ser/Thr (X puede ser cualquier aminoácido excepto prolina). La segunda, parte de la unión de una N-acetilgalactosamina al grupo hidroxilo de una serina o treonina (YaremayBertozzi, 2001).

Herramientas de la Glicoproteómica

El análisis de la glicoproteómica puede resultar sumamente complejo debido a las extensas combinaciones y a la flexibilidad en las estructuras de glicanos presentes en una glicoproteína, al rango dinámico en la conformación espacial de las proteínas y a la relación compleja del glicano con la proteína correspondiente (Pan *et al.*, 2011b).

Metodológicamente, el estudio del glicoproteoma consiste en el aislamiento de glicoproteínas, su enriquecimiento (glicoproteínas/glicopéptidos), detección, digestión proteolítica, identificación de la proteína y sus sitios de glicosilación ó estructuras de glicanos, mediante el uso de técnicas de MS (Huang *et al.*, 2005; Pan *et al.*, 2011a) (Figura 2).

Al igual que la proteómica, la glicoproteomica hace uso de técnicas cromatográficas (intercambio iónico, exclusión molecular, interacción hidrofóbica y afinidad) y electroforéticas (1D y 2D PAGE), para la separación de glicoproteínas. La cromatografía de afinidad con lectinas es por excelencia la técnica utilizada para el enriquecimiento y purificación de glicoproteínas. Ésta se basa en la unión específica y reversible de las lectinas a los glicanos ligados a las proteínas (Cummings, 1994; Taketa, 1998). Las lectinas también se usan para detectar glicoproteínas en muestras complejas (lectino-detección). Las proteínas que son separadas mediante 1D ó 2D PAGE pueden ser transferidas a membranas, para posteriormente ser detectadas mediante técnicas de inmunodetección pero usando lectinas marcadas.

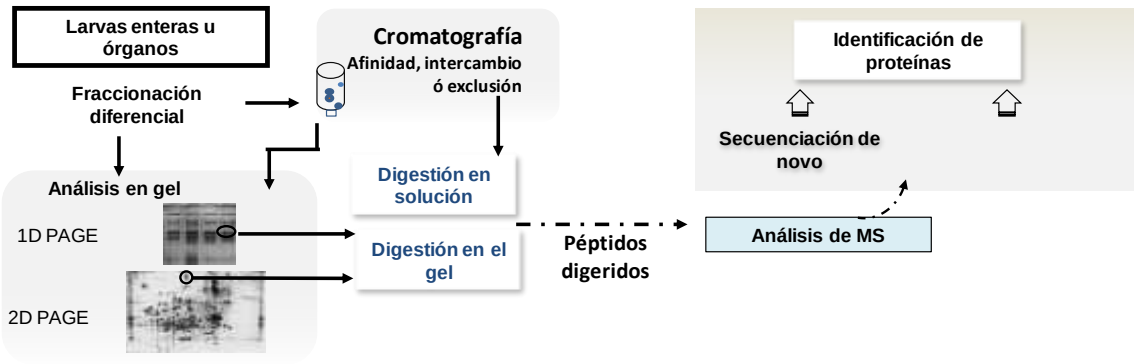

Figura 3. Esquema general del estudio de la proteómica.

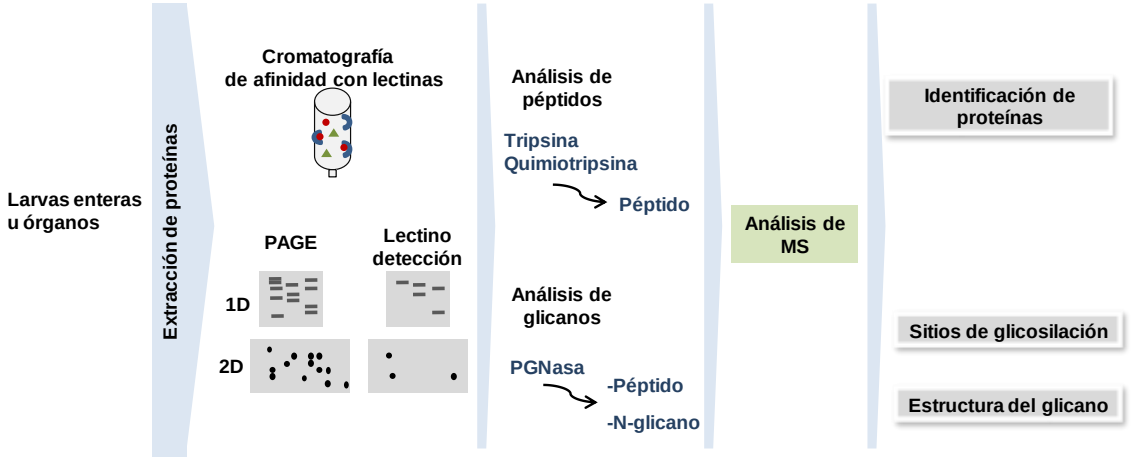

Figura 4. Esquema general del estudio de la glicoproteómica

CONTRIBUCIÓN DE LA PROTEÓMICA Y LA GLICOPROTEÓMICA EN EL ESTUDIO DE INSECTOS PLAGA

Como lo describe (Agrawal, 2011) en su revisión, 72 proyectos de secuenciación del genoma de insectos se encuentran vigentes; de éstos, sólo 2 han sido completados, 45 se encuentran fase de montaje y 25 se encuentran en curso (NCBI Entrez Genome Project-Corriente mayo de 2011). Gracias a la secuenciación del genoma de insectos y a los adelantos en MS, el estudio del proteoma y glicoproteoma de insectos ha aumentado notablemente en los últimos diez años (Tabla 1).

A la fecha el proteoma de *Drosophila melanogaster* es el más estudiado; alrededor del 63% de las proteínas codificadas en su genoma ha sido secuenciado (Brunner *et al.*, 2007). El proteoma de *D. melanogaster* ha sido estudiado a través del análisis de diferentes tipos de células y estados de desarrollo larval del insecto. Para ello se usa el fraccionamiento multidimensional con el fin de reducir la complejidad de las muestras (Veraksa, 2010). Estudios similares se han hecho en insectos como *Acyrthosiphon pisum*, *Apis mellifera*, *Bomyx mori, Tribollium castaneum,* entre otros (Morris *et al.*, 2009; Pauchet *et al.*, 2008; Popova-Butler y Dean, 2009; Zhang *et al.*, 2011)

La proteómica en insectos puede ser utilizada para investigar diferencias en la expresión de proteínas en respuesta al desarrollo del insecto utilizando líneas celulares y para estudiar la expresión de proteínas en órganos específicos (Shi y Paskewitz, 2006). En insectos plaga, la mayor parte de las investigaciones se dirigen al estudio del proteoma intestinal (Morris *et al.*, 2009; Pauchet *et al.*, 2008; Popova-Butler y Dean, 2009; Zhang *et al.*, 2011). Lo anterior debido a que este sitio se considera, el principal objetivo de estudio para el control de insectos plaga. A través de la proteómica ha sido posible explorar los componentes del intestino de insectos y lograr una mejor comprensión de su funcionamiento. Así mismo, la proteómica ha facilitado la identificación de

receptores potenciales en el intestino que puedan ser usados como blancos para moléculas insecticidas. Por ejemplo, la identificación de proteínas receptoras en el intestino de insecto de las toxinas Cry de *Bacillus thuringiensis* ha sido posible gracias a la proteómica (Fernandez-Luna *et al.*, 2010).

Por su parte la glicoproteomica no sólo ha facilitado el estudio de la glicosilación en insectos, sino que también ha permitido la identificación de receptores de lectinas insecticidas. Las lectinas con diferente especificidad se usan para capturar glicoproteínas de insectos con diferentes perfiles de glicanos. Lo anterior se logra inmovilizando a las lectinas en diferentes soportes químicos y poniéndolas en contacto con extractos obtenidos del insecto. De esta manera se han logrado purificar glicoproteínas provenientes de *D. melanogaster* (Diptera), *Tribollium castaneum* (Coleoptera), *Bombyx mori* (Lepidoptera), *Apis mellifera* (Hymenoptera) y *Acyrthosiphon pisum* (Hemiptera), etc.) (Vandenborre *et al.*, 2011a; Vandenborre *et al.*, 2010).

Las glicoproteínas capturadas y sus glicanos se identifican y caracterizan por MS. Varios estudios revelan que muchas de estas glicoproteínas están involucradas en procesos tales como comunicación celular, respuesta a estrés, transporte trans membranal e incluso se han encontrado enzimas relacionadas con una amplia gama de procesos metabólicos y funciones celulares (Vandenborre *et al.*, 2011a; Vandenborre *et al.*, 2010).

La glicoproteómica también ha ayudado a caracterizar el perfil de glicosilación de insectos. En insectos, como en otras especies, la glicosilación de proteínas varía de acuerdo a la etapa de desarrollo y al estado reproductivo (Li *et al.*, 2007). Por ejemplo, en *D. melanogaster* el perfil de N-glicanos varía durante su desarrollo, sugiriendo que la regulación específica en el perfil de glicosilación de una proteína puede estar asociada con su función biológica en determinada etapa del desarrollo del insecto.

Por lo tanto, la proteómica y glicoproteómica permiten estudiar como la expresión de proteínas es modulada en respuesta a un conjunto de factores tales como infecciones, alimentación, etapa de desarrollo, entre otros. Lo anterior, acompañado con la determinación de la función de proteínas ó glicoproteínas, son piezas clave para comprender mecanismo celular y por tanto la fisiología de insectos.

Tabla 1. Estudios del perfil proteómico y glicoproteómico en insectos

Perfil	Insecto	Muestra	Método	Referencia
Proteómico	*Drosophila melanogaster*	intestino hemolinfa embriones	2D PAGE MALDI-TOF	(Li *et al.*, 2007) (Karlsson *et al.*, 2004)
	Tribolium castaneum	intestino	2D PAGE MALDI-TOF	Morris et al., 2009
	Anopheles gambiae	cerebro, hemolinfa glándulas salivales	2D PAGE, MS SDS-PAGE LC-MS/MS MALDI-TOF,	Paskewitz and Shi, 2005 Chun et al., 2000 Kalume et al., 2005
	Anopheles Stephensi	intestino medio	2D PAGE	Kotsyfakis et al., 2005 Prevot et al., 1998
	Aedes aegypti	tejido larval cuerpos de grasa Vesículas de membrana del borde del cepillo de células epiteliales.	2D PAGE, MALDITOF 2D PAGE 2D PAGE LS/LS MudPIT	Biron et al, 2005 Shih and Fallon, 2001 Popova-Butler et al., 2009
	Bombyx mori	hemolinfa, intestino medio y cuerpos de grasa	2D PAGE, MALDI-TOF	Wang et al., 2004
	Papilio xuthus	intestino medio	2D PAGE MALDI-TOF-MS 2D PAGE LC-MS/MS	Zhang et al., 2010 (Zhu *et al.*, 2009)
	Helicoverpa armigera	Plasma intestino	2D PAGE MS/MS novo	(Pauchet *et al.*, 2008)

Glicoproteómico	*Drosphophila melanogaster*	Insecto completo	Cromatografía con lectinas	(Vandenborre *et al.*, 2010)
	Tribollium castaneum *Bombyx mori* *Apis mellifera* *Acyrthosiphon pisum*	Larvas e insectos adultos completos	Cromatografía con lectinas y LC-MS/MS	(Vandenborre *et al.*, 2011a)

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CAPITULO II

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

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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).

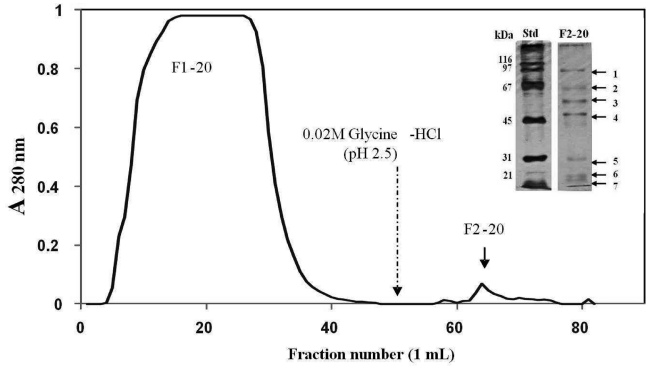

Figure 1. Affinity chromatography using PF2-Sepharose of *Z. subfasciatus* midgut protein extracts from 20-day-old larvae. PF2-Sepharose was prepared and packed, animals were dissected, and proteins were extracted as described in the Materials and Methods. The gel was equilibrated with PBS (pH 7.2) and 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

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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).

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obectus       ------------------IISQESGKKEAFGTLGMIYAMMAIGLLGFVVWAHHMFTVGMDV 43
subfasciatus  ---------------ISHIISQESGKGEAFGTLGMIYAMMAIGLLGFVVWAHHMFTVGMDV 46
tenuicornis   HPEVYILILPGFGMISHIISQESGKKETFGSLGMIYAMMAIGLLGFVVWAHHMFTVGMDV 60
                        ******** *:**:****************************

obectus       DTRAYFTSATMIIAVPTGIKVFSWLATFHGTQILNSPVTLWALGFVFLFTVGGLTGVILA 103
subfasciatus  DTRAYFTSATMIIAVPTGIKVFSWMATFHGTQILYKPVTLWALGFVFLFTVGGLTGVILA 106
tenuicornis   DTRAYFTSATMIIAVPTGIKIFSWLATLHGTQINYSPSMIWALGFVFLFTIGGLTGVILA 120
              ********************:***:**:*****  .*  :**********:*********

obectus       NSSIDIILHDTYYVVAHFHYVLSMGAVFAIMAGIVQWFPLFTGLTLNNFFLKTQFITMFL 163
subfasciatus  NSSIDIVLHDTYYVVAHFHYVLSMGAVFAIMAGIIQWFPLFTGLTLNDYYLKIQFFMMFI 166
tenuicornis   NSSIDVILHDTYYVVAHFHYVLSMGAVFAIMAGLVQWYPLFTGLILNNKLLKIQFLVMFI 180
              *****::**************************::**:****** **:  ** **: **:

obectus       GVNLTFFPQHFLGLSGMPRRYSDYPDAFTIWNAISSIGSIISLTSIIFFLFILWESLSSQ 223
subfasciatus  GVNVTFFPQHFLGLSGMPRRYSDYPDAFTIWNIISSIGSMISLVSIIFFLFILWEAFSMQ 226
tenuicornis   GVNLTFFPQHFLGLAGMPRRYSDYPDAYSTWNIVSSIGSLISLLAIIFFLFIIWDSMISS 240
              ***:**********:************:: ** :*****:*** :*******:*:::  .

obectus       RKSLSTLNVTTSIEWFQALPPS------------------------------------ 245
subfasciatus  RKSISSLSMTTSIEWLQYQPPAE------------------------------------ 249
tenuicornis   RKSINSLNMVTSLEWLQNMPPAEHSYSELPMLSNF------------------------ 275
              ***:.:*.:.**:**:*  **:
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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

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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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CAPITULO III

An amylase as a receptor for PF2 lectin binding in the midgut of *Zabrotes subfasciatus*

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An amylase as a receptor for PF2 lectin binding in the midgut of *Zabrotes subfasciatus*

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Abstract

Alpha-amylases are important enzymes for insect carbohydrate metabolism and larval survival. For this reason, these enzymes are targets for controlling starch-dependent insects. PF2 (*Olneya tesota*) is a lectin that is toxic to *Z. subfasciatus* larvae. In this study, we evaluated the recognition of PF2 by α-amylases from *Z. subfasciatus* midgut and the effects of PF2 on amylase enzyme activity. PF2 binding caused a 30% decrease in of total *amylase* activity *in vitro*. α-amylase isoforms were isolated from midgut tissues using ion exchange chromatography. Three amylase isoforms were confirmed by in-gel activity; however, only one interacted with anti-amylase antiserum and PF2. The identity of Z*. subfasciatus* α-amylase was confirmed by LC- MS/MS. Our findings strongly suggest that one a-amylase isoform is glycosylated, in such a way that PF2 binding partially prevents starch digestion.

1. Introduction

Zabrotes subfasciatus is an insect that has an important role in the postharvest loss of the common bean (*Phaseolus vulgaris*). Larvae grow and feed inside seeds of grains, which causes severe damage to the seeds and reduction in their nutritional quality (Hall et al., 1997).

Legumes can contain large amounts of enzyme inhibitors, tannins and lectins that can be used against insect pests (Daoust et al., 1985). Lectins, in particular, are proteins or glycoproteins that can reversibly recognize specific mono- or oligosaccharides (Sharon and Lis, 2004). The insecticidal activity of plant lectins is associated with their binding to glycoproteins present in the insect midgut that are important in maintaining the normal function of the gut (Chrispeels and Raikhel, 1991; Du et al., 2000). Others have reported identifying insect midgut receptors for lectins including enzymes such as, aminopeptidase, aldehyde dehydrogenase, α -amylase, alpha-mannosidase, 3-hydroxyacyl-coa dehyrogenase (Cristofoletti et al., 2006; Vandenborre et al., 2011).

Alpha-amylases play a key role in the carbohydrate metabolism of insects inhibition of amylase activity has been shown an effective control mechanism for insect pests (Shade et al., 1994). Alpha-amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are hydrolytic enzymes that catalyze the hydrolysis of α-D-(1,4)-glucan linkage in starch components, glycogen and various other related carbohydrates (Franco et al., 2000; Strobl et al., 1998)(Strobl et al., 1998). Recently, an α-amylase was identified in midgut brush border membrane vesicles of *Anopheles albimanus* that interacted with *Bacillus thuringiensis* Cry4Ba and Cry11Aa toxins (Fernandez-Luna et al., 2010).

Z. subfasciatus larvae, like other insect pests of beans, have a diet rich in polysaccharides, such as starch, and larval survival depends to a large extent on the effectiveness of their α-amylases in starch digestion (Shade et al., 1994). Previously, we reported that PF2 lectin of *Olneya tesota* seeds produced 100% mortality to *Z. subfasciatus larvae* when incorporated into an artificial diet at a level 0.5% w/w (Lagarda-Diaz et al., 2009). The aim of this study was to evaluate the recognition of PF2 by α-amylases from Z*. subfasciatus* and the effect of PF2 on amylase enzyme activity.

2. Materials and methods

2.1. Insects.

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

2.2. Plant Material.

Seeds of *O. tesota* were collected from mature trees located in the Sonora Desert, in 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 in paper bags.

2.3. PF2 Lectin Purification.

PF2 lectin was purified according to Vazquez-Moreno et al., 2000. Briefly, *O. tesota* seeds were grounded and meals defatted by hexane extraction. Hexane was removed by aeration under a chemical hood. 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 800g 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. Briefly, the crude extract (15 mL) was injected onto an agarose-fetuin column (10 mm × 100 mm), previously equilibrated with PBS [0.02 M KH2PO4/K2HPO4, 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 lectin 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.

*2.4. Preparation of gut lumen soluble proteins***.**

Midguts of 400 larvae (20 days old), selected as described by Rodriguez-Quiroz et al., 2000 were cold-immobilized and dissected in cold 250 mM NaCl solution. Larval midguts were separated using surgical tweezers and gut portions resected and retained (posterior to proventriculus and anterior the Malpighian tubules segments). Only actively feeding larvae with food filling the gut tract were chosen. Midguts were were rinsed in 250 mM NaCl 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 lumen soluble proteins (crude extract) was kept at −80 °C, and the protein concentration estimated using bovine serum albumin as a standard (Lowry et al., 1951).

*2.5. in vitro Effect of PF2 on α-Amylase activity***.**

The effect of PF2 on α-amylase activity of *Z. subfasciatus* (ZSA) larvae was determined using of the Bernfeld method (Noelting and Bernfeld, 1948). The crude extract (2 µl) of midgut of 20 days-old larvae was incubated with varying concentrations of PF2 (25µg/ml, 50 µg/ml and 100µg/ml) in 100mM acetate buffer 20 mM NaCl and 0.2mM $CaCl_2$, pH 5.8 at 30°C. After 3 min of incubation with PF2, 25µl of 1% soluble starch was added, and this preparation was further

incubated at 30°C for 15 min. The assay was stopped by the addition of 100 µl DNS (3,5-dinitrosaliciylic acid) and heated in boiling water for 10 min, cooled and diluted with 1 ml of water and absorbance was measured at 540 nm. The α-amylase activity was expressed in mg maltose liberated/10 min/37°C. The α-amylase inhibitory activity was expressed as a relative a-amylase activity without pre-incubation with the PF2. Assays were carried out in triplicate.

2.6. PF2-Sepharose Affinity Chromatography.

Purified PF2 was cross-linked to glycidol-activated Sepharose 4BCl (PF2-Sepharose) (Hermanson et al., 1992). The crude extract from 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.

2.7. Polyacrylamide Gel Electrophoresis and amylases detection.

The elution obtained by PF2-Sepharose affinity chromatography was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE). An immunoblot assay was performed as described by (Towbin et al., 1979). Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane at 2.5 mA/cm^2 for 40 min, using a semidry blotter (Labconco). Membranes were blocked for 1.5 h with PBS containing 2% bovine serum albumin (BSA). The presence of α-amylase was detected using rabbit polyclonal α-amylase antiserum (1:500) (AmyAnti), (Roche) for 4h and followed by incubation with biotinylated goat anti-rabbit antibody (Bio Rad) for 2h and setreptavidin-peroxidase for 1.5h (Sigma Chemicals Co., St Louis, MO, USA). The color reaction was developed at room temperature (RT) by addition of peroxidase substrate, 0.075% 3,3′-diaminobenzidine 4HCl.

2.8. Isolation of α-amylases.

The crude extract containing the gut lumen soluble proteins was submitted to ammonium sulfate fractionation. Alpha-amylases were isolated from the supernatant fraction of 50% to 80% saturation of ammonium sulfate. The precipitated protein was extensively dialyzed against 40mM Tris-HCL, pH 8.5, and applied to a HiTrap Q HP ion exchange column, on an AKTA chromatography system. Bound proteins were eluted using a linear salt gradient from 0-500 mM sodium chloride gradient in 40mM Tris-HCL, pH 8.6. The elution was monitored by absorbance at 280 nm and the α-amylases-containing fractions were pooled. Protein concentration was determined using the Bradford method using bovine serum albumin as a standard.

*2.9. In gel assay and Immunoblotting***.**

Alpha-amylase activity was also detected in an 8% SDS-PAGE in semi denaturing conditions using a Biorad Mini II apparatus. The pool of amylases obtained by ion exchange chromatography was diluted two-fold in electrophoresis sample buffer (without 2-mercaptoethanol) and subjected to electrophoresis (samples were not previously boiled). Electrophoresis was carried out at 4°C using pre-cooled buffers and continued for 120 min at 150V after the tracking dye had run off gels. After electrophoresis, one half of the gel was used for detection of α-amylase activity as follows; the gel was transferred to a 2.5% (w/v) solution of Triton X-100 for 20 min at room temperature, then, incubated with 1% solution of soluble starch in 100mM acetate buffer, 20 mM NaCl and 0.2mM $CaCl_2$, pH 5.8 at 30°C for 30 min, followed by 2 min wash with distilled water. The gel was stained with a solution of 1.3% (w/v) I_2, 3 % (w/v) KI. Iodine excess was washed off with cold distilled water. Light bands against the dark back-ground indicated the presence of α-amylase activity. The proteins on the second half of gel were transferred to a nitrocellulose membrane as described before. One half of the membrane was immunoblotted using AmyAnti (1:500), followed by treatment with biotinylated anti-rabbit Ig (BioGenex) and the

second membrane section was incubated with biotinylated PF2. After the incubation with the streptavidin-peroxidase complex for 1.5 h, the color reaction was developed at RT by addition of peroxidase substrate, 0.075% 3,3′-diaminobenzidine 4HCl.

2.10. Separation of Proteins by Two-dimensional Gel Electrophoresis and Immunoblotting Assay.

Amylases isolated by ion exchange column were mixed with re-hydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 60 mM DTT, 1% carried ampholytes and trace of bromophenol blue) and homogenized for 5 min in a vortex. Insoluble material was removed by centrifugation (10,000 x g for 2 min at 4ºC). 25 µg of protein in 125µl of re-hydration buffer were applied on 7 cm IPG strips pH 3-10 (BioRad, Hercules, CA) for 16 h rehydration at room temperature. Isoelectric focusing (IEF) was performed on a Protean IEF Cell (BioRad) for 8,000 V-h at 50 µA per strip. After IEF the strips were equilibrated for 30 min in a solution containing 0.375M 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 run, the strips were electrophoresed in a SDS-PAGE (12%), by running at 200V and 4°C. SDS-PAGE 2D separated proteins were transferred onto a nitrocellulose membrane at 0.8 mA/cm2 for 45 min, using a semi-dry blotter (Labconco, USA). The presence of α-amylase was detected using rabbit polyclonal α-amylase antiserum (1:500) (AmyAnti) as described before.

2.11. 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 band was 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 (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 (8cm × 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% H2O, 10% methanol, 0.5% formic acid, and 0.01% trifluoroacetic acid) and buffer B [98% methanol, 2% H2O, 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 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 ±1.5 Da. All matched peptides were confirmed by visual examination of the spectra. All spectra were analyzed using insect protein database from NCBI. 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 (Keller et al., 2002)

3. Results and discussion

3.1. Reduction of α-amylase activity by PF2 lectin.

We previously found that PF2 is toxic to ZSA larvae, and we thought this related to the prevention of starch digestion, thus we conducted *in vitro* α-amylase inhibition assay with the PF2 lectin. Our results show PF2 inhibits 30 % of the

enzymatic activity at several different PF2 concentrations (0.05, 0.1 and 1 ug of PF2) (Figure 1). These findings also indicate that only a fraction of the amylases present are glycosylated with the carbohydrates recognized by PF2. Toxicity of the activity of insect enzymes by lectins has been previously observed, for example, GNA lectin reduces the α-glucosidase activity in *L. oleracea* larvae, and almost 50% of the sucrose-isomaltase activity in rats (Ficthes and Gatehouse, 1998; Pusztai et al., 1990). In contrast, lectins also can increase activity of some enzymes by increasing the number of enzyme active sites by altering substrate accessibility or affinity (Erickson et al., 1985; You et al., 1992). This was the case for BmoLL lectin that increased the *in vitro* α-amylase activity of *Callosobruchus maculates* (Macedo et al., 2007).

3.2. PF2 binding of amylase.

Affinity chromatography followed by inmunodetection with Anti-Amy was used to examine the ability of PF2 to bind to amylases from *Z. subfasciatus*. The detection with AmyAnti showed a band of approximately 50 kDa (Figure 2A) that coincides with the mass reported for *Z. subfasciatus* amylase (Pelegrini et al., 2006). Moreover, when we isolated α-amylases from crude gut tissue extract using ion exchange chromatography, α-amylase fractions were eluted with 0.31-0.36 M NaCl and amylase was detected by zymogram analysis (Figure 2B). The results indicate three activity bands (due to starch degradation). Others have reported that *Z. subfasciatus* larvae possess three α-amylase isoforms, where the major constitutive amylase shows a faster electrophoretic mobility than two minor isoforms (Silva et al., 2001). Other isoforms of *Z. subfasciatus* amylase can be induced by variations to the diet and during different larvae development stages (Grossi de Sa and Chrispeels, 1997; Silva et al., 2001; Silva et al., 1999). We examined the presence of α-amylases in the midgut tissue extracts from *Z. subfasciatus* larvae using AmyAnti. α-amylase detection with AmyAnti showed only one band with amylase activity (Figure 2B). The weakness of the signal of AmyAnti likely reflects that amylases are partially folded under semi-denaturing conditions, and their structural epitopes are not always accessible to the

antiserum. In addition, an assay with porcine α-amylase (Roche) was used to test the antibody specificity with similar results (data not shown). As a way to confirm if the bands with amylase activity corresponded to glycoproteins of midgut recognized by PF2, lectin-detection with biotinylated PF2 was used (Figure 2B). The recognition pattern of PF2 was similar to that observed for AmyAnti detection. PF2 recognized only one of the bands that showed amylase activity in the zymogram(α-3), and also recognized one more band that interestingly, was not recognized by AmyAnti. Finally, the isolated amylase fraction was applied to 2D SDS-PAGE followed by an inmunodetection using AmyAnti. The 2D detection pattern showed three isoforms of approximately 50 kDa (Figure 3), with isoelectric points (pI) close to 7; this coincides with the estimated pI for *Z. subfasciatus* amylase (Grossi de Sa and Chrispeels, 1997).

3.3. Mass spectrometry identification of amylase.

The band recognized by both PF2 lectin and AmyAnti was subjected to in gel protease digestion for LC-MS/MS analysis. Seven peptides matched an amylase of *Z. subfasciatus* (Table 1); this confirmed that a *Z. subfasciatus* α-amylase could be glycosylated and this oligosaccharide could be recognized by PF2 lectin. The α-amylase reported for this insect has a potential N-glycosylation site, N442, close to the C-terminus and an O-glycosylation site at the T425 residue (Grossi de Sa and Chrispeels, 1997). The glycosylation of α-amylases has been observed only for porcine pancreatic, human salivary and some bacterial amylases (Matsushita et al., 2002). Recently, it was reported that the GNA lectin recognizes α-amylases from several insects including *Acyrthosiphon pisum*, *Tribollium castaneum, Apis mellifera and Bombyx mori* (Vandenborre et al., 2011).

In conclusion, the decrease in the amylase activity in the presence of PF2 can be caused by the binding of PF2 to a glycosylated amylase that results in the inhibition of its enzymatic activity. However, there are other amylase isoforms not recognized by the PF2 lectin that retain their enzymatic activity.

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Figure 1. PF2 inhibits *Z. subfasciatus* α-amylase activity. . Inhibition shows the percentage of one unit of α-amylase activity for crude larval gut extract incubated in the presence of various concentrations of PF2, relative to incubation of crude (CE) without PF2.

Figure 2. Detection of α-amylase. (**A)** Inmunodetection of α-amylase using rabbit polyclonal α-amylase antiserum. Midgut protein extract from 20 days-old larvae was separated by affinity chromatography using PF2-Sepharose, the eluted proteins were subjected to (12%) SDS-PAGE and transferred onto a nitrocellulose membrane and detected using α-amylase antiserum. (**B)** In gel assay of *Z. subfasciatus* α-amylases. Samples containing 45µg of total protein were resolved by SDS-PAGE on an 8% gel. (a) Gel assay of α-amylase activity; (b) Inmunodetection of α-amylases using rabbit polyclonal α-amylase antiserum; (c) Lectin-detection with biotinylated PF2. * The protein band that was sequenced by LC-MS/MS.

Figure 3. Inmunodetection of α-amylases using rabbit polyclonal α-amylase antiserum in a 2D gel. The isolated of amylase was obtained by ion exchange chromatography.

Figure 1

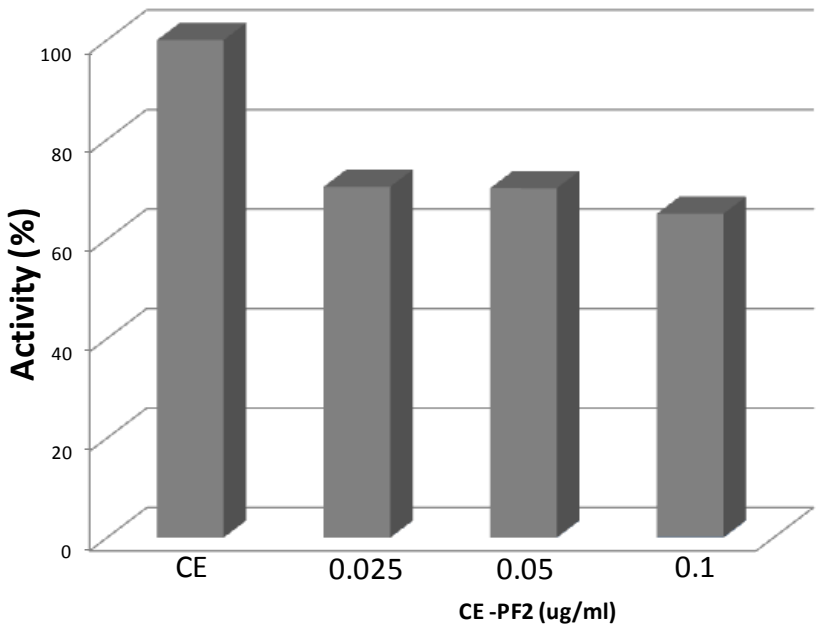

Figure 2

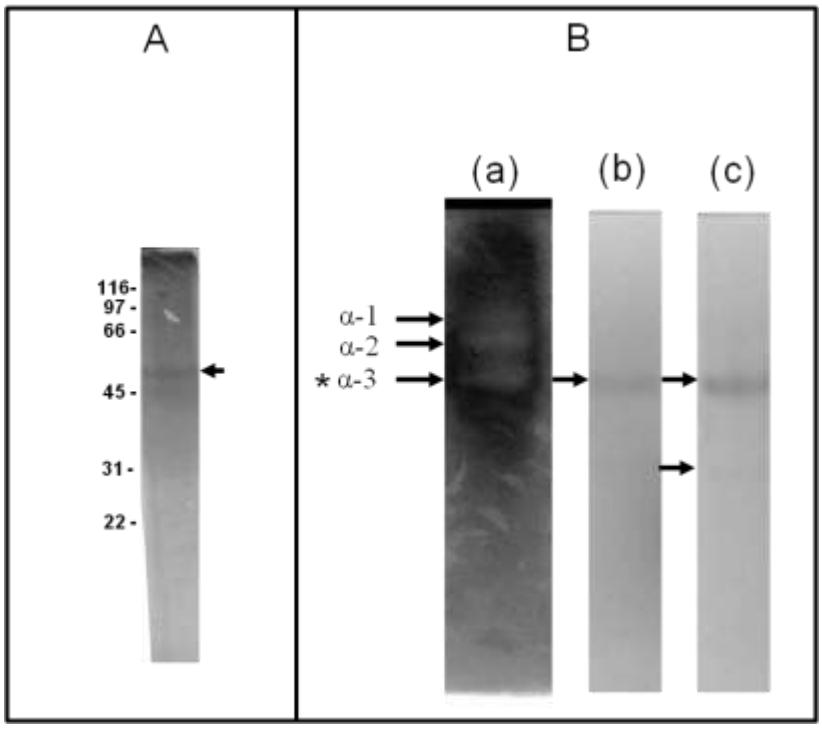

Figure 3

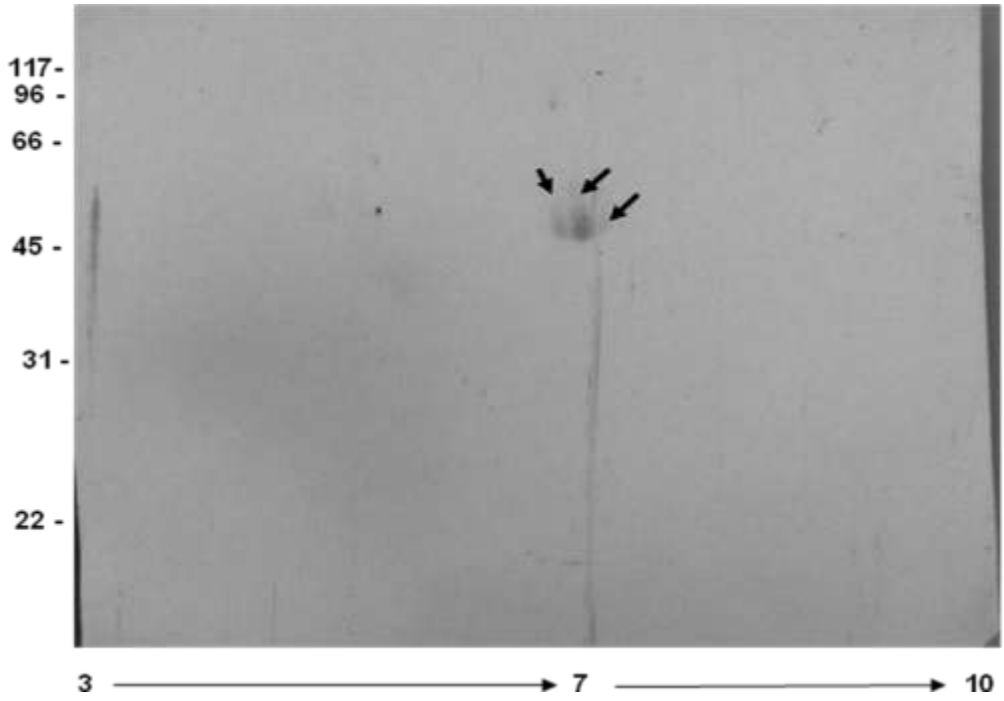

Table 1. Confirmation of α-amylase from *Z. subfasciatus* by LC-MS/MS using searching in insect database from NCBI.

Band	accesión number GenBank ID	description	Organism	Peptide(s)	Coverage (%)	Glycosylation sities
α-3	AAF73435.1	Amylase	*Zabrotes subfasciatus*	(k)NSNFQPGR(n) (k)ECETFLGPK(g) (r)YQPLSYQLT(t) (r)SGDEGALADMIKR(c) (r)VYADVVFNHMAAK(g) (r)NCQLVGLPDLDQSK(q) (k)MANAFMLAHPYAEIPK(l)	17	1

CAPITULO IV

Membrane glycoproteins interacting with PF2 lectin of the midgut larval of *Zabrotes subfasciatus* associated to the insecticidal mechanism of PF2 lectin from *Olneya tesota*

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Membrane glycoproteins interacting with PF2 lectin of the midgut larval of *Zabrotes subfasciatus* associated to the insecticidal mechanism of PF2 lectin from *Olneya tesota*

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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 glycoconjugates results in anti-physiological and insecticidal effects. In order to evaluate the effect of insecticidal lectins in pests, it is important to know the gut composition, distribution and physiological role of the glycoconjugates. PF2 lectin from *Olneya tesota* has insecticidal activity against *Zabrotessubfasciatus* larvae (bean pest). In this study, the binding of PF2 to midgut glycoproteins was evaluated at different larval stages (16, 20 and 25 days old) using 2D-gel electrophoresis and lectin blotting developed with biotinylated PF2. Lectin interaction revealed differences in glycoprotein patterns at different larval stages. Analysing of LC- MS/MS and searching of insect databases showed that several glycoproteins could act as targets for PF2 recognition; these included an ATPase, actin, α-amylase, putative IDGF (Imaginal disc growth factor) and a porin. Each of these proteins is physiologically important in such a way that could be associatedto the insecticidal mechanism of PF2.

Keywords: PF2 lectin; *Olneyatesota*, wild legume,*Zabrotessubfasciatus*; midgut membrane insect proteins, 2D SDS-PAGE.

1. 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(Lopez-Perez et al., 2007). Some plant defense proteins (lectins, α-amylase and protease inhibitors, arcelins, vicilins) are highly selective for specific pests and their use as insecticidal agents could reduce the impact on non-target species including mammals(Daoust et al., 1985). Defense proteins must resist enzymatic proteolysis within the insect gut and operate in a relatively hostile environment (Brunelle et al., 2004; Peumans and van Damme, 1995; Zhu-Salzman and Salzman, 2001).

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). In order to evaluate the effect and mechanisms of insecticidal proteins, there is a large need to increase our understanding of the composition, distribution and physiological roles of the glycoconjugates present in the intestinal tract of insects. A glycoproteomic approach combined with advances in mass spectrometry and protein sequencing has been used to determine potential receptors for insecticidal proteins, particularly lectins, naturally present in plants (Vandenborre et al., 2011b). 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., 2011a). Recently it was reported that PF2 lectin of *Olneya tesota* seeds produced 100% mortality to *Z. subfasciatus* when incorporated into an artificial diet at a level 0.5% w/w. In addition, studies 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).

The aim of this study was to evaluate the recognition of PF2 by midgut glycoproteins at different larval stages (16, 20 and 25 days-old) using 2D-gel electrophoresis and lectin blotting with biotinylated PF2. Analysis was completed by following studies using NanoLC- MS/MS and glycoprotein identification by available insect data bases.

2. Materials and methods

2.1. Insect rearing.

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

2.2. Sample preparation.

Larvae (200) from each instar, (16, 20 and 25 days old),selected using the method of Rodriguez-Quiroz et al. 2000,were cold-immobilized and dissected in a cold 40 mM Tris Base solution. Larval midguts were surgically separated using tweezers. Gut portions taken were posterior to proventriculus and anterior the Malpighian tubules segments. Only actively feeding larvae with food filling the gut tract were chosen. The midgut proteins were extracted sequentially with ReadyPrep Sequential Extraction Kit (Bio-Rad). Briefly, midgut tissues were homogenized in a cold 40 mMTris Base solution containing a complete cocktail of protease inhibitors (Roche) and subsequently centrifuged at 434902*g*for 20 min at 4ºC.The pellet was resuspended in the reagent 2 containing 8 M urea, 4% CHAPS, 2 mM TBP, 40 mMTris, 0.2% ampholytes and vortexed for 5 minutes and subsequently centrifuged at 100000 rpm for 20 min at 4 ºC. The

supernatants containing the membrane proteins were kept at -80 ºC and protein concentration estimated using DC Protein Assay (Bio Rad).

*2.3. Separation of Proteins by Two-dimensional Gel Electrophoresis and Immunoblotting Assay***.**

Gut lumen proteins were mixed with iced-cold re-hydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 60 mM DTT, 1% carried ampholytes, and trace of bromophenol blue) and homogenized for 5 min in a vortex. Insoluble material was removed by centrifugation (10,000 *x g* for 2 min at 4ºC). Gut proteins (150µg) were resuspended in 150µl of re-hydration buffer and applied on 7cm IPG strips pH 3-10 (BioRad, Hercules, CA) for 16 h rehydration at RT. Membrane-bound proteins were re-hydrated in the extraction buffer (8 M urea, 4% CHAPS, 2 mM TBP, 40 mM Tris, 0.2% ampholytes). In addition, samples of 16 days-old larvae were applied on 7 cm IPG strips pH 4-7.

Isoelectric focusing (IEF) was performed on a Protean IEF Cell (BioRad) for 10,000 V-hr at 50 µA per strip. After IEF the strips were equilibrated for 30 min in a solution containing 0.375M 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, the strips were placed on the stacking gel and covered with 0.5% agarose and SDS-PAGE (12% acrylamide), and run under constant voltage (200 V).

Immunoblot assay was performed as described by(Towbin et al., 1979).SDS-PAGE separated proteins were transferred onto a nitrocellulose membrane at 0.8 mA/cm2 for 2.5 h, using a semi-dry blotter (Labconco, USA). Membranes were blocked overnight with PBST (0.02 M KH2PO4/K2HPO4, 0.9% NaCl, 0.1% (v/v) tween, pH 7.2) containing 2.5% (w/v) BSA. The membranes were then washed with PBST and further incubated independently for 3 h with biotinylated PF2 lectin (8µg/ml) followed by streptavidin-peroxidase for 1.5 h. The color

reaction was developed at RT by addition of peroxidase substrate, 0.075% of 3, 3´-diaminobenzidine 4 HCl (DAB).

2.4. Nanoscale Liquid Chromatography−Tandem Mass Spectrometry (NanoLC−MS/MS).

For spectrometry analysis, sampleswere sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona, Tucson, Arizona). The protein band wasmanually excised from the gel, destained and in-gel-digested with commercial Proteotmax. 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 (8cm × 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% H2O, 10% methanol, 0.5% formic acid, and 0.01% trifluoroacetic acid) and buffer B [98% methanol, 2% H2O, 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 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 ±1.5 Da. All matched peptides were confirmed by visual examination of the spectra. All spectra were analyzed using insect protein database from NCBI. 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 (Keller et al., 2002).

3. Results and discussion

2D-gel electrophoresis is an important tool for proteomic analysis particularly for the comparison of changes in protein expression during different stages of the development of an organism. The insect midgut is important because it is the site of most of digestion and absorption of nutrients (Pauchet et al., 2008). Membrane-bound midgut proteins from *Z. subfasciatus* larvae were extracted and resolved by 2D SDS-PAGE (Figure 1). 2D-gels revealed differences in the pattern of proteins at different larval stages as shown by the varied and highly complex set of proteins distributed in the acidic to neutral range.

Lectin-detection with biotinylated PF2 reveled differences in glycoprotein patterns at different stages, the range of proteins detected were of >21 to <79-kDa. The greatest abundance of glycoproteins with affinity to PF2 was found in midgut larvae at day 20.In addition, lectin blotting revealed unique glycoproteins for the various larval ages. Diverse post-translational modifications might shift proteins in horizontal and vertical directions in 2D-gels. In such a case, proteins may appear as a horizontal ‘‘chain’’ of spots. Glycosylation can change the weight of protein, as well as the isoelectric point (pI) (Morris et al., 2009). Possible examples of post-translational modifications, seen as horizontal ‘‘chains’’, could be found in spots 1a-4a (Figure 1A). All spots hadclose molecular weights, but have a different pI. Each larval stage presented similar “chain of spots”.

Since insecticidal effect of PF2 was observed at very early stages of development (Lagarda-Diaz et al., 2009), spots 6-9 (16 day-old larvae) (Figure 1A) were excised, protease digestedand submitted for identification by Nano LC-MS/MS. Search in insect databases showed several glycoproteins. Using insect

databases of NCBI, we identified that the spots matched a putative Imaginal disc growth factor (IDGF) from *Diaprepes abbreviates* and a putative mitochondrial porin from *Graphocephala atropunctata* (Table 1). These results extend our understanding of the 2D profile of membrane proteins and provide the first comparison by 2D SDS-PAGE of the level of protein expression at different larval stages of *Z. subfasciatus.*

IDGF proteins are members of the family of growth promoting glycoproteins, referred to as the imaginal disc growth factors. Six of these proteins have been reported from the dipterans, *Drosophila*, two from the lepidopterans, *Bombyx mori* and *Pieris rapae,* and one putative protein from the coleopteran *Diaprepes abbreviates* (Huang et al., 2006; Kawamura et al., 1999; Wang et al., 2009).

IDGF proteins are abundant in the fat body and it has been suggested that are transported to hemolymph and from there spread to the other organs such as midgut. In *Droshophila, IDGF* transcripts are revealed in the anterior midgut invagination and the posterior midgut invagination. The expression of these proteins can be variable during development of the insect; in *Droshophila*, IDGF was highly expressed on the mid-larval stages (Kawamura et al., 1999). In lepidoterans such as *B. mori,* the expression of IDGF is high during the fouth instar and decreases in the fifth instar (Wang et al., 2009). In this work, these proteins were observed only in larvae from 16 days old. Throughout all developmental stages, IDGF proteins are expressed suggesting that they are important proteins having multiple functions during the entire life cycle. It has been suggested that in *Bombyx* wing disc cells the IDGF proteins help insulin bind to its receptor and that the disruption of critical developmental pathways, such as reduction of the signaling of the insulin receptor, may provide a mean for the development of novel management methods to reduce growth and/or survival of the insect (Wang et al., 2009).

A putative porin also was identified in larvae (16 days old). Porins are major channel-forming proteins found in the mitochondrial outer membrane. Many metabolites, as well as ATP and ADP, pass through these channels (Graham and Craigen, 2005). The mechanisms that regulate the expression of porins have been extensively studied; however, their biological role in the adaptive responses is poorly understood.

Since most of the proteins were resolved with an acid pI (3-7) and because the insecticidal effect of PF2 was observed at very early stages, the samples from 16 days-old larvae were further resolved on 7cm IPG strips pH 4-7. Lectin (PF2) interaction revealed greatest abundance of glycoproteins, some of which were submitted toMS analysis for identification. Among the MS-identified glycoproteins,characteristic membrane proteins such as ATP synthase, NADH-ubiquinone reductase subunit, bellwether (ATP binding) and actin were located (Table 1).

In insects, including A*cyrthosiphon pisum, D. melanogaster, Apis mellifera, B. mori* and *Tribolium castaneum* the interaction of GNA lectin with an ATP synthase subunit was reported (Vandenborre et al., 2011b). In our work, we identified an ATP synthase corresponding to F1-ATPase_beta and alpha region and V-ATPasesubunit (Vacuolar H (+) - ATPase). F-ATP synthase is an enzyme composed of an integral membrane component called Fo and a peripheral catalytic moiety called F1. Membrane-bound F1 catalyzes both synthesis and hydrolysis of ATP in reactions that are coupled to proton translocation through the FoF1 ATP synthase beta subunit (Wang et al., 1999).The presence of F1-ATPase has been reported in the cytoplasmic surface of apical plasma membranes of insect cells and in lepidopteran midgut. On the other hand, the V-ATPases have similar structure and mechanism of action than F-ATPase, and several of their subunits evolved from common ancestors. In contrast to F-ATPases, whose primary function in eukaryotic cells is to form ATP at the expense of the proton-motive force, V-ATPases function exclusively as ATP-

dependent proton pumps(Nelson, 1992). In the last few years V-ATPases have also been found in the cellmembranes of insects, wherethey appear to be vital for specialized physiological roles of these cells(Wolfersberger, 1992; Zhuang et al., 1999). In the larval midgut of the lepidopteran, *Manduca sexta*, the V-ATPases are located in the goblet-cell membranesurface.These cell are input for the alkalinization of the gut lumen andplaying a vital role in proton secretion as well asmaintaining the ion balance within the insect(Azuma et al., 1995). In addition an extensively glycosylated V-ATPase was isolated from midgut and Malpighian tubules of the tobacco hornworm, *Manduca sexta* (Merzendorfer et al., 1999).

We found that PF2 recognizes an actin in the *Z. subfasiatus* midgut. Actin is one of the three major components of the cytoskeleton and it is involved in important cellular processes such as cell motility, cell division, vesicle movement, differentiation, and proliferation (Bayyareddy et al., 2009).The interaction of lectins (GNA)with actin has been reported by others in *A. pisum* and *A. mellifera* (Vandenborre et al., 2011b). Actin was also reported as a binding protein for Cry toxin from *Bacillus thuringiensis* (Hoebt-Schmidt) in some insects, and henceis thought to be involved in the mode of action of this toxin (Bayyareddy et al., 2009; Chen et al., 2010; Krishnamoorthy et al., 2007; McNall and Adang, 2003).Since actin is a protein that plays a key role in cellular metabolism, the union of PF2 might affect its operationand result in high cost for the insect.

Another interesting finding was the occurrence ofan α-amylase like a membrane-bound protein in *Z. subfasciatus*. A-amylaseis initially found as an integral protein in the membrane of epithelial cells; the enzyme is subsequently processed to become soluble and secreted into the midgut lumen by exocytosis. It has been reported that soluble amylase and membrane-bound α-amylase are virtually identical(Santos and Terra, 1985). We found that PF2 recognizes both α-amylases. Recently, it was reported that the GNA lectin recognizes α-amylases from several insects including *A. pisum*, *T. castaneum, A. mellifera*

and B. mori (Vandenborre et al., 2011b).Also, an α-amylase present in midgut brush border membrane vesicles of mosquito *Anopheles albimanus* was reported as a receptor forCry4Ba and Cry11Aa toxinsof *B. thuringiensis* (Fernandez-Luna et al., 2010).

The detection of inner mitochondrial proteins that bind PF2 lectins might not be physiologically relevant, as this localization is not compatible with action mechanisms already known for lectins. However, the internalization of insecticidal lectins into insect midgut has been reported. This uptake mechanism, clathrin-mediated endocytosis, caveolae-mediated endocytosis, is suggested for vacuolar H1-ATPase (V-HATPase), phosphoinositide 3-kinases (PI3Ks)-dependent endocytosis, and actin-independent endocytosis (Shahidi-Noghabi et al., 2011). Further research on uptake mechanisms for PF2 in the midgut cell is necessary to determine the possible roles for PF2in insecticidal mechanisms.

In conclusion, we identified membrane-bound glycoproteins presentin *Z. subfasciatus* midgut, some of which appear to be present only at a specific developmental stage (IDGF and porin). This work suggests that PF2 acts on *Z. subfasciatus* through the simultaneous interaction with target glycoproteins.The use of the PF2 lectin assisted us in focusing the study to target glycoproteins important for the physiology of larvae, which also could be associated with the insecticidal mechanism of PF2.

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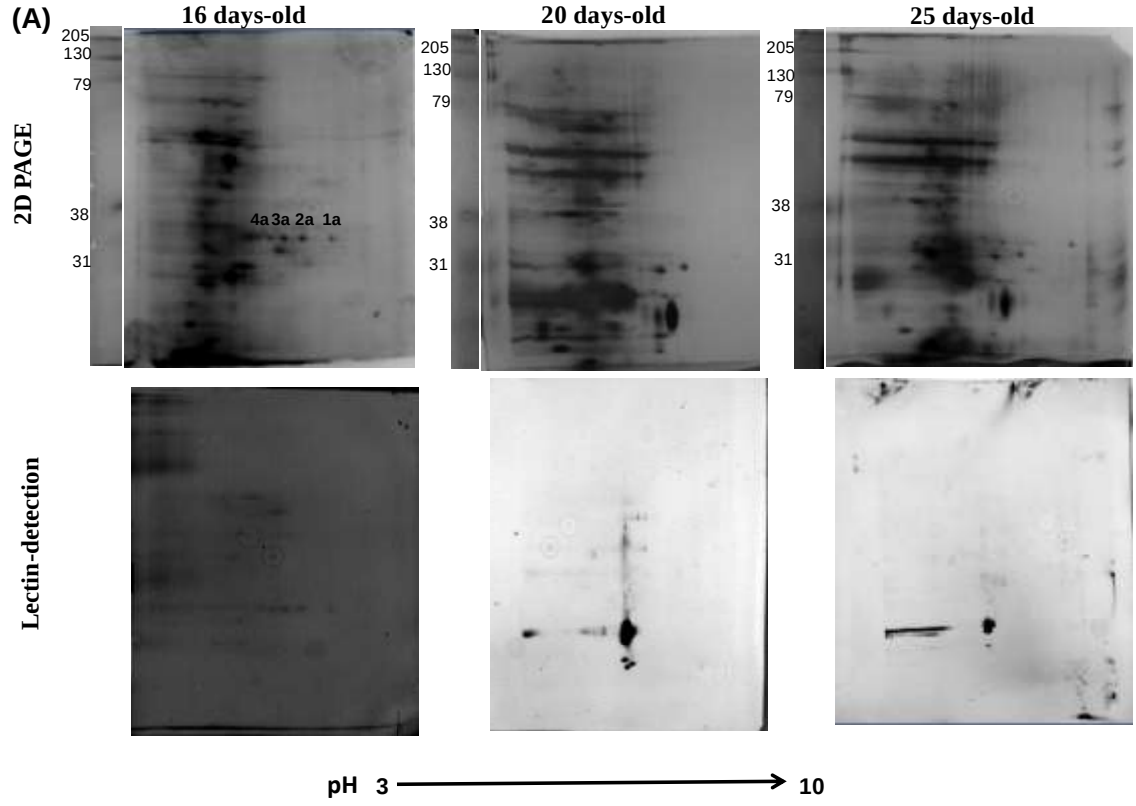
Figure 1.Membrane-bound proteins from *Z. subfasciatus* larval midgut. Lectin-detection of proteins separated by 2DE in 7cm IPG strip pH 3-10 (A) and pH 4-7 (B). For 2DE, proteins (150ug) were resolved by iso-electric focusing using pH 3–10 and 4-7 strips followed by separation on SDS-12%PAGE gels. Gels were silver stained. Proteins separated were transferred onto a PVDF membrane and detected with biotinylated PF2 lectin followed by treatment with streptavidin-peroxidase complex and DAB. Lane M contained molecular weightmarkers.

Figure 1


(A)
16 days-old
20 days-old
25 days-old
2D PAGE
205
130
79
38
31
4a 3a 2a 1a
Lectin-detection
pH 3
10

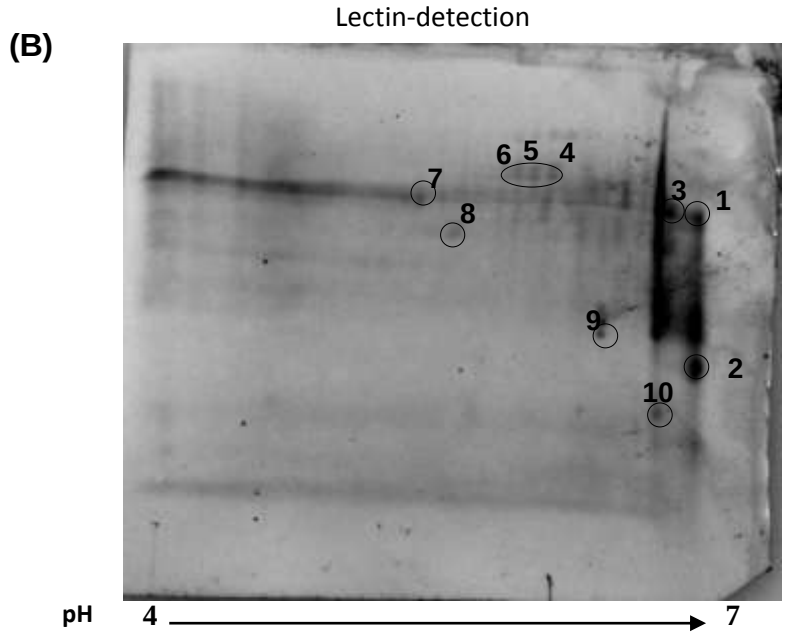

(B)
Lectin-detection
6 5 4
7
8
3
1
9
2
10
pH 4
7

Table 1.Identification of midgut membrane proteins (of intermediate solubility) from *Z. subfasciatus* obtained by searching in insect databases of NCBI.

Spot	Accession number GenBank ID	Description	Predicted function	Organism	Predicted molecular mass (kDa)	Peptides hits
3a	AAV68692.1	IDGF protein	imaginal disc growth factors	*Diaprepesabrevviatus*	49	1
2a,3a	ABD98775.1	Mitochondrialporin	voltage-gated anion channel activity	*Graphocephalaatropunctata*	30	1
3b	AAF73435.1	alphaamylase	catalyses the breakdown of starch into sugars	*Zabrotessubfasciatus*	53	10
9b	NP_001164361.1	ATP Synthase -beta subunit	produces ATP from ADP in the presence of a proton gradient across the membrane	*Triboliumcastaneum*	55	67
	AAF59391.1	ATP Synthase-beta, isoform A		*Drosophila melanogaster*	55	60
3b,7b	AAF54837.1	Vacuolar H (+)4 ATPase B subunit isoform B	couple ATP hydrolysis to the build up of a H+ gradient, but V-type ATPases do not catalyze the reverse reaction	*D. melanogaster*	55	3, 2
2b	AAF51998.1	Vacuolar H (+) ATPase E subunit, isoform A		*D. melanogaster*	26	4
3b	AAF46903.1	Bellwether (ATP binding)	produces ATP from ADP in the presence of a proton gradient across the membrane	*D. melanogaster*	59	14

7b	ACF54724.1	beta-actin	involved in the formation of filaments that are a major component of the cytoskeleton	*Mantichorulasemenowi*	42	3
	AAF46098.1	Actin 5C, isoform A		*D. melanogaster*	42	2
	NP_001172074.1	Actin related protein		*Apismellifera*	42	3
4b	AAF46356.1	NADH: ubiquinonereductasesub unitprecursor, isoform A	play an important role in electrontransfer processes and in various enzymatic reactions	*D. melanogaster*	26	4
3b		Hypothetical protein TcasGA2_TC008728		*T. castaneum*	60	16
7b		Hypothetical protein TcasGA2_TC015046		*T. castaneum*	53	7
3b,7b		Hypothetical protein TcasGA2_TC012133		*T. castaneum*	55	3,2
4b		Hypothetical protein TcasGA2_TC006252		*T. castaneum*	79	4
2b		Hypothetical protein TcasGA2_TC010367		*T. castaneum*	27	4