

First report on the involvement of sulfated N-glycans and protein content of *Hylesia metabus* urticating setae in lepidopterism and the insect's possible mechanisms of defense

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REPORT

ABSTRACT

Hylesia metabus moth inhabits swampy areas of the Orinoco river delta, and in other regions of South America. The adult female contains urticating abdominal setae that upon contact with the skin causes severe dermatitis syndrome known as "lepidopterism", also associated to inflammation, focal hemorrhages, insomnia, restlessness, and febrile state. During matting flights, the adult females of *H. metabus* release large amount of setae reaching highly distant geographic areas. Also, the positive phototropism of the moth causes them to strike against the light sources increasing the probability of its contact with humans. In this work, information was provided on the structural identification of some of the *H. metabus* toxin protein and oligosaccharide components responsible for the clinical symptoms of lepidopterism. Five proteases were identified, two of which are N-glycosylated with neutral bi-antennary oligosaccharides, agalactosylated and fucosylated, and also sulfated, this last modification not previously reported in insects. It was demonstrated that the proteolytic activity of one of the proteases is related to the erosion of the blood vessels and the focal hemorrhages characteristic of lepidopterism, providing the first report on N-sulfation in insects and their causal role in the clinical setting in humans. The results have potential applications for rational drug design and pest control. In addition, methodological contributions were included for the differentiation between sulfated and phosphorylated N-glycans. This work received the Annual Award of the Cuban Academy of Sciences for the year 2017.

Keywords: *Hylesia metabus*, insects, lepidopterism, serine proteases, sulfated N-glycans, vitellogenin, chitinase

RESUMEN

Primer reporte de la implicación de los N-glicanos sulfatados y la composición proteica de las setas urticantes de *Hylesia metabus* en el lepidopterismo y en los posibles mecanismos de defensa. La polilla *Hylesia metabus* habita en zonas pantanosas del delta del río Orinoco, y en otras regiones de Suramérica. La hembra adulta contiene setas abdominales urticantes que por contacto con la piel causan dermatitis severa conocida como lepidopterismo, también asociado con inflamación, hemorragias focales, insomnio, intranquilidad, y estado febril. Durante los vuelos de apareamiento, la hembra adulta de *H. metabus* libera grandes cantidades de setas que pueden dispersarse con el viento hasta áreas geográficas muy distantes. Además, el fototropismo positivo de la polilla hace que se golpeen contra las fuentes de luz y se incremente la liberación de las setas y su contacto con seres humanos. En este trabajo se brinda información sobre la identidad estructural de algunos de los componentes proteicos y los oligosacáridos de la toxina de *H. metabus*, responsables de los síntomas clínicos del lepidopterismo. Se identificó a cinco proteasas, dos de estas N-glicosiladas con oligosacáridos bi-antennarios neutros, agalactosilados y fucosilados, y también sulfatados, esta última modificación no reportada en insectos. Se demostró que la actividad proteolítica de una de las proteasas causa la erosión de los vasos sanguíneos y las hemorragias focales propias del lepidopterismo. Este fue el primer reporte sobre la N-sulfatación en insectos y su rol causal en el cuadro clínico de lepidopterismo. Los resultados pudieran aplicarse en el diseño racional de drogas y el control de plagas. Se hicieron aportes metodológicos para la diferenciación entre N-glicanos sulfatados y fosforilados. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba en el año 2017.

Palabras clave: *Hylesia metabus*, insectos, lepidopterismo, proteasas serino-dependientes, N-glicanos sulfatados, vitellogenina, quitinasa

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Introduction

Hylesia metabus is a neotropical moth endemic of the North-Eastern region in South America (mainly, in Venezuela, Suriname, French and English Guyana, Trinidad and Tobago and Brazil), specifically in swampy regions rich in mangroves. The *H. metabus* females bear abdominal hair-like structures commonly known as setae, which contain urticating substances of unknown composition. When the human skin gets into contact with setae, a severe acute dermatological reaction is triggered, characterized by a harsh and intense ardor which is followed by the appearance of erythematous vesicles, vascular degeneration, focal hemorrhages, local acute inflammation, insomnia, restlessness and mild febrile symptoms [1]. The onset of the symptoms occurs 6 to 12 hours following the contact with setae, and they could last for up to 5 to 10 days, with the worst reactions lasting even for months. The severity of the symptoms is not mediated by a previous exposure and for now there is no known effective medical treatment available. Neither antihistamine nor steroidal drugs are effective against the severe reactions described for the exposure to the *H. metabus* setae, a condition named as 'lepidopterism'.

The life cycle of *H. metabus* lasts approximately 100 days and has four stages i.e., eggs, larvae, pupae and adults [2]. Large amounts of setae are produced by adult *H. metabus* females in the mating season. The female insect recovers the nest with setae (200-300 eggs in a single oviposition) to protect it during the following 26 days until hatching. Furthermore, *H. metabus* females have evolved this strategy to compensate the vulnerability of their larvae, which are devoid of fully activated setae. By these means, they compensate for population losses of over 90 % which will not reach the adult phase due to the attack of larvae by natural predators, like bacteria, fungi, parasitoid flies and hornets. In this sense, Osborn *et al.* isolated over 29 different bacterial strains from *H. metabus* larvae, with *P. aeruginosa* the most lethal among them [3].

Setae are light structures than can be spread apart through the wind, therefore reaching vast and highly distant geographic areas. Significantly, setae are able to preserve its urticating properties for months under environmental conditions, something that makes difficult to control its action long after the mating season [4]. Moreover, the insect displays positive phototropism, obligating the villagers to turn off every light source to avoid the release of the setae by the crash of insects against light bubbles. Hence, while not live-threatening for humans, the cyclic incidence of setae affects all the areas of human activity, thereby producing huge economic losses. More importantly, lepidopterism is recognized as a health problem in Venezuela, this insect regarded as the most noxious of *Hylesia* spp.

In this setting, a joint research project was launched between the Institute of Scientific Research of Venezuela (IVIC) and the Center for Genetic Engineering and Biotechnology (CIGB) of Havana, Cuba, issued to the X Bipartite Commission Cuba-Venezuela, aimed to study the protein components of the *H. metabus* setae and its relationship with lepidopterism, including

the defense mechanism of this insect. Since most of the insect's progeny is guaranteed at the nesting reproductive phase, we hypothesized that it could be probably the more likely source to obtain setae for its characterization. It was interesting to investigate whether the protein composition of the *H. metabus* nest setae could be related to the symptoms seen in lepidopterism, to take advantage of the natural activity of the identified proteins for developing therapeutic approaches. This work received the Annual Award of the Cuban Academy of Sciences for the year 2017.

Main results

The first task was to address a successful characterization of the setae proteins. This was particularly troublesome, since neither the genome of this organism is sequenced nor its proteins annotated, further hampering the successful analysis by protein homology to assign biological functions. There was also no prior characterization of the N-glycosylation pattern of this insect, making impossible its comparison with human glycosylation patterns. Therefore, a protein isolation and characterization approach was implemented, involving the isolation of setae proteins from the insect nest and studying them with the aid of SDS-PAGE and Mass Spectrometry (MS) procedures. Specifically, MALDI-MS and ESI-MS/MS techniques were applied, followed by permethylation analysis, analysis with a combination of exoglycosidases and NP-HPLC. The exact molecular mass of residues and moieties was determined with an error below 1 ppm. Protein sequences were further aligned with TFASTS and MSBLAST software.

The work was started by analyzing one of two fractions isolated by precipitation with ammonium sulphate 60 % (HM-PT60) and 80 % (HM-PT80) at the IVIC, which contained most of the proteolytic activity of the toxic extract from setae [5]. Both fractions were previously characterized by means of several chromogenic substrates, displaying kallikrein-like activity [6]. Specifically, the fraction HM-PT60 showed a 35 kDa band with positive Schiff's staining [5], something that might be related to lepidopterism and worth of attention. This determined the selection of HM-PT60 fraction for further characterization.

Up to nine proteins of the *H. metabus* were identified by homology. It was found for the first time that up to 65 % of the protein content of the *H. metabus* nest setae contained five type S1A serine proteases. When this fraction was inoculated in a lepidopterism model in guinea pigs (*Cavia porcellus*), the proteolytic activity was the one mediating tissue and blood vessel damage, which led to focal hemorrhages [7]. It was plausible that the combination of the five identified proteases could degrade more target proteins that eventually produce the severe acute dermatological reactions seen in lepidopterism.

Regarding the glycosylation pattern, it was described for the first time for this insect that two of these proteases are in fact N-glycosylated, showing a mix of neutral and sulfated G0F N-glycans. Seeking for an explanation on the role of this type of glycosylation on the toxicity seen, it was evident that neutral G0F structure is not harmful for the im-

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immune system, since it is also found in the N-linked structures of IgG immunoglobulins. Nevertheless, the sulfated N-glycans could transform common structural motifs into unique structures with pretty defined biological functions that can be recognized by receptors or lectins. Since there were no previous reports on post-translational modifications implying sulfate moieties in N-glycans in insects, and this modification could represent an energetic burden for the biosynthetic machinery on every living organism, it was inferred that it could certainly play a biological role in lepidopterism and possibly as a defense mechanism against predators. This was confirmed when these N-glycans were inoculated in the *C. pocellus* model of lepidopterism, where a potent inflammatory response was entangled, stimulating the secretion of TNF- α from activated macrophages. The *H. metabus* toxin deglycosylated with PNGase F, desulfated with *Helix pomatia* sulfatase and the protein reduced and alkylated, all these induced just mild focal hemorrhages and fibrin depositions [7]. This last allowed us to question whether sulfated N-glycans could be mediating other adverse effects caused by insects in humans. Moreover, the maximal urticating activity of setae is achieved with the maximal sexual maturity in the insect's adult stage. Therefore, it could be also possible that the secretory pathway associated to abdominal setae could process all the setae proteins, inserting on them sulfated N-glycans on potential N-glycosylation sites. If this were true, then female insects could exacerbate the inflammatory responses regardless the protease activity of setae.

Another protein was identified, from the hemolymph, of approximately 180 kDa, known as Vitelogenin, a major constituent of egg yolk [8]. It plays an important role in defense mechanisms, due to its capacity to recognize pathogen-associated molecular patterns (PAMPs) sequences [9], also bearing lytic activity that can destabilize the cellular membrane of microorganisms [10, 11].

A chitinase protein was identified in setae, highly homologous with insects' chitinases [2]. This type of enzymes is involved in remodeling the exoskeleton of insects, but is also functional on the chitin structures of the entomopathogenic fungi (i.e., *Beauveria bassiana*) [12], and, therefore, provides a plausible antifungal defense mechanism. Observations of

behavioral experiments conducted by Rodríguez *et al.* [13] with depredatory ants (*Pheidole* spp.) evidenced that these scavenger insects avoided the contact with *H. metabus* eggs recovered by setae, while uncovered eggs were successfully transported [13]. Additionally, if the chitin of the same insect gets affected by its own chitinase activity, fragments could be generated thereby contributing to boost the immune response against the setae toxins in humans.

A 30-kDa protein was identified, corresponding to the insect's hemolymph, which has been reported of displaying binding activity for N-glycans, and could be involved in anti-fungal responses.

The last protein identified was microvitelogenin, with no putative role in the defense mechanisms described herein. Curiously, there are some reports on the toxicity in humans of proteins derived from insects' hemolymph [14].

Overall, the proteins identified in this study, together with knowledge on the structuring of *H. metabus* nest by the female insect is strong evidence on the development of an adaptive mechanism for egg protection in a single oviposition by this species.

Scientific relevance and impact of results

This was the very first characterization of the *H. metabus* egg nest setae composition, providing molecular information to understand the mechanisms involved in lepidopterism. In this regard, this was the first report on setae composition as a mix of SIA proteases. Moreover, this was the first description of sulfated N-glycans structures in insects, demonstrated by orthogonal analytical techniques, and their involvement in the development of an inflammatory response typical of lepidopterism. From this work, three new structures of sulfated N-glycans were described at the GlycoBase (Available at: <http://glycobase.ucd.ie>; accession numbers 27955, 27956 and 27957). There was also contributed the first report on using the isotopic distribution of the m/z 97 ion to differentiate between sulfate and phosphate moieties of N-glycans, of methodological value for the general study of N-glycans regardless their origin. This work also tribute to two international publications in renowned academic journals, a M.Sc. Thesis of Tambara Y. at the CIGB and the PhD. Thesis of Cabrera G, at the Faculty of Biology, University of Havana, Cuba.

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