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Spatial and temporal synthesis of *Mamestra configurata* peritrophic matrix through a larval stadium

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ABSTRACT

The structure and synthesis of the *Mamestra configurata* peritrophic matrix (PM) was examined at various time points during a larval stadium. Bright field and confocal fluorescence microscopy revealed major differences between the PM of feeding and molting larvae. The PM from feeding larvae was thinner and composed of approximately 5–10 layers. In contrast, mid-molt larvae had a chitinaceaous PM composed of multiple thick layers which filled most of the midgut lumen. PM synthesis initiates in the anterior midgut, based on the expression of genes encoding chitin synthase-2 (CHS-2), coincident with the incorporation of the major structural PM proteins (McIIM1, McIIM2 and McPM1). This is followed by reinforcement with other PM proteins (McIIM3 and McIIM4) as it moves toward the posterior of the midgut.

Chitin deacetylase (McCDA1) was associated only with the anterior PM. Collectively, these findings indicate that the structural properties of the PM differ along the length of the midgut. Genes encoding chitinolytic enzymes (McCHI and McNAG) were expressed and exochitinase activity was present when the PM had degraded (pre-molt) and when the new PM was forming (mid-molt), indicating that they are involved in either PM turnover and/or maintenance dependent upon the stage.

1. Introduction

The alimentary canal of most insects contains an acellular, porous tube lining the midgut epithelium, known as the peritrophic matrix (PM). The PM is an essential component of the insect digestive system as anything that is ingested (food, pathogens, toxins, etc.) must either pass through or in some way interact with this structure. The PM surrounds the food bolus and compartmentalizes digestion. It also protects the underlying epithelial cells from mechanical damage by rough food particles, from chemical damage by toxins and from infection by pathogens (reviewed in Richards and Richards, 1977; Lehane, 1997; Terra, 2001; Hegedus et al., 2009).

The PM is composed of chitin and proteins. Chitin is a polymer of *N*-acetylglucosamine (GlcNac) that assembles into micro-fibrils and eventually into larger chitin bundles. The bundles organize into a 'fishing-net' like structure to form the basal network of the PM (Lagermalm et al., 1950; Mercer and Day, 1952). PM chitin is synthesized by the midgut specific chitin synthase 2 (CHS-2), modified by enzymes such as chitin deacetylases (CDA), and degraded by chitinolytic enzymes, mainly endochitinase (CHI) and the exochitinase, *N*-acetylglucosaminidase (NAG) (Cohen, 2010). Integral PM proteins, called peritrophins, interact with the chitin scaffold and contribute to the structural and functional features of the PM such as integrity, elasticity and permeability (Tellam et al., 1999; Toprak et al., 2010c).

In dipteran larvae, there is evidence that the PM is secreted from cardial invaginations to form a multi-laminate membrane (Binnington, 1988). This is often referred to as a Type II PM. PM synthesis in lepidopteran larvae, noted as having a Type I PM, and many other insects, is somewhat more complicated. Adang and Spence (1981) reported that the PM in *Trichoplusia ni* was produced along the entire midgut epithelium beginning with the secretion of an amorphous material that matured into cross-linked fibrous network. Subsequently, the *Heliothis virescens* PM was shown to be secreted from a specialized band of cells at the junction

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between the foregut and midgut (Ryerse et al., 1992). In *Ostrinia nubilalis*, a single layer PM formed in the anterior midgut, which became progressively thicker as new layers were added along the length of the midgut (Harper and Hopkins, 1997). This hybrid model, which is something between the traditional definition of Type I and Type II PM formation (Wigglesworth, 1972), appears to be more commonplace in lepidopterans (Hopkins and Harper, 2001) with some types of peritrophins being incorporated into the PM along the entire midgut (Harper and Granados, 1999) and others only in the anterior region (Bolognesi et al., 2001; Campbell et al., 2008). As such, to accurately define the processes involved in PM formation in any insect, it is necessary to examine the sites of both PM chitin and peritrophin synthesis along the entire midgut.

Previous studies have examined PM formation in the feeding stages, while turnover and formation of PM during the molt has largely been ignored. Recently, we demonstrated that the PM in molting *Mamestra configurata* larvae differed significantly in appearance from the PM found in feeding larvae (Toprak et al., 2010b). The PM of these molting larvae consisted of a highly opaque mass containing chitinaceous material surrounded by a thin, translucent sleeve. It has been observed that the morphology of the PM also differs along the length of the midgut (Harper and Hopkins, 1997; Harper and Granados, 1999) and the midgut epithelium can be separated into three, morphologically-distinct regions, the anterior, middle and posterior (Wigglesworth, 1929; Cioffi, 1979; Harper and Hopkins, 1997). However, the structure and composition of the PM along the length of the midgut has not been addressed in detail.

Mamestra configurata, commonly known as the bertha armyworm, is a major noctuid pest of cruciferous oilseed crops (*Brassica napus* and *B. rapa*) in Canada and the USA (Mason et al., 1998). This species has been used to develop the current structural model of the lepidopteran PM (Hegedus et al., 2009). In this study, we examined the macroscopic and microscopic morphology of the PM in the three regions of the midgut throughout an entire *M. configurata* larval stadium. In addition, the temporal and spatial expression patterns of genes encoding enzymes involved in chitin metabolism (McCHS-2, McCHI, McNAG and McCDA1) and peritrophins (McIIM1-4, McPM1) was determined. Finally, the occurrence of peritrophins and McCDA1 in PM derived from the three midgut regions was examined.

2. Materials and methods

2.1. Insects

M. configurata larvae were reared at 21 ± 1 °C under a 16 h light/8 h dark photoperiod on artificial diet (Bucher and Bracken, 1976).

2.2. Microscopy

Midguts and PMs were dissected from feeding mid-stadium 4th instar larvae, pre-molt larvae that had ceased feeding, mid-molt larvae between 4th and 5th instar (when the separation of the old cuticle reached the mid-point of the body) and early 5th instar larvae (within 1 h of commencement of feeding). PMs were isolated from the midgut tissue and rinsed in insect Ringer's solution (153 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2 2H_2O$). Whole PMs were examined using a Leica dissecting microscope at 6.3× magnification. For confocal microscopy, the PMs were cut into $^1/_3$ sections (anterior, middle and posterior) and the sections individually incubated in 300 μl of 10 μg/ml wheat germ agglutinin – AlexaFluor 488 conjugate (Molecular Probes, Life Technologies) in 1× PBS (pH 8.0) for 30 min and then washed in 1× PBS (pH 8.0) for 5 min in the dark. The PM sections were then mounted on slides in 25% glycerol using a 0.75 mm spacer to avoid crushing the PMs. PM sections were visualized using a 20× objective lens (NA of 0.8) on an inverted Zeiss LSM 710 laser scanning confocal microscope with a chameleon two-photon laser tuned to 720 nm at 2–20% laser power. The acquired images were de-convolved using Autoquant X2.2 (Media Cybernetics, Rockville, MD, USA) and visualized using Imaris 7.4 software (Bitplane, Zurich, Switzerland).

2.3. Analysis of gene expression

Expression of genes encoding structural peritrophins and enzymes involved in chitin metabolism was examined by reverse-transcription quantitative PCR (qPCR). Total midgut RNA was purified using the Illustra RNAspin mini kit with an on-column DNAse treatment (GE Healthcare, Little Chalfont, UK) from the following stages: early (12–24 h after molting) and middle (36–48 h after molting) stage feeding larvae, pre-molt (6–8 h after larvae stopped feeding and turned pale green) and mid-molt (3–5 min period when shedding of the old cuticle reached the midpoint of the body) 4th to 5th instar larvae. For each stage, the midgut was cut into three approximately equal sections (anterior, middle, and posterior) prior to total RNA extraction. Single-strand cDNA was synthesized from 1.2 μg of purified total RNA using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Four pairs of qPCR primers for each gene were designed using Primer Select (DNASTAR Lasergene) in combination with manual selection. When sufficient gene characterization was available, the primer pairs were designed to span introns. An initial qPCR was conducted using the SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) with middle stage feeding and mid-molt larval cDNA with 0.25 μM of each primer set using a BioRad CFX 96 Real-Time PCR system to test the efficiency of primer sets for each gene (Supplementary Table 1). cDNAs were diluted 1:10 and 6 μl was used as a template in a 20 μl qPCR reaction under the following conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. A melt curve of denatured double-stranded amplicon was established to test the purity of the products using the following conditions: 95 °C for 15 s, 60 °C for 1 min, 0.3 °C increments for 15 s each until reaching 95 °C. Amplification of the *M. configurata* actin (*McACT*) gene was used as a control to ensure equivalent amounts of cDNA template in all samples. Of the three housekeeping genes that were assessed (*McACT, M. configurata tubulin* and *M. configurata ribosomal protein S3*), *McACT* had the most stable spatial and temporal expression level across the tissue samples used in this study (Supplementary Table 2). For each gene of interest, transcript levels were calculated using the ΔΔCT method of quantification (BioRad CFX Manager 2.1) with *McACT* as the endogenous reference gene and the mid-feeding, middle midgut portion sample as the template standard for relative gene expression determinations. Data were obtained from each of two biological replicates with three technical replicates for each biological replicate. A no cDNA template negative control was included in each qPCR assay.

2.4. Western blot analyses

PMs were isolated from 20 5th instar mid-feeding larvae in insect Ringer's solution with protease inhibitor cocktail as previously described (Toprak et al., 2008). Each PM was cut into $^1/_3$ sections by length (anterior, middle and posterior) and the sections vigorously rinsed in Ringer's plus protease inhibitor solution for 1 min to remove the food bolus. Identical sections from 20 larvae were pooled in 200 μl of Ringer's plus protease inhibitor solution in 1.5 ml centrifuge tubes and incubated on ice. The PM samples were then pelleted (14,000 × g for 2 min) and resuspended in 100 μl of