Insect Biochemistry and Molecular Biology 56 (2015) 21–28

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Chitin is a necessary component to maintain the barrier function of the peritrophic matrix in the insect midgut

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ARTICLE INFO

Article history:
Received 7 September 2014
Received in revised form
10 November 2014
Accepted 13 November 2014
Available online 20 November 2014

Keywords:
Aedes aegypti
Chitin
Diflubenzuron
Peritrophic matrix
Permeability
Red flour beetle
RNA interference
Tribolium castaneum

ABSTRACT

In most insects, the peritrophic matrix (PM) partitions the midgut into different digestive compartments, and functions as a protective barrier against abrasive particles and microbial infections. In a previous study we demonstrated that certain PM proteins are essential in maintaining the PM's barrier function and establishing a gradient of PM permeability from the anterior to the posterior part of the midgut which facilitates digestion (Agrawal et al., 2014). In this study, we focused on the effects of a reduction in chitin content on PM permeability in larvae of the red flour beetle, *Tribolium castaneum*. Oral administration of the chitin synthesis inhibitor diflubenzuron (DFB) only partially reduced chitin content of the larval PM even at high concentrations. We observed no nutritional effects, as larval growth was unaffected and neutral lipids were not depleted from the fat body. However, the metamorphic molt was disrupted and the insects died at the pharate pupal stage, presumably due to DFB's effect on cuticle formation. RNAi to knock-down expression of the gene encoding chitin synthase 2 in *T. castaneum* (*TcCHS-2*) caused a complete loss of chitin in the PM. Larval growth was significantly reduced, and the fat body was depleted of neutral lipids. *In situ* PM permeability assays monitoring the distribution of FITC dextrans after DFB exposure or RNAi for *TcCHS-2* revealed that PM permeability was increased in both cases. RNAi for *TcCHS-2*, however, led to a higher permeation of the PM by FITC dextrans than DFB treatment even at high doses. Similar effects were observed when the chitin content was reduced by feeding DFB to adult yellow fever mosquitos, *Aedes aegypti*. We demonstrate that the presence of chitin is necessary for maintaining the PM's barrier function in insects. It seems that the insecticidal effects of DFB are mediated by the disruption of cuticle synthesis during the metamorphic molt rather than by interfering with larval nutrition. However, as DFB clearly affects PM permeability, it may be suitable to increase the efficiency of pesticides targeting the midgut.

1. Introduction

In response to a feeding stimulus, the midgut cells of many insects secrete a peritrophic matrix (PM)[1], which lines the epithelium and envelopes the gut contents. It partitions the gut into three distinct compartments, i.e. the endoperitrophic space (containing the gut content), the ectoperitrophic space (between the midgut epithelium and the PM) and the PM itself. The PM is composed of chitin fibrils and PM associated proteins including invertebrate intestinal mucins (Tellam, 1996). Some of these proteins are tightly bound to the chitin fibrils, due to the presence of CBM14 family chitin-binding domains (peritrophin A-type or ChtBD2 family) (Cantarel et al., 2009; Jasrapuria et al., 2010; Lehane, 1997; Shao et al., 2005; Shen and Jacobs-Lorena, 1998; Tellam et al., 1999). They are referred as PM proteins (PMPs). As the number of CBM14 domains varies among different PMPs, it has been proposed that the occurrence of different numbers may influence their ability to associate with chitin microfibrils or to cap the ends of chitin microfibrils. In literature, mainly four functions have been attributed to the PM of insects: i) protection from mechanical damage, ii) facilitation of digestion by partitioning of digestive enzymes and/or digestion products, iii) neutralization of ingested toxins by binding or retention, and iv) protection from invasive parasites, bacteria and viruses (Hegedus et al., 2009).

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http://dx.doi.org/10.1016/j.ibmb.2014.11.005
0965-1748/

In recent years, experimental evidence has been provided for the latter three functions: An RNAi study performed in the red flour beetle, *Tribolium castaneum*, indicated that two PMPs are crucial for the barrier function of the PM and the formation of an anterior-to-posterior PM permeability gradient, which has been suggested to facilitate digestion (Agrawal et al., 2014). In the yellow fever mosquito, *Aedes aegypti*, the peritrophic matrix protein, AelMUCI, has been shown to bind toxic heme *in vitro* using multiple heme-binding sites, which have been suggested to promote heme aggregation mitigating its toxic effects (Devenport et al., 2006). In the fruit fly, *Drosophila melanogaster*, it has been demonstrated that the PM protects from oral infections with entomopathogenic bacteria. Mutant flies defective in the *drosocrystallin* gene, which encodes a protein essential for proper PM formation, showed decreased survival rates after oral infections, and were also more susceptible to bacterial extracts containing Monalysin, a pore-forming toxin produced by *Pseudomonas entomophila* (Kuraishi et al., 2011). The PM barrier function is further compromised by intestinal pathogens that express hydrolytic enzymes, such as chitinase produced by the ookinete of the malaria parasite, *Plasmodium falciparum* (Shahabuddin et al., 1993), or enhancin, which is formed after *Mamestra configurata* nucleopolyhedrovirus infection (Toprak et al., 2012). Also plant cysteine proteases have been shown to act on the PM by degrading particular PMPs in the midgut of *Spodoptera frugiperda* (Fescemyer et al., 2013; Pechan et al., 2000). Bacterial endochitinases or baculovirus-encoded enhancins, have been further shown to increase the toxic effects of *Bacillus thuringiensis* (Bt) Cry delta-endotoxins (Bischoff and Slavicek, 1997; Rees et al., 2009; Regev et al., 1996).

As the PM is evidently essential for insect survival, several strategies were utilized to use PM compromising agents for the purpose of insect control. For instance, perturbation of the PM structure and function by different lectins and lectin-like proteins such as wheat germ agglutinin (WGA) and Labramin has been demonstrated in different lepidopteran species including the European corn borer (*Ostrinia nubilalis*) and the Mediterranean flour moth, *Ephestia kuehniella* (Harper et al., 1998; Hopkins and Harper, 2001; Martinez et al., 2012). Similar to these effects, the chitin-binding fluorophor Calcofluor white (CFW) has been reported to disrupt the PM function by displacing PM proteins (Wang and Granados, 2000). In *Trichoplusia ni* and *Spodoptera exigua*, disruption of PM formation by CFW significantly retarded larval development, increased their susceptibility to baculoviral infection and resulted in high mortality (Wang and Granados, 2000; Zhu et al., 2007b).

Next to chitin binding compounds, inhibitors of chitin biosynthesis such as diflubenzuron (DFB) have also been shown to impair PM formation. In the locust, *Locusta migratoria*, DFB partially blocks chitin synthesis in the midgut, which is paralleled by a decrease in the PM's protein content (Clarke et al., 1977). In adult blow flies, *Calliphora erythrocephala*, the PM formation rate measured either in units of length or volume was reduced after treatment with DFB (Becker, 1978). DFB also affected the production of the peritrophic membrane in adult *Tenebrio molitor* beetles when added to the diet. The loss of body weight and reduction in lifespan of the treated beetles was suggested to be due to DFB-induced alterations of the PM (Soltani, 1984). In larvae of *Ae. Aegypti*, however, chitin labeling of the PM by WGA remained unchanged after treatment with the DFB derivative novaluron (Farnesi et al., 2012), confirming previous reports on chitin quantitation in *Anopheles quadrimaculatus* larvae that were exposed to DFB (Zhu et al., 2007a). However, novaluron-treatment affected the morphology of the midgut and the PM in the mosquito larvae, in that the epithelium appeared thicker as individual cell were enlarged, and the PM did not form a continuous lining.

The effects of a reduction in the PM's chitin content have not been investigated with respect to the permeability and barrier function of the PM so far. To examine these effects we have chosen the model beetle and stored product pest, *T. castaneum*. The larvae of this beetle form a “type I” PM which is secreted by the entire midgut (Hegedus et al., 2009; Tellam, 1996). In a previous study, we have shown that oral application of DFB results in abortive molting, hatching defects and a reduction in the chitin amounts of about 50%, not only in eggs and the larval cuticle, but also in the PM, which was consequently less stained by CFW (Merzendorfer et al., 2012). Utilizing an *in situ* permeability assay based on the feeding of fluorescein isothiocyanate (FITC) dextrans, which we previously developed to assess physiological functions of PMPs in the larval midgut of *T. castaneum* (Agrawal et al., 2014), we now demonstrate that DFB disrupts the PM of *T. castaneum* leading to an increased permeability of the PM for large particles that are completely retained in the gut lumen of control animals. This effect, however, was less pronounced compared to larvae, that have been injected with dsRNA specific for *TcCHS-2* to knock-down chitin synthesis in the midgut of *T. castaneum* as reported previously (Arakane et al., 2008). Similar effects on the PM permeability are observed in adult yellow fever mosquitos, *Ae. aegypti* after feeding blood plasma supplemented with FITC dextrans and DFB.

2. Materials and methods

2.1. Insect breeding, insecticide treatment and permeability assays

In this study the *T. castaneum* strain GA-1 (Haliscak and Beeman, 1983) was used. Beetles were reared in whole wheat flour containing 5% (w/w) brewer's yeast at 30 °C as described previously (Beeman and Stuart, 1990). For some experiments, the wheat flour was supplemented with diflubenzuron (DFB, PESTANAL, Sigma—Aldrich, St. Louis, USA) as described previously (Merzendorfer et al., 2012). Larvae with an average body weight of 1.5 mg were placed on the control diet, or a diet containing either 100 ppm or 1000 ppm DFB, and continuously fed for six days. Larval body weights were determined shortly before exposure to DFB or dsRNA injection, as well as three and six days after starting the treatment. For PM permeability assays, fluorescein isothiocyanate (FITC) dextrans of different molecular masses were added to the wheat flour at concentrations reported before (Agrawal et al., 2014). *Ae. aegypti* mosquitoes of the white-eyed khW strain were maintained under long day conditions (16 h light, 8 h dark) at 27 ± 3 °C) as described previously (Kaufmann et al., 2009). To obtain eggs, females were starved for 12 h and then were allowed to feed on anaesthetized BALB/C mice. For the analysis of PM permeability in female adult mosquitos, pre-warmed (37 °C) blood plasma from pig supplemented with fluorescein isothiocyanate (FITC) or FITC-dextrans was fed using funnels covered by chicken skin.

2.2. Synthesis of dsRNA probes

Total RNA was prepared from pools of 4 larvae using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed with the Superscript cDNA synthesis kit (Invitrogen) using 1 μg of total RNA and oligo-dT primers. The cDNA served as a template in a PCR amplifying fragments of *TcVER* of *TcCHS-2* with gene-specific primers listed in Table S1. After ligating the PCR products into pGEM-T (Promega), the derived plasmid was used to transform *E. coli* DH5α cells. High copy number plasmids containing either *TcVER* or *TcCHS-2* fragments were isolated using the Plasmid Mini Kit (Qiagen) and used as templates for another PCR with gene-specific primers containing T7 RNA polymerase binding sites at their 5′-ends (see Table S1). After agarose gel electrophoresis, the PCR