Journal of Insect Physiology 54 (2008) 1413–1422

Contents lists available at ScienceDirect

Journal of Insect Physiology

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

Peritrophic membrane role in enhancing digestive efficiency Theoretical and experimental models

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ARTICLE INFO

Article history:
Received 14 May 2008
Received in revised form 23 July 2008
Accepted 4 August 2008

Keywords:
Peritrophic membrane
PM function
Enzyme recycling
Calcofluor
Nutritional parameters

ABSTRACT

The peritrophic membrane (PM) is an anatomical structure surrounding the food bolus in most insects. Rejecting the idea that PM has evolved from coating mucus to play the same protective role as it, novel functions were proposed and experimentally tested. The theoretical principles underlying the digestive enzyme recycling mechanism were described and used to develop an algorithm to calculate enzyme distributions along the midgut and to infer secretory and absorptive sites. The activity of a *Spodoptera frugiperda* microvillar aminopeptidase decreases by 50% if placed in the presence of midgut contents. *S. frugiperda* trypsin preparations placed into dialysis bags in stirred and unstirred media have activities of 210 and 160%, respectively, over the activities of samples in a test tube. The ectoperitrophic fluid (EF) present in the midgut caeca of *Rhynchosciara americana* may be collected. If the enzymes restricted to this fluid are assayed in the presence of PM contents (PMC) their activities decrease by at least 58%. The lack of PM caused by calcofluor feeding impairs growth due to an increase in the metabolic cost associated with the conversion of food into body mass. This probably results from an increase in digestive enzyme excretion and useless homeostatic attempt to reestablish destroyed midgut gradients. The experimental models support the view that PM enhances digestive efficiency by: (a) prevention of non-specific binding of undigested material onto cell surface; (b) prevention of excretion by allowing enzyme recycling powered by an ectoperitrophic counterflux of fluid; (c) removal from inside PM of the oligomeric molecules that may inhibit the enzymes involved in initial digestion; (d) restriction of oligomer hydrolases to ectoperitrophic space (ECS) to avoid probable partial inhibition by non-dispersed undigested food. Finally, PM functions are discussed regarding insects feeding on any diet.

1. Introduction

The peritrophic membrane (PM) is an anatomical structure surrounding the food bolus in most insects with the remarkable exception of Hemiptera and Thysanoptera that have instead a lipid membrane (the perimicrovillar membrane, Kitajima, 1975; Lane and Harrison, 1979; Silva et al., 2004) ensheathing their midgut microvilli. PM is made of proteins (peritrophins) interlocked with chitin fibrils. This anatomical structure is sometimes also called peritrophic matrix, in spite the fact that matrix in biology does not convey the idea of a sheath and suggests a substance that fills a space, like the mitochondrial matrix. The vast literature available on PM is comprehensively reviewed by Peters (1992), by Jacobs-Lorena and Oo (1996) on hematophagous Diptera and three others reviews that emphasize PM structural aspects and PM roles in midgut epithelium protection (Tellam, 1996; Lehane, 1997; Tellam et al., 1999).

Since the insect midgut epithelium lacks a mucus coating, PM functions were supposed to be analogous to that of the mucus that lubricates the mucosa, protecting it from mechanical damage, and to trap bacteria and parasites. Thus, insects deprived of PM may have the midgut cells damaged by coarse food and may be liable to microorganism invasion in some reported cases (Peters, 1992; Tellam, 1996; Lehane, 1997). Nevertheless, taking into account the theory of evolution, it seems unlikely that the ancestral mucus that is found in most animals is replaced by a complicated multi-molecular structure to realize the same protective function.

According to Terra (2001) ancestral insects had their midgut cells covered with a mucus similar to that found in most animals. Later on, the peritrophins evolved from mucins by acquiring chitin-binding domains. The parallel evolution of chitin secretion by midgut cells permitted the formation of the chitin–protein network characteristic of PM structure (see reviews above). Thus, the specific functions of PM (those not played also by mucus) must

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doi:10.1016/j.jinsphys.2008.08.002

depend on the fact that PM compartmentalizes the midgut lumen into an endoperitrophic space (EDS) (inside PM) and an ectoperitrophic space (ECS) (space between PM and midgut epithelium). Work done with the larvae of *R. americana* showed the significance and importance of the midgut compartments in regulating the initial, intermediate and final stages of polymer digestion (Terra et al., 1979; Ferreira and Terra, 1984). This prompted a large number of papers aimed to study the compartmentalization of digestive events in model insects pertaining to different insect orders. These studies describing the spatial organization of digestive events and determining the PM permeability were reviewed several times (Terra and Ferreira, 1994, 2003, 2005).

Based on those studies, Terra (2001) proposed that the PM functions distinct from those of the gastrointestinal mucus may be divided into primary and secondary functions. Primary functions are those probably evolved under selective pressures, whereas secondary ones are consequences of the chemical properties of PM components (enzyme immobilization and toxin binding). The primary functions found in all insects are: (a) prevention of non-specific binding of undigested material onto cell surface; (b) prevention of excretion by allowing enzyme recycling powered by an ectoperitrophic counterflux of fluid; (c) removal of oligomeric molecules that may inhibit the enzymes involved in initial digestion from inside PM. Primary functions that are restricted to panorpoid insects (dipterans and lepidopterans) are: (a) restriction of oligomer hydrolases to ectoperitrophic space to avoid probable partial inhibition by polymeric food (because of non-productive binding) and putative non-specific binding by non-dispersed undigested food; (b) restriction of monomer production to cell surface causing increased concentration of the final products close to the carriers responsible for their absorption.

From the PM functions proposed, only the prevention of enzyme excretion has some experimental support. Both *R. americana* and *Musca domestica* present a decreasing trypsin gradient along midgut contents (putatively generated by the recycling mechanism) and excrete less than 15% of midgut luminal trypsin after each gut emptying. When the larvae were fed a diet with excess protein, the trypsin gradient along midgut contents becomes less discernible and trypsin excretion increases to 40%. This is exactly what would be expected if the recycling mechanism existed and an increase in undigested dietary protein prevents trypsin from diffusing into the ectoperitrophic space and moving into anterior midgut by the countercurrent (CC) flux of fluid. Subsequently, dye experiments showed the existence of the appropriate fluid fluxes (Terra and Ferreira, 1994, 2005). More recently, experimental evidence that a recycling mechanism also occurs in Lepidoptera and Coleoptera was described (Peterson et al., 1994; Borhegyi et al., 1999; Ferreira et al., 2002).

This paper was carried out to provide experimental and theoretical support for the proposals of PM function described above. For this, a theoretical model for enzyme recycling was advanced and experimental models were developed to detail the recycling mechanism and to test the other proposals. The results confirmed the PM functions proposed.

2. Materials and methods

2.1. Insects

Stock cultures of the yellow mealworm, *Tenebrio molitor* (Coleoptera), were cultured under natural photoregime conditions on wheat bran at 24–26 °C and a relative humidity of 70–75%. Fully grown larvae (each weighing about 0.12 g), having midguts full of food, of both sexes were used.

R. americana (Diptera: Sciaridae) are continuous feeders usually found under decaying plants in banana orchards near the southeast coast of Brazil. The larvae were a gift from Dr. Roberto V. Santelli (University of São Paulo) and we have used only feeding larvae at the end of the 2nd period of the 4th instar (Terra et al., 1973).

Larvae of *M. domestica* (Diptera, Cyclorrhapha, Muscidae) were reared in a mixture of fermented commercial pig food and rice hull (1:2, v/v) (Targa and Peres, 1979). The larvae used in this study were actively feeding individuals at third instar.

Spodoptera frugiperda (Lepidoptera: Noctuidae) were laboratory reared according to Parra (1986). The larvae were individually contained in glass vials with a diet based on kidney bean (*Phaseolus vulgaris*), wheat germ, yeast, and agar and were maintained under a natural photoregime at 25 °C. Fifth (last) instar larvae of both sexes were used in the experiments.

2.2. Hydrolase assays

Aminopeptidase N was assayed in 100 mM Tris–HCl buffer (pH 7.5) for *S. frugiperda* samples and 100 mM phosphate–sodium buffer (pH 8) for *R. americana* samples, using as substrate 1 mM L-Leu-*p*-nitroanilide and following the release of *p*-nitroaniline according to Erlanger et al. (1961). *Aminopeptidase A* was determined with 0.25 mM Asp-β-naphthylamide as substrate (according to Hopsu et al., 1966) in 100 mM Tris–HCl buffer pH 7.5. *Amylase* activity was measured by determining the appearance of reducing groups (Noelting and Bernfeld, 1948) from 0.5% soluble starch in media containing 10 mM NaCl in 100 mM citrate–sodium phosphate buffer pH 5.0 (*M. domestica*), pH 6.5 (*T. molitor*) or 100 mM glycine–NaOH buffer pH 9.5 (*S. frugiperda*). β-*N*-*Acetylglucosaminidase* was determined by following the increase of *p*-nitrophenolate (according to Terra et al., 1979) produced from 1.25 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 50 mM citrate–sodium phosphate buffer pH 6.0. *Carboxypeptidase A* activity was measured with carbobenzoxy-glycyl-L-phenylalanine as substrate in 100 mM Tris–HCl buffer pH 8 and accompanying the increase of Phe (Nicholson and Kim, 1975). *Chymotrypsin* was assayed with 1 μM *N*-succinyl-Ala-Ala-Phe 7-amido-4 methyl-coumarin in 100 mM Tris–HCl buffer (pH 8.5). The substrate is dissolved in dimethyl sulfoxide and then diluted 100 times with buffer. The reaction is stopped with 30% acetic acid and the fluorescence was detected in fluorimeter, with excitation at 380 nm and detection at 460 nm (Alves et al., 1996). In samples containing calcofluor, chymotrypsin was assayed with 0.5 mM succinyl-Ala-Ala-Phe-*p*-nitroanilide in 100 mM Tris–HCl buffer (pH 8.5). The reaction was stopped as before and the absorbance was determined at 410 nm. *Dipeptidase* and *maltase* were assayed with 5 mM Gly-Leu (buffer: 100 mM Tris–HCl pH 8) and 7mM maltose (buffer: 50 mM citrate–sodium phosphate pH 5) as substrate and determining the appearance of leucine (Nicholson and Kim, 1975) and glucose (Dahlqvist, 1968), respectively. β-*Glucosidase* was measured by following the increase of glucose (Dahlqvist, 1968) from 0.15 mM amygdalin in 100 mM citrate–sodium phosphate buffer pH 5.5. At this condition, the measured β-glucosidase activity corresponds to the one that was immunocytolocalized in *T. molitor* midgut (Ferreira et al., 2002). *Trypsin* was assayed with 10 μM carbobenzoxy-Arg-7-amido-4 methyl coumarin in 100 mM Tris–HCl buffer pH 8.0 (*T. molitor*), pH 9.0 (*M. domestica*) or pH 7.5 (*S. frugiperda*). The previous preparation of substrate and details of detection were similar to those of chymotrypsin described above. Trypsin was also assayed with α-*N*-benzoyl DL-Arg-*p*-nitroanilide. Other conditions as before.

All assays were performed at 30 °C in media of the indicated pH values and incubations have been carried out for at least four different periods of time and the initial rates of hydrolysis have