Journal of Insect Physiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Insect Physiology

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

Physiology of digestion and the molecular characterization of the major digestive enzymes from *Periplaneta americana*

Fábio K. Tamaki [a,1], André C. Pimentel [a,1], Alcides B. Dias [a], Christiane Cardoso [a], Alberto F. Ribeiro [b], Clélia Ferreira [a], Walter R. Terra [a,*]

[a] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 26077, 05513-970 São Paulo, Brazil
[b] Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, C.P. 11461, 05513-970 São Paulo, Brazil

ARTICLE INFO

Article history:
Received 26 June 2014
Received in revised form 22 August 2014
Accepted 23 August 2014
Available online xxxx

Keywords:
Proteases
Carbohydrases
Digestive enzymes sequences
Secretory mechanism
Enzyme immunocytolocalization

ABSTRACT

Cockroaches are among the first insects to appear in the fossil record. This work is part of ongoing research on insects at critical points in the evolutionary tree to disclose evolutionary trends in the digestive characteristics of insects. A transcriptome (454 Roche platform) of the midgut of *Periplaneta americana* was searched for sequences of digestive enzymes. The selected sequences were manually curated. The complete or nearly complete sequences showing all characteristic motifs and highly expressed (reads counting) had their predicted sequences checked by cloning and Sanger sequencing. There are two chitinases (lacking mucin and chitin-binding domains), one amylase, two α- and three β-glucosidases, one β-galactosidase, two aminopeptidases (none of the N-group), one chymotrypsin, 5 trypsins, and none β-glucanase. Electrophoretic and enzymological data agreed with transcriptome data in showing that there is a single β-galactosidase, two α-glucosidases, one preferring as substrate maltase and the other aryl α-glucoside, and two β-glucosidases. Chromatographic and enzymological data identified 4 trypsins, one chymotrypsin (also found in the transcriptome), and one non-identified proteinase. The major digestive trypsin is identifiable to a major *P. americana* allergen (Per a 10). The lack of β-glucanase expression in midguts was confirmed, thus lending support to claims that those enzymes are salivary. A salivary amylase was molecularly cloned and shown to be different from the one from the midgut. Enzyme distribution showed that most digestion occurs under the action of salivary and midgut enzymes in the foregut and anterior midgut, except the posterior terminal digestion of proteins. A counter-flux of fluid may be functional in the midgut of the cockroach to explain the low excretory rate of digestive enzymes. Ultrastructural and immunocytochemical localization data showed that amylase and trypsin are released by both merocrine and apocrine secretion mainly from gastric caeca. Finally, a discussion on Polyneoptera digestive physiology is provided.

1. Introduction

Cockroaches, which are among the first neopteran insects to appear in the fossil record, are extremely generalized in most morphological features. They are usually omnivorous and are included in the sub-order Blattodea that together with Mantodea (mantids) form the Order Dictyoptera. After extensive molecular phylogenetic analyses, Inward et al. (2007) showed that termites are social cockroaches, no longer deserving classification as a separate order (Isoptera) from cockroaches. Actually, termites pertain to a sister family (Termitidae) of that of the woodroach *Cryptocercus* (Cryptocercidae) (Lo et al., 2000). The branch Cryptocercidae-Termitidae is a sister of Blattidae, forming Blattoidea that is a sister of Blaberoidea (Blattelidae plus Blaberidae), which in addition to Polyphagoidea form the Blattodea.

The organization of the digestive process in the different insect orders that corresponds to the basic plans of the ancestral forms was reviewed several times (Terra, 1988, 1990; Terra and Ferreira, 1994, 2012). Dictyoptera is supposed to be derived from the Polyneoptera ancestors. Hence, its basic digestive organization should be alike that of the Polyneoptera ancestor. One of the aims of this paper is to provide support in this direction.

Data on compartmentalization of midgut pH and digestion in Dictyoptera are fragmentary and contradictory (see Elpidina et al., 2001 and references therein). In part this is a consequence

* Corresponding author. Fax: +55 11 3838 2186.
E-mail address: warterra@iq.usp.br (W.R. Terra).
[1] These authors contribute equally to this paper.

http://dx.doi.org/10.1016/j.jinsphys.2014.08.007
0022-1910/

of differences among the cockroaches. For example, starch digestion occurs mainly in foregut and anterior midgut and protein digestion takes place in posterior midgut in *Nauphoeta cinerea* (Blaberidae) (Elpidina et al., 2001), whereas in several other cockroach species there is not a marked difference in starch digestion in foregut and midgut (Vinokurov et al., 2007). There is a lack of data on cockroach terminal digestion of both proteins and carbohydrates and on the presence (or absence) of a midgut countercurrent flux of fluid to propel digestive enzyme recycling (reviews: Terra, 1988, 1990; Terra and Ferreira, 1994, 2012). Thus, a re-examination of *Periplaneta americana* (Blattidae) digestive enzyme compartmentalization attempting to those details and using only one sex (males) would reduce variability and result in a broader picture of cockroach digestive physiology.

There are numerous papers dealing with cockroach digestive enzymes, including from *P. americana*, such as chitinase (Powning and Irzykiewicz, 1963), glycosidases (Scrivener et al., 1989; Koffi et al., 2012), peptidases (Baumann, 1990; Lopes and Terra, 2003; Hivrale et al., 2005) and also some works regarding the distribution of polymer hydrolases in cockroach gut, exemplified by Elpidina et al. (2001) and Vinokurov et al. (2007). Nevertheless, there is a lack of data on digestive enzyme sequences.

In this paper, we combined enzymological and transcriptomic data to identify the sequences of the major digestive enzymes. Furthermore, enzyme distribution studies showed how the digestive process is spatially organized and that a countercurrent flux of fluid may be functional in the *P. americana* midgut to explain the low excretory rate of digestive enzymes. Finally, secretory mechanisms of digestive enzymes were identified by immunocytolocalization of trypsin and amylase.

2. Materials and methods

2.1. Animals and enzyme samples

P. americana (Dictyoptera) were laboratory reared feeding on chayote (*Sechium* sp.) and oats (*Avena sativa*) and maintained under a natural photoregime at room temperature. Only adult males were used in the experiments, because females do not routinely feed, lay eggs (that may affect results), and have variable sizes. Adult males were immobilized in a carbon dioxide chamber for 10 min. The antennae, legs and wings were removed, the insects placed on ice and then dissected in cold 220 mM NaCl. The rinsed guts were transferred to a glass slide. The gut was divided into foregut (crop), midgut caeca, midgut and ileum + colon. Midgut was separated into sections of identical lengths: anterior and posterior midgut. Midgut sections were separated into tissue and contents.

Midgut sections of tissue and contents, as well as foreguts and ileum, were homogenized in double distilled water with the aid of a Potter–Elvehjem homogenizer and centrifuged at 20,000×*g* for 30 min at 4 °C. The supernatants were filtered in glass wool and the pellets (except those of midgut contents) were resuspended in double distilled water. The pellets were regarded as cell membrane fraction, because the hypotonic homogenization medium ruptures all cells. The samples were stored at −20 °C until use. No enzyme inactivation was detected on storage.

2.2. Protein determination, enzymatic assays, excretion rates, effect of pH on enzymatic activity and luminal pH determination

Protein was determined according to Bradford (1976) using ovalbumin as a standard.

Enzymatic assays were performed at 30 °C in specific conditions (Table 1). Proteinase profiles were determined using synthetic substrates (Table 1) or the natural substrate casein-FITC (Twining, 1984), combined or not with inhibitors: 17 μM SBTI, 100 μM benzamidine, 100 μM chymostatin or 20 μM TPCK.

The effect of pH on enzymatic activities were studied in 50 mM sodium citrate–phosphate (pH 4.0–7.0) or 50 mM sodium phosphate (pH 7.0–8.0) for α-glucosidase, β-glucosidase, β-galactosidase, cellobiase, and trehalase. For amylase, trypsin, maltase and aminopeptidase assays were performed in 100 mM sodium acetate (pH 3.0–4.0), 100 mM piperazine (pH 4.0–6.0), 100 mM HEPES (pH 6.0–8.0), 100 mM TAPS (pH 8.0–9.0) and 100 mM CAPS (pH 9.0–10.0). All buffers included 100 mM NaCl.

The pH of midgut contents sections were separately determined using a universal pH indicator (E. Merck, Darmstadt, pH 4.0–10.0). For this, midgut section contents were dispersed in 5 μL of dissecting saline and then added to 5 μL of a 5-fold dilution of the pH indicator. The resulting colored solutions were compared with suitable standards.

Excretion rates were calculated using the equation {[Activity Ileum + Colon × (Volume Midgut/Volume Ileum + Colon)]/Activity Midgut}.

2.3. Resolution of midgut enzymes by chromatography and polyacrylamide-gel-electrophoresis in native conditions

Midgut proteins were resolved by ion-exchange chromatography in a High-Q column connected to a Econo-pac System (Bio-Rad, USA) in 20 mM Tris–HCl pH 7.5. Elution was carried by a linear gradient of 0–0.6 M NaCl, followed by a washing step with 10 mL 1 M NaCl. Fractions of 1 mL were collected and assayed with different substrates for peptidases in the presence and absence of specific enzyme inhibitors.

Midgut homogenates were applied onto a 7.5% polyacrylamide gel (8 cm), and proteins were electrophoretically resolved at 4 °C using 2.5 mA per cylinder (Hedrick and Smith, 1968). Afterwards, gels were washed for 1 h in 20 mM citrate–phosphate pH 6.0 to remove the Tris–HCl buffer (which inhibits carbohydrases) at 4 °C and subsequently sliced in a 50 mM citrate–phosphate pH 6.0 buffer. Samples were collected and proteins were eluted for 3 h at 4 °C, before being assayed with different substrates for carbohydrases (Table 1).

2.4. SDS–PAGE and Western blotting

SDS–PAGE was carried in 12% (w/v) polyacrylamide gel electrophoresis containing 0.1% SDS in a discontinuous pH system (Laemmli, 1970), in a Mini-Protean II equipment (Bio-Rad (U.S.A.). Samples were mixed with sample buffer containing: 60 mM Tris–HCl buffer pH 8.8, 2.5% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromophenol blue and 0.36 mM 2-mercaptoethanol. The samples were heated at 95 °C for 3 min, before being loaded onto gel. Electrophoresis was carried out at 200 V and silver-stained (Blum et al., 1987).

Western blottings were performed after SDS–PAGE separation of midgut homogenate proteins (see above) and electrophoretic transfer to nitrocellulose membrane filter (pore size 0.45 μm, Bio-Rad, U.S.A.). The transfer efficiency was verified using a pre-stained molecular mass marker (Bio-Rad, U.S.A.). After blocking the membrane with milk in TBS-T (Tris-buffered saline containing 0.05% Tween 20), it was reacted for 4 h at room temperature with polyclonal *Tenebrio molitor* anti-amylase (Cristofoletti et al., 2001) or polyclonal *Musca domestica* anti-trypsin (Jordão et al., 1996) sera, both diluted 500-fold in TBS-T. After washing 3 times with TBS-T, the membrane was reacted with anti-rabbit IgG coupled with peroxidase diluted 1000-fold in TBS-T for 2 h at room temperature. After extensive washing, the membranes were maintained in 0.08% 4-chloro-1-naphthol in TBS containing 0.1%

Please cite this article in press as: Tamaki, F.K., et al. Physiology of digestion and the molecular characterization of the major digestive enzymes from *Periplaneta americana*. Journal of Insect Physiology (2014), http://dx.doi.org/10.1016/j.jinsphys.2014.08.007