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Spatial characterization of proteolytic enzyme activity in the foregut region of the adult necrophagous fly, *Protophormia terraenovae*

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ABSTRACT

The spatial distribution of proteolytic enzymes in the adult foregut of *Protophormia terraenovae* was studied in the context of protein digestion and regurgitation. Based on substrate specificity, pH optima, and use of specific protease inhibitors, all adults tested displayed enzyme activity in the foregut consistent with pepsin, trypsin and chymotrypsin. Chymotrypsin-like and trypsin-like enzyme activity were detected in all gut fluids and tissues tested, with chymotrypsin displaying the highest activity in saliva and salivary gland tissue, whereas maximal trypsin activity was evident in the crop. Pepsin-like activity was only evident in crop fluids and tissues. The activity of all three enzymes was low or undetectable (pepsin) in the fluids and tissue homogenates derived from the esophagus and cardia of any of the adults assayed. Fed adult females displayed higher enzyme activities than fed males, and the activity of all three enzymes were much more prevalent in fed adults than starved. The pH optimum of the trypsin-like enzyme was between pH 7.0 and 8.0; chymotrypsin was near pH 8.0; and maximal pepsin-like activity occurred between pH 1.0 and 2.0. Regurgitate from fed adult females displayed enzyme activity consistent with the proteolytic enzymes detected in crop gut fluids. Enzymes in regurgitate were not derived from food sources based on assays of bovine liver samples. These latter observations suggest that adult flies release fluids from foregut when encountering dry foods, potentially as a means to initiate extra-oral digestion.

1. Introduction

Several species of cyclorrhaphous Diptera feed as larvae on a wide range of carrion, devouring the food source while formed in large heterogeneous feeding aggregations or maggot masses (Rivers et al., 2011). Such feeding assemblages quickly consume an animal corpse in a matter of days under favorable environmental conditions. As carrion is an ephemeral, patchy, nutrient-rich resource, it draws the attention of many organisms beyond just arthropods. Among the initial colonizers are a variety of microorganisms, typically obligate or facultatively anaerobic bacteria (Thompson et al., 2013). Bacteria are not passive inhabitants of the carrion community; rather they actively compete with the fly larvae for the finite pool of nutrients (Tomberlin et al., 2011; Thompson et al., 2013) and appear to be essential to the chemical signaling that attracts adult calliphorids and presumably other Diptera to the decomposing carcass (Ma et al., 2012; Tomberlin et al., 2012). Saprophagous fly larvae are well equipped for the challenges posed by bacterial competitors, especially by virtue of their highly adapted digestive system (Greenberg, 1968; Rockstein and Kamal, 1954; Lemos and Terra, 1991; Mumcuoglu et al., 2001).

Much less is known about the digestive adaptations of adult calliphorids and related flies (Terra and Ferreira, 1994). The digestive enzymes of only a limited number of necrophagous adults have been studied in any detail, with the most extensive investigations involving *Phormia regina* (Meigen) (Diptera: Calliphoridae) and *Musca domestica* L. (Muscidae) (reviewed in Terra, 1988; Stoffolano and Haselton, 2013). Adults of both species feed by imbibing liquid foods, or salivating or regurgitating the contents of the foregut onto dry food, and then ingesting the liquefied materials (Stoffolano et al., 2008; Terra, 1988). Enzymatic digestion is thought to occur entirely in the fly digestive tract, with chemical digestion being initiated by salivary amylases within the crop (Stoffolano and Haselton, 2013; Terra, 1988). In *M. domestica*, protein processing and destruction of bacteria are initiated once the

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bolus travels through the anterior midgut, relying predominately on a trypsin enzyme and lysozyme activity, respectively, in an acidic gut environment (Terra et al., 1988). The foregut of *P. regina* is similar in that it appears principally designed for carbohydrate digestion, although the crop is the site for limited protein and lipid processing (Stoffolano and Haselton, 2013). These observations are consistent with the view that non-hematophagous Diptera use the crop to store large quantities of nutrients, especially protein, and to serve as a site for chemical digestion, especially carbohydrates. However this region is thought to lack the ability to produce digestive enzymes (Stoffolano and Haselton, 2013). Hence, any enzymes found in the crop are presumed to originate from salivary glands. Sinha (1976), however, reported the presence of several proteases in the foregut of adult *Sarcophaga ruficornis* F. (Diptera: Sarcophagidae) and *M. domestica*, each occurring only in tissues and fluids of the crop. Considering that necrophagous flies rely on the precursor step of bubbling or droplet regurgitation before ingestion when encountering dry food (Stoffolano and Haselton, 2013), and the tremendous importance of a protein meal to egg provisioning for anautogenous species or those relying on income breeding strategies (Rivers and Dahlem, 2014), it is entirely plausible that the foregut of at least some species contains proteolytic enzymes. The presence of such enzymes in the foregut would suggest that protein digestion is initiated in regurgitate, analogous to a form of extra-oral digestion, and may contribute to the elimination of bacteria prior to the bolus entering the midgut.

This study focused on characterizing the spatial activity of proteolytic enzymes in the adult foregut of the necrophagous fly, *Protophormia terraenovae* Robineau-Desvoidy (Diptera: Calliphoridae). The fly was especially well suited for the study since females are anautogenous and known to feed by depositing regurgitate on food prior to ingestion (Harlow, 1956; Rivers, unpublished). Here, we examined proteolytic enzyme activity in different regions of the adult male and female foregut, comparing enzyme activity under a range of conditions (fed vs. starved, varying pH, and in the presence of inhibitors). We also assayed regurgitate from fed adult females for enzyme activity to determine whether extra-oral digestion may initiate food processing.

2. Materials and methods

2.1. Fly rearing

Larvae of *P. terraenovae* were purchased from Forked Tree Ranch (Bonners Ferry, Idaho, USA) and raised in colony for five generations before use in this study. Adults were reared in wire mesh cages (30 cm × 30 cm × 30 cm) at 25 °C, 70–75% RH under a long-day (LD 15:9 h) photoperiod and fed bovine liver and sugar cubes *ad libitum*. Larvae were fed fresh bovine liver (liver was placed on sand in open plastic containers [30 cm × 20 cm × 10 cm]) throughout development under the same conditions as adults in environmental chambers (Model I30BLLC8, Percival Scientific, Boone, IA, USA).

2.2. Collection of gut fluids and tissues

Gut fluids and tissues were isolated from adult flies that were either previously fed *ad libitum* bovine liver for 48 h at 25 °C (LD 15:9 h), or that had been deprived of all food (but not water) for 24 h under the same conditions. The latter flies are referred to as ‘starved’. One hundred adult flies were placed in mesh cages with 25 g fresh bovine liver and water. At the end of the feeding or starvation period, all adult flies were 72 h old after adult emergence at 25 °C. Each fly was immediately processed by placing on ice, the sex determined by examining external genitalia under a stereo-dissecting microscope (Zeiss STEMI 2000, Gottingen, Germany), a longitudinal incision was made along the pronotum to the last abdominal tergite, then the entire length of the digestive tract (including salivary glands) from the esophagus to the rectum was removed with forceps, and placed on a chilled glass microscope slide. The gut tube was sectioned with a micro-scalpel into the esophagus, crop, anterior midgut (cardia), and salivary glands using the morphological guide of Boonsriwong et al. (2011). Each section was quickly dipped in 5 μl chilled Dulbecco’s phosphate buffered saline (pH 7.2) (Sigma Chemical Co., St. Louis, MO, USA), and then the tissue was transferred to a microcentrifuge tube containing 25 μl chilled buffer (on ice). The initial rinse for each tissue type was pooled so that gut fluids from 50 adult flies were stored together at −80 °C. Similarly, gut sections from adult flies were pooled together in chilled Dulbecco’s phosphate buffered saline (pH 7.2). In all cases, tissues and fluids of adult males were kept separate from females. Tissues were centrifuged (3000 rpm at 4 °C for 5 min) (Sorvall RC2-B) to form a loose pellet. The supernatant was discarded, the pellet subjected to homogenization using a sterile micro-pestle, and the resulting homogenate centrifuged at 10,000 rpm at 4 °C for 15 min. Supernatants from each tissue section homogenized were stored frozen at −80 °C until used for protein determinations and enzyme assays.

2.3. Collection of regurgitate

Several species of calyptrate flies salivate or regurgitate the contents of the crop to form a bubble that is deposited on food or other substrates, and then is consumed later (Terra, 1988). In order to determine whether the exuded fluid contains proteolytic enzyme activity, regurgitate was obtained by placing fed adult females singly into a plastic 15 ml polystyrene tube containing a 4″ × 1″ piece of sterile nylon membrane (Osmonics, Denver, CO, USA), and then capped. Individual flies were observed under a stereo-dissecting microscope for food bubble formation and subsequent deposition on the nylon membrane. Following regurgitation, the flies were removed, and the fly spot carefully isolated by cutting away excess membrane. Fly regurgitate was easily identifiable by darkened circular spots on an otherwise pristine white membrane and readily distinguishable in morphology from defecatory stains (Benecke and Barksdale, 2003; Fujikawa et al., 2011). Excised spots were placed in 25 μl chilled Dulbecco’s phosphate buffered saline (pH 7.2), vortexed at maximum speed for 20 s, and then the membrane removed. Regurgitate from 50 flies were pooled before stored frozen at −80 °C.

The method outlined has the admitted problem of possible contamination from the adult flies walking across the surface of the membranes before depositing regurgitate. The most likely source of contamination is the bovine liver that each fly was fed. To determine background enzyme levels, a 1 g piece of fresh bovine liver (previously frozen, then thawed) was placed in 1 ml chilled Dulbecco’s phosphate buffered saline (pH 7.2), homogenized in a sterile glass tissue homogenizer, and then centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was transferred to a sterile microcentrifuge tube, and then a 1 μl aliquot was pipetted onto a sterile nylon membrane. The ‘liver’ spot was excised and processed as described for fly regurgitate, and the procedure repeated so that 50 spots were pooled before frozen at −80 °C.

2.4. Protein determination

Protein content of gut fluids, homogenized tissues (following centrifugation), regurgitate, and bovine liver was determined using a Coomassie protein assay kit per the manufacturer’s instructions (Thermo Scientific, Rockford, IL, USA). The assay relies on colori-