

11 Biochemistry and Molecular Biology of Digestion

Walter R Terra

University of São Paulo, São Paulo, Brazil

Clélia Ferreira

University of São Paulo, São Paulo, Brazil

© 2012 Elsevier B.V. All Rights Reserved

11.1. Introduction	366
11.2. Overview of the Digestive Process	366
11.2.1. Initial Considerations	366
11.2.2. Characterization of Digestive Enzymes	366
11.2.3. Classification of Digestive Enzymes	368
11.2.4. Phases of Digestion and Their Compartmentalization in the Insect Gut	368
11.2.5. Role of Microorganisms in Digestion	368
11.3. Midgut Conditions Affecting Enzyme Activity	369
11.4. Digestion of Carbohydrates	372
11.4.1. Initial Considerations	372
11.4.2. Amylases	372
11.4.3. β -Glucanases	374
11.4.4. Xylanases and Pectinases	375
11.4.5. Chitinases and Lysozymes	376
11.4.6. α -Glucosidases	378
11.4.7. β -Glucosidases, β -Galactosidases, and Myrosinases	378
11.4.8. Trehalases	380
11.4.9. Acetylhexosaminidases, β -Fructosidases, and α -Galactosidases	381
11.5. Digestion of Proteins	382
11.5.1. Initial Considerations	382
11.5.2. Serine Proteinases	382
11.5.3. Cysteine Proteinases	385
11.5.4. Aspartic Proteinases	386
11.5.5. Aminopeptidases	387
11.5.6. Carboxypeptidases and Dipeptidases	388
11.6. Digestion of Lipids and Phosphates	388
11.6.1. Overview	388
11.6.2. Lipases	389
11.6.3. Phospholipases	389
11.6.4. Phosphatases	390
11.7. Microvillar Membranes	390
11.7.1. Isolation, Chemistry, and Enzymology	390
11.7.2. Microvillar Proteins	391
11.8. The Peritrophic Membrane	392
11.8.1. The Origin, Structure, and Formation of the Peritrophic Membrane	392
11.8.2. The Physiological Role of the Peritrophic Membrane	394
11.9. Organization of the Digestive Process	397
11.9.1. Evolutionary Trends of Insect Digestive Systems	397
11.9.2. Digestion in the Major Insect Orders	398
11.10. Digestive Enzyme Secretion Mechanisms	404
11.11. Concluding Remarks	406

11.1. Introduction

The growth of knowledge in the biochemistry of insect digestion had a bright start during the first decades of the last century, but slowed down after the development of synthetic chemical insecticides in the 1940s. Later on, with the environmental problems caused by chemical insecticides, new approaches for insect control were investigated. Midgut studies were particularly stimulated after the realization that the gut is a very large and relatively unprotected interface between the insect and its environment. Hence, an understanding of gut function was thought to be essential when developing methods of control that act through the gut, such as the use of transgenic plants to control phytophagous insects.

Applebaum (1985), in his review on the biochemistry of digestion, described the beginning of the renewed growth of the field. He discussed contemporary research showing that most insect digestive enzymes are similar to their mammalian counterparts, but that insect exotic diets require specific enzymes. In the next decade it became apparent that even enzymes similar to those of mammals have distinct characteristics, because each insect taxon deals with food in a special way (Terra and Ferreira, 1994). Since then, the field of digestive physiology and biochemistry has progressed dramatically at the molecular level (Terra and Ferreira, 2005).

The aim of this chapter is to review the recent and spectacular progress in the study of insect digestive biochemistry. To provide a broad coverage while keeping the chapter within reasonable size limits, only a brief account with key references is given for work done prior to 2000. Papers after 2000 have been selected from those richer in molecular details, and, when they were too numerous, representative papers were chosen, especially when abundant in references to other papers. Throughout, the focus is on providing a coherent picture of phenomena and highlighting further research areas. Amino acid residues are denoted by the one-letter code, if in peptides, for the sake of brevity. When mentioned in text with a position number, amino acid residues are denoted by the three-letter code to avoid ambiguity. For consistency, traditional abbreviations, like BAPA for benzoyl-arginine p-nitroanilide, have been changed, in the example to B-R-pNA, because the one-letter code for arginine is R.

The chapter is organized into four parts. The first part (sections 11.2 and 11.3) tries to establish uniform parameters for studying insect digestive enzymes, providing an overview of the biochemistry of insect digestion, and discusses factors affecting digestive enzymes *in vivo*. The second part (sections 11.4–11.7) reviews digestive enzymes and microvillar proteins, with the emphasis on molecular aspects, whereas the third part (sections 11.8 and 11.9) describes the details of the digestive biochemical process alongside insect evolution. Finally, the fourth

part (section 11.10) discusses data on digestive enzyme secretion mechanisms.

11.2. Overview of the Digestive Process

11.2.1. Initial Considerations

Digestion is the process by which food molecules are broken down into smaller molecules that are absorbed by cells in the gut tissue. This process is controlled by digestive enzymes, and is dependent on their localization in the insect gut.

11.2.2. Characterization of Digestive Enzymes

Enzyme kinetic parameters are meaningless unless assays are performed in conditions in which enzymes are stable. If researchers adopt uniform parameters and methods, comparisons among similar and different insect species will be more meaningful. A rectilinear plot of product formation (or substrate disappearance) versus time will ensure that enzymes are stable in a given condition. Activities (velocities) calculated from this plot are reliable parameters. According to the International Union of Biochemistry and Molecular Biology, the assay temperature should be 30°C, except when the enzyme is unstable at this temperature or altered for specific purposes. Owing to partial inactivation, the optimum temperature is not a true property of enzymes, and therefore should not be included in the characterization. Optimum enzyme pH should be determined using different buffers to discount the effects of chemical constituents of the buffers and their ionic strength on enzyme activity. The number of molecular forms of a given enzyme should be evaluated by submitting the enzyme preparation to a separation process (gel permeation, ion-exchange chromatography, hydrophobic chromatography, electrophoresis, gradient ultracentrifugation, etc.), followed by assays of the resulting fractions. Substrate specificity of each molecular form of a given enzyme should be evaluated and substrate preference quantified by determining V_m/K_m ratios for each substrate, keeping the amount of each enzyme form constant. Substrate preference expressed as the percentage activity towards a given substrate in relation to the activity upon a reference substrate may be misleading because, in this condition, enzyme activities are determined at different substrate saturations. The isoelectric points of many enzymes can be determined after staining with specific substrates following the separation of the native enzymes on isoelectrofocusing gels.

If enzyme characterization is performed as part of a digestive physiology study, emphasis should be given to enzyme compartmentalization, substrate specificity, and substrate preference, in order to discover the sequential action of enzymes during the digestive process.

Knowledge of the effect of pH on enzyme activity is useful in evaluating enzyme action in gut compartments (**Figure 1**) with different pH values. Finally, the determination of the molecular masses of digestive enzymes, associated with the ability of enzymes to pass through the peritrophic membrane, allows estimation of the pore sizes of the peritrophic membrane. Molecular masses determined in non-denaturing conditions are preferred, since in these conditions the enzymes should maintain their *in vivo* aggregation states (not only their quaternary structures if present). The method of choice in this case is gradient ultracentrifugation.

Complete enzymological characterization requires purification to homogeneity, and sequencing. Furthermore,

details of the catalytic mechanisms, including involvement of amino acid residues in catalysis and substrate specificity, should be determined. This permits the classification of insect digestive enzymes into catalytic families, and discloses the structural basis of substrate specificities; it will also enable us to establish evolutionary relationships with enzymes from other organisms.

Cloning cDNA sequences encoding digestive enzymes enables the expression of large amounts of recombinant enzymes that may be crystallized or used for the production of antibodies. Antibodies are used in Western blots to identify a specific enzyme in protein mixtures, or to localize the enzyme in tissue sections in a light or electron microscope. Enzyme crystals used for resolving

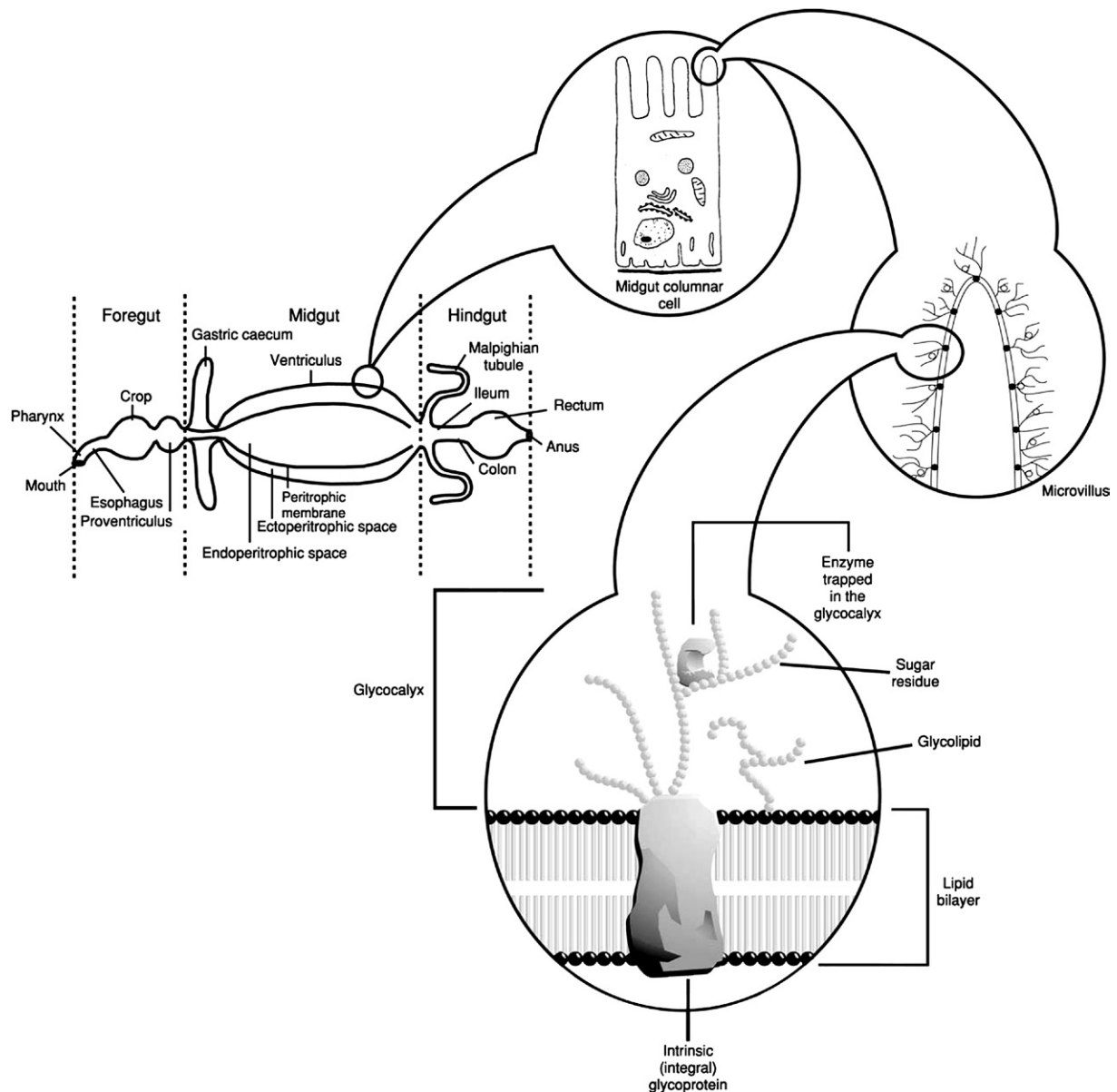


Figure 1 Diagrammatic representation of insect gut compartments. Glycocalyx: the carbohydrate moiety of intrinsic proteins and glycolipids occurring in the luminal face of microvillar membranes.

three-dimensional (3D) structures (via X-ray diffraction or nuclear magnetic resonance, NMR) need amounts of purified enzymes that frequently are difficult to isolate from insects by conventional separation procedures. However, detailed 3D structures are necessary to understand enzyme mechanisms and the binding of inhibitors to enzyme molecules. Alternatively, cDNA may be amenable to site-directed mutagenesis for structure–function studies. Site-directed mutagenesis tests the role of individual amino acid residues in enzyme function or structure. Such knowledge is a prerequisite in developing new biotechnological approaches to control insects via the gut. Finally, interference RNA may be used to suppress the expression of one enzyme, in order to test hypotheses regarding its physiological role (see Chapter 2).

11.2.3. Classification of Digestive Enzymes

Digestive enzymes are hydrolases. The enzyme classification and numbering system used here is that recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Enzyme Commission).

Peptidases (peptide hydrolases, EC 3.4) are enzymes that act on peptide bonds and include the proteinases (endopeptidases, EC 3.4.21–24) and the exopeptidases (EC 3.2.4.11–19; see also Chapter 10 in this volume). Proteinases are divided into subclasses on the basis of catalytic mechanism, as shown with specific reagents or effect of pH. Specificity is only used to identify individual enzymes within subclasses. Serine proteinases (EC 3.4.21) have a serine and a histidine in the active site. Cysteine proteinases (EC 3.4.22) possess a cysteine in the active site, and are inhibited by mercurial compounds. Aspartic proteinases (EC 3.4.23) have a pH optimum below 5, due to the involvement of a carboxyl residue in catalysis. Metalloproteinases (EC 2.3.24) need a metal ion in the catalytic process. Exopeptidases include enzymes that hydrolyze single amino acids from the N-terminus (aminopeptidases, EC 3.4.11) or from the C-terminus (carboxypeptidases, EC 3.4.16–18) of the peptide chain, and those enzymes specific for dipeptides (dipeptide hydrolases, EC 3.4.13) (**Figure 2**).

Glycosidases (EC 3.2) are classified according to their substrate specificities. They include the enzymes that cleave internal bonds in polysaccharides and are usually named from their substrates – for example, amylase, cellulase, pectinase, and chitinase. They also include the enzymes that hydrolyze oligosaccharides and disaccharides. Oligosaccharidases and disaccharidases are usually named based on the monosaccharide that gives its reducing group to the glycosidic bond, and on the configuration (α or β) of this bond (**Figure 2**).

Lipids are a large and heterogeneous group of substances that are relatively insoluble in water but readily

soluble in apolar solvents. Some contain fatty acids (fats, phospholipids, glycolipids, and waxes), while others lack them (terpenes, steroids, and carotenoids). Ester bonds are hydrolyzed in lipids containing fatty acids before they are absorbed. The enzymes that hydrolyze ester bonds comprise: (1) carboxylic ester hydrolases (EC 3.1.1), such as lipases, esterases, and phospholipases A and B; (2) phosphoric monoester hydrolases (EC 3.1.3), which are the phosphatases; and (3) phosphoric diester hydrolases (EC 3.1.4), including phospholipases C and D (**Figure 2**).

11.2.4. Phases of Digestion and Their Compartmentalization in the Insect Gut

Most food molecules to be digested are polymers, such as proteins and starch, and are digested sequentially in three phases. Primary digestion is the dispersion and reduction in molecular size of the polymers, and results in oligomers. During intermediate digestion, these undergo a further reduction in molecular size to dimers; in final digestion, they become monomers. Digestion usually occurs under the action of digestive enzymes from the midgut, with little or no participation of salivary enzymes.

Any description of the spatial organization of digestion in an insect must relate the midgut compartments (cell, ecto-, and endoperitrophic spaces) to each phase of digestion, and hence to the corresponding enzymes. To accomplish this, enzyme determinations must be performed in each midgut luminal compartment and in the corresponding tissue. Techniques of sampling enzymes from midgut luminal compartments and enzymes trapped in cell glycocalyx have been reviewed elsewhere (*Terra and Ferreira, 1994*). Microvillar enzymes are discussed in detail in [section 11.7](#).

Frequently, initial digestion occurs inside the peritrophic membrane (see [sections 11.8.1 and 11.8.2](#)), intermediate digestion in the ectoperitrophic space, and final digestion at the surface of midgut cells by integral microvillar enzymes or by enzymes trapped into the glycocalyx (**Figure 1**). Exceptions to this rule, and the procedures for studying the organization of the digestive process, will be detailed below.

11.2.5. Role of Microorganisms in Digestion

Most insects harbor a substantial microbiota, including bacteria, yeast, and protozoa. Microorganisms might be symbiotic or fortuitous contaminants from the external environment. They are found in the lumen, adhering to the peritrophic membrane, attached to the midgut surface, or within cells. Intracellular bacteria are usually found in special cells, mycetocytes, which may be organized in groups, mycetomes. Microorganisms produce and secrete their own hydrolases, and cell death will result in the release of enzymes into the intestinal milieu. Any

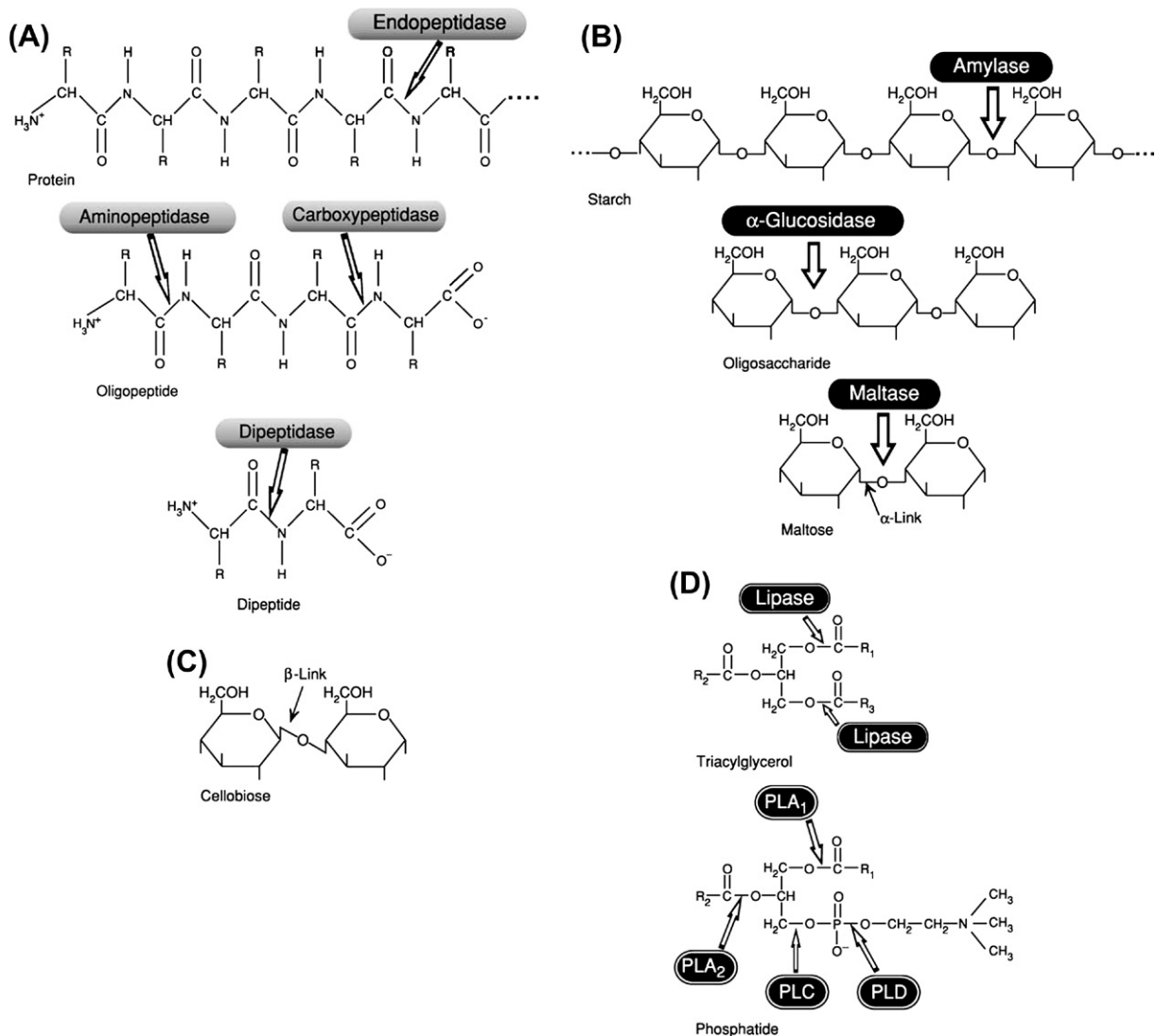


Figure 2 Digestion of important nutrient classes. Arrows point to bonds cleaved by enzymes. (A) Protein digestion; R, different amino acid moieties; (B) starch digestion; (C) β -linked glucoside; (D) lipid digestion; PL, phospholipase; R, fatty acyl moieties. Reprinted with permission from Terra, W.R., 2009. Digestion. In: Resh, V.H., Cardé, R.T. (eds), *Encyclopedia of Insects*, 2nd edn, Academic Press, San Diego, CA, pp. 271–273; ©Elsevier.)

consideration of the spectrum of hydrolase activity in the midgut must include the possibility that some of the activity may derive from microorganisms. Despite the fact that digestive enzymes of some insects are thought to be derived from the microbiota, there are relatively few studies that show an unambiguous contribution of microbial hydrolases. Best examples are found among wood- and humus-feeding insects like termites, tipulid fly larvae, and scarabid beetle larvae. Although these insects may have their own cellulases (see section 11.4.3), only fungi and certain filamentous bacteria developed a strategy for the chemical breakdown of lignin. Lignin is a phenolic polymer that forms an amorphous resin in which the polysaccharides of the secondary plant cell wall are embedded, thus becoming protected from enzymatic attack (Terra *et al.*, 1996; Brune, 1998; Dillon and Dillon, 2004).

Microorganisms play a limited role in digestion, but they may enable phytophagous insects to overcome biochemical barriers to herbivory – for example, detoxifying flavonoid alkaloids and the phenolic aglycones of plant glycosides. They may also provide complex-B vitamins for blood-feeders and essential amino acids for phloem feeders, produce pheromone components, or withstand the colonization of the gut by non-indigenous species (including pathogens) (Dillon and Dillon, 2004; Genta *et al.*, 2006a).

11.3. Midgut Conditions Affecting Enzyme Activity

The pH of the contents of the midgut is one of the important internal environmental properties that affect digestive enzymes. Although midgut pH is hypothesized

to result from adaptation of an ancestral insect to a particular diet, its descendants may diverge, feeding on different diets, while still retaining the ancestral midgut pH condition. Thus, there is not necessarily a correlation between midgut pH and diet. In fact, midgut pH correlates well with insect phylogeny (Terra and Ferreira, 1994; Clark, 1999).

The pH of insect midgut contents is usually in the 6–7.5 range. Major exceptions are the very alkaline midgut contents (pH 9–12) of Lepidoptera, scarab beetles, and nematoceran Diptera larvae; the very acid (pH 3.1–3.4) middle region of the midgut of cyclorrhaphous Diptera; and the acid posterior region of the midgut of heteropteran Hemiptera (Terra and Ferreira, 1994; Clark, 1999). pH values may not be equally buffered along the midgut. Thus, midgut contents are acidic in the anterior midgut and nearly neutral or alkaline in the posterior midgut in Dictyoptera, Orthoptera, and most families of Coleoptera. Cyclorrhaphan Diptera midguts have nearly neutral contents in the anterior and posterior regions, whereas in middle midgut the contents are very acid (Terra and Ferreira, 1994).

A pH in the ectoperitrophic space lower than in the midgut lumen was reported in some lepidopteran larvae. This is an artefact caused by a halt in alkaline secretion by the isolated midgut tissue (Grigorten *et al.*, 1993). Nevertheless, the pH in the immediate neighborhood of the negatively-charged microvillar glycocalyx is expected to be lower than in the bulk solution, because of proton retention (Quina *et al.*, 1980).

The high alkanity of lepidopteran midgut contents is thought to allow these insects to feed on plant material rich in tannins, which bind to proteins at lower pH, reducing the efficiency of digestion (Berenbaum, 1980). This explanation may also hold for scarab beetles and for detritus-feeding nematoceran Diptera larvae that usually feed on refractory materials such as humus. Nevertheless, mechanisms other than high gut pH must account for the resistance to tannin displayed by some locusts (Bernays *et al.*, 1981) and beetles (Fox and Macauley, 1977). One possibility is the effect of surfactants such as lysolecithin, which is formed in insect fluids due to the action of phospholipase A on cell membranes (Figure 2), and which occurs widely in insect digestive fluids (De Veau and Schultz, 1992). Surfactants are known to prevent the precipitation of proteins by tannins even at pH as low as 6.5 (Martin and Martin, 1984). Present knowledge is not sufficient to relate midgut detergency to diet or phylogeny, or to both.

Tannins may have deleterious effects other than precipitating proteins. Tannic acid is frequently oxidized in the midgut lumen, generating peroxides, including hydrogen peroxide, which readily diffuses across cell membranes and is a powerful cytotoxin. In some insects (e.g., *Orgyia leucostigma*), tannic acid oxidation and the generation of

peroxides are suppressed by the presence of high concentrations of ascorbate and glutathione in the midgut lumen (Barbehenn *et al.*, 2003). Dihydroxy phenolics in an alkaline medium are converted to quinones that react with lysine ϵ -amino groups. This leads to protein aggregation and a decrease in lysine availability for the insect. Other compounds (e.g., oleuropein, alkylate lysine residues in proteins) cause the same problems as dihydroxy phenolics. These phenomena are inhibited in larvae of several lepidopteran species by secreting glycine into the midgut lumen. Glycine competes with lysine residues in the denaturing reaction (Konno *et al.*, 2001). In some insects, tannins reduce the overall efficiency of conversion of ingested matter to body mass by an unknown mechanism. Nevertheless, the performance of these insects remains unchanged, because of compensatory feeding (Barbehenn *et al.*, 2009).

A high midgut pH may also be of importance, in addition to its role in preventing tannin binding to proteins, in freeing hemicelluloses from plant cell walls ingested by insects. Hemicelluloses are usually extracted in alkaline solutions for analytical purposes (Blake *et al.*, 1971), and insects such as the caterpillar *Erinnyis ello* are able to digest hemicelluloses efficiently without affecting the cellulose from the leaves they ingest to any degree (Terra, 1988). This explanation is better than the previous one in accounting for the very high pH observed in several insects, since a pH of about 8 is sufficient to prevent tannin binding to proteins (Terra, 1988).

The acid region in the cyclorrhaphous Diptera midgut is assumed to be involved in the process of killing and digesting bacteria, which may be an important food for maggots. This region is retained in Muscidae that have not diverged from the putative ancestral bacteria-feeding habit, as well as in the flesh-feeding Calliphoridae and the fruit-feeding Tephritidae (Terra and Ferreira, 1994). The acid posterior midgut of Hemiptera may be related to their lysosome-like digestive enzymes (cysteine and aspartic proteinase) (see sections 11.5.3 and 11.5.4).

Few papers have dealt with midgut pH buffering mechanisms. Dow (1992) described a carbonate secretion system, which may be responsible for the high pH found in Lepidoptera midguts (Figure 3). Phosphorus NMR microscopy has been used to show that valinomycin leads to a loss of alkalization in the midgut of *Spodoptera litura* (Skibbe *et al.*, 1996). As valinomycin is known to transport K^+ down its concentration gradient, this result gives further support to the model described in Figure 3. Midgut alkalization in nematoceran Diptera occurs by mechanisms similar to those of lepidopteran larvae (Okech *et al.*, 2008), whereas no data are available for scarab beetles. Terra and Regel (1995) determined pH values and concentrations of ammonia, chloride, and phosphate in the presence or absence of ouabain and vanadate in *Musca*

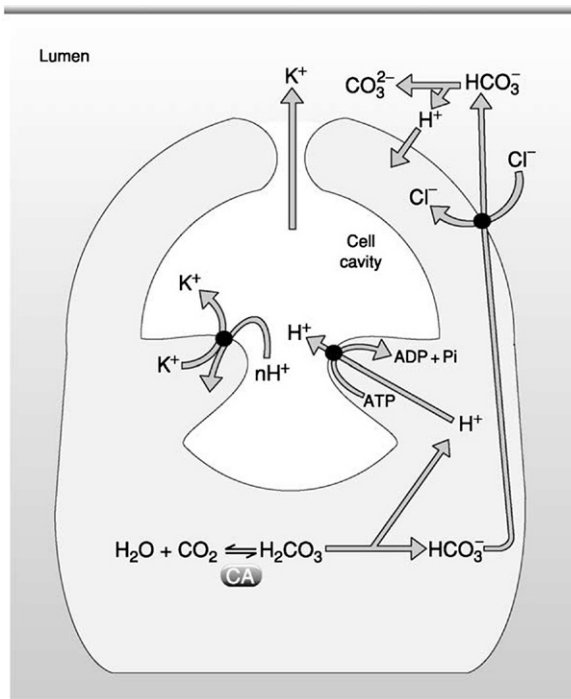


Figure 3 A model for generation of high gut pH by the goblet cells of lepidopteran larvae. Carbonic anhydrase (CA) produces carbonic acid that dissociates into bicarbonate and a proton. The proton is pumped by a V-ATPase into the goblet cell cavity, from where it is removed in exchange with K^+ that eventually diffuses into lumen. Bicarbonate is secreted in exchange with chloride and loses a proton due to the intense field near the membrane, forming carbonate and raising the gut pH. Data from Dow (1992).

domestica midguts. From the results, they proposed that middle midgut acidification is accomplished by a proton pump of mammalian-like oxyntic cells, whereas the neutralization of posterior midgut contents depends on ammonia secretion (**Figure 4**).

Redox conditions in the midgut are regulated and may be the result of phylogeny, although data are scarce. Reducing conditions are observed in clothes moth, sphinx moths, owl moths, and dermestid beetles (Appel and Martin, 1990), and in Hemiptera (Silva and Terra, 1994). Reducing conditions are important to open disulfide bonds in keratin ingested by some insects (clothes moths, dermestid beetles) (Appel and Martin, 1990), to maintain the activity of the major proteinase in Hemiptera (see section 11.5.3), and to reduce the impact of some plant allelochemicals, such as phenol, in some herbivores (Appel and Martin, 1990). In spite of this, the artificial lowering of *in vivo* redox potentials did not significantly impact digestive efficiency of the herbivore *Helicoverpa zea*, although the reducing agent used (dithiothreitol) inhibited some proteinases *in vitro* (Johnson and Felton, 2000). Midgut antioxidant enzymes in *Spodoptera littoralis* are upregulated in response to increased oxidative stress caused by oxidizable allelochemicals (Krishnan and Kodrik, 2006).

Although several allelochemicals other than phenols may be present in the insect gut lumen, including alkaloids, terpene aldehydes, saponins, and hydroxamic acids (Appel, 1994), data on their effect on digestion are lacking.

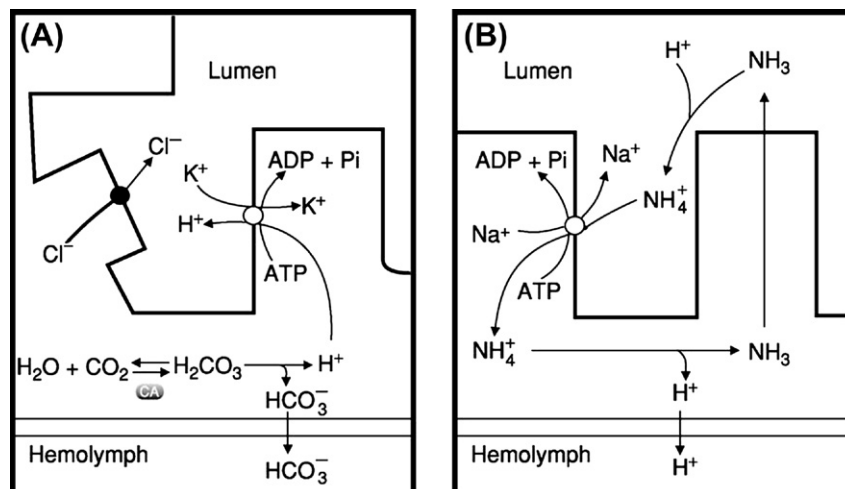


Figure 4 Diagrammatic representation of ion movements, proposed as being responsible for maintenance of pH in the larval midgut contents of *Musca domestica*. Carbonic anhydrase (CA) in cup-shaped oxyntic cells in the middle of the midgut (A) produces carbonic acid which dissociates into bicarbonate and a proton. Bicarbonate is transported into the hemolymph, whereas the proton is actively translocated into the midgut lumen acidifying its contents to pH 3.2. Chloride ions follow the movement of protons. NH_3 diffuses from anterior and posterior midgut cells (B) into the midgut lumen, becoming protonated and neutralizing their contents to pH 6.1–6.8. NH_4^+ is then exchanged for Na^+ by a microvillar Na^+/K^+ -ATPase. Inside the cells, NH_4^+ forms NH_3 , which diffuses into midgut lumen, and proton that is transferred into the hemolymph. Reprinted with permission from Terra, W.R., Regel, R., 1995. pH buffering in *Musca domestica* midguts. *Comp. Biochem. Physiol. A* 112, 559–564; ©Elsevier.

11.4. Digestion of Carbohydrates

11.4.1. Initial Considerations

Polysaccharides are major constituents of cell walls and energy reserves, such as starch granules within plant cells and glycogen within animal cells. For phytophagous insects, disruption of plant cell walls is necessary in order to expose storage polymers in cell contents to polymer hydrolases. Cell wall breakdown may be achieved by mastication, but more frequently it is the result of the action of digestive enzymes. Thus, even insects unable to obtain nourishment from the cellulosic and non-cellulosic cell wall biochemicals would profit from having enzymes active against these structural components. Cell walls are disrupted by β -glucanases, xylanases, and pectinases (plant cells), lysozyme (bacterial cells), or chitinase and β -1,3-glucanase (fungal cells). The carbohydrates associated with cellulose are frequently named “hemicelluloses” and the enzymes that attack them “hemicellulases.” Thus, the hemicellulases include β -glucanases other than cellulases, xylanases, and pectinases. Following the loss of cell wall integrity, starch digestion is initiated by amylases. A complex of carbohydrases converts the oligomers resulting from the action of the polymer hydrolyzing enzymes into dimers (such as sucrose, cellobiose, and maltose, which also occur free in some cells), and finally into monosaccharides like glucose and fructose.

11.4.2. Amylases

α -Amylases (EC 3.2.1.1) catalyze the endohydrolysis of long α -1,4-glucan chains such as starch and glycogen. Amylases are usually purified by glycogen–amylase complex formation followed by precipitation in cold ethanol, or, alternatively, by affinity chromatography in a gel matrix linked to a protein amylase inhibitor. In sequence, isoamylases can be resolved by anion exchange chromatography (Terra and Ferreira, 1994).

Most insect amylases have molecular weights in the range 48–60 kDa, pI values of 3.5–4.0, and K_m values with soluble starch around 0.1%. pH optima generally correspond to the pH prevailing in midguts from which the amylases were isolated. Insect amylases are calcium-dependent enzymes, and are activated by chloride with displacement of the pH optimum. Activation also occurs with anions other than chloride, such as bromide and nitrate, and it seems to depend upon the ionic size (Terra and Ferreira, 1994).

The best-known insect α -amylase, and the only one whose 3D structure has been resolved, is the midgut α -amylase of *Tenebrio molitor* larvae. The enzyme has three domains. The central domain (domain A) is a $(\beta/\alpha)_8$ -barrel that comprises the core of the molecule

and includes the catalytic amino acid residues (Asp 185, Glu 222, and Asp 287) (*T. molitor* α -amylase numbering throughout). Domains B and C are almost opposite each other, on each side of domain A. The substrate-binding site is located in a long V-shaped cleft between domains A and B. There, six saccharide units can be accommodated, with the sugar chain being cleaved between the third and fourth bound glucose residues. A calcium ion is placed in domain B, and is coordinated by Asn 98, Arg 146, and Asp 155. This ion is important for the structural integrity of the enzyme, and also seems to be relevant due its contact with His 189. This residue interacts with the fourth sugar of the substrate bound in the active site, forming a hinge between the catalytic site and the Ca^{2+} -binding site. A chloride ion is coordinated by Arg 183, Asn 285, and Arg 321 in domain A (Strobl *et al.*, 1998a). Domain C is placed in the C-terminal part of the enzyme, contains the so-called “Greek key” motif, and has no clear function (Figure 5). These structural features are shared by all known α -amylases (Nielsen and Borchert, 2000), although not all α -amylases have a chloride-binding site (Strobl *et al.*, 1998a). The most striking difference between mammalian and insect α -amylases is the presence of additional loops in the vicinity of the active site of the mammalian enzymes (Strobl *et al.*, 1998a).

Asp 287 is conserved in all α -amylases. Comparative studies have shown that Glu 222 is the proton donor and Asp 185 the nucleophile, and that Asp 287 is important, but not a direct participant in catalysis. It is proposed that its role is to elevate the pK_a of the proton donor (Nielsen and Borchert, 2000).

Chloride ion is an allosteric activator of α -amylases, leading to a conformation change in the enzyme that changes the environment of the proton donor. This change causes an increase in the pK_a of the proton donor, thus displacing the pH optimum of the enzyme and increasing its V_{max} (Levitzki and Steer, 1974). According to Strobl *et al.* (1998a), the increase in V_{max} is a consequence of the chloride ion being close to the water molecule that has been suggested to initiate the cleavage of the substrate chain. The nucleophilicity of this water molecule might be enhanced by the negative charge of the ion.

There is a large and growing number of complete sequences of insect α -amylases registered in the GenBank. Examples may be found among Hymenoptera (Da Lage *et al.*, 2002), Coleoptera (Strobl *et al.*, 1997; Titarenko and Chrispeels, 2000), Diptera (Grossmann *et al.*, 1997; Charlab *et al.*, 1999), and Lepidoptera (Da Lage *et al.*, 2002; Pytelkova *et al.*, 2009). All the sequences have the catalytic triad (Asp 185, Glu 222, and Asp 287), the substrate-binding histidine residues (His 99, His 189, and His 286), and the Ca^{2+} -coordinating residues (Asn 98, Arg 146, and Asp 155) (Figure 5). From the residues found to be involved in chloride binding, Arg 183

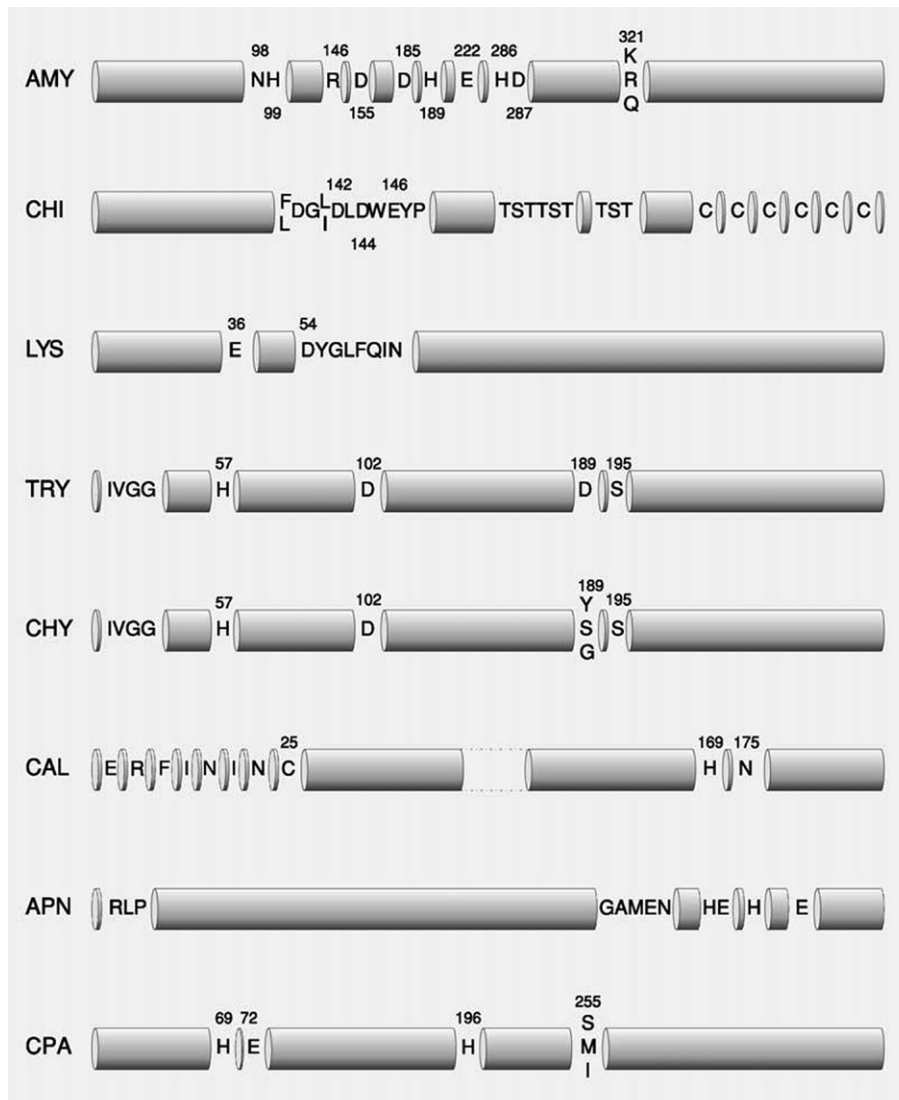


Figure 5 Conserved residues in the primary structures of major insect digestive enzymes. AMY (amylase) follows *Tenebrio molitor* amylase numbering; CHI (chitinase), molting-fluid *Manduca sexta* chitinase numbering. TRY (trypsin) and CHY (chymotrypsin) follow the bovine chymotrypsin numbering; CAL (cathepsin L), papain numbering; APN (aminopeptidase N) does not have a consensual numbering; CPA (carboxypeptidase A), mammalian CPA numbering.

and Asn 285 are conserved, whereas position 321 varies. According to D'Amico *et al.* (2000), all known chloride-activated α -amylases have an arginine or lysine residue at position 321. Insect α -amylase sequences have arginine at position 321, except those of *Zabrotes subfasciatus* and *Anthonomus grandis*, which have lysine, and the lepidopteran α -amylases, which have glutamine. This agrees with the observation that most insect α -amylases are activated by chloride, with the remarkable exception of lepidopteran amylases (Terra and Ferreira, 1994). The few coleopteran and hymenopteran α -amylases reported not to be affected by chloride (Terra and Ferreira, 1994) deserve reinvestigation. It is possible that another anion is replacing chloride as an activator, as shown for some hemipteran amylases (Hori, 1972). It is worth noting

that there is more Arg + Lys in the lepidopteran amylase than in *T. molitor* amylase (Pytelkova *et al.*, 2009). This is thought to be an adaptation to the higher pH milieu of lepidopteran midguts.

The “action pattern” refers to the number of bonds hydrolyzed during the lifetime of a particular enzyme–substrate complex. If more than one bond is hydrolyzed after the first hydrolytic step, the action pattern is said to be processive. The degree of multiple attack is the average number of hydrolyzed bonds after the first bond is broken. *Rhynchosciara americana* amylase has a degree of multiple attack between that of the amylase of *Bacillus subtilis* (1.7) and porcine pancreas (6). Amylases from larvae and adults of *Sitophilus zeamais*, *S. granarius*, and *S. oryzae*, and larvae of *Bombyx mori*, have action patterns similar

to that of porcine pancreas amylase (Terra and Ferreira, 1994). These studies need to be taken further, including the determination of the affinities corresponding to each subsite in the active center. Such studies, especially if combined with crystallographic data, may describe in molecular detail the reasons why amylases act differently toward starches of distinct origins.

There is a variety of natural compounds that affect amylases, including many plant protein inhibitors (Franco *et al.*, 2002). Crystallographic data have shown that these protein inhibitors always occupy the amylase active site (Strobl *et al.*, 1998b; Payan, 2004). In the case of the *Amaranth* α -amylase inhibitor, a comparison of *T. molitor* amylase-inhibitor complex with a modeled complex between porcine pancreatic α -amylase and the inhibitor identified six hydrogen bonds that can be formed only in the *T. molitor* amylase-inhibitor complex (Pereira *et al.*, 1999). This was the first successful explanation of how a protein inhibitor specifically inhibits α -amylases from insects, but not from mammalian sources. As will be discussed with details for trypsins (see section 11.5.2.1), specific amylases are induced when insect larvae are fed with α -amylase inhibitor-containing diets (Silva *et al.*, 2001; Pytelkova *et al.*, 2009). The mechanisms underlying this induction are unknown.

11.4.3. β -Glucanases

β -Glucanases are enzymes acting on β -glucans. These are major polysaccharide components of plant cell walls, and include β -1,4-glucans (cellulose), β -1,3-glucans (callose), and β -1,3;1,4-glucans (cereal β -glucans) (Bacic *et al.*, 1988). The cell walls of certain groups of fungi have β -1,3;1,6-glucans (Bacic *et al.*, 1988).

11.4.3.1. Cellulases Cellulose is by far the most important β -glucan. It occurs in the form of β -1,4-glucan chains packed in an ordered manner to form compact aggregates which are stabilized by hydrogen bonds. The resulting structure is crystalline and not soluble. According to work done with microbial systems, cellulose is digested by a combined action of two enzymes. An endo- β -1,4-glucanase (EC 3.2.1.4), with an open substrate-binding cleft, cleaves bonds located within chains in the amorphous regions of cellulose, creating new chain ends. An exo- β -1,4-glucanase (EC 3.2.1.91) processively releases cellobiose from the ends of cellulose chains in a tunnel-like active site. Surface loops in cellobiohydrolase prevent the dislodged cellulose chains from readhering to the crystal surface, as the enzyme progresses into crystalline cellulose (Rouvinen *et al.*, 1990; Kleywegt *et al.*, 1997). Cellobiohydrolase structure is modular, comprising a catalytic domain linked to a distinct cellulose-binding domain, which enhances the activity of the enzyme towards insoluble cellulose (Linder and Teeri, 1997).

Cellulose digestion in insects is rare, presumably because the dietary factor that usually limits growth in plant feeders is protein quality. Thus, cellulose digestion is unlikely to be advantageous to an insect that can meet its dietary requirements using more easily digested constituents. This is exemplified by lepidopterans that, even ingesting plant material, lack cellulase genes (Watanabe and Tokuda, 2010). Nevertheless, cellulose digestion occurs in several insects that have, as a rule, nutritionally poor diets such as wood or humus (Terra and Ferreira, 1994). The role of symbiotic organisms in insect cellulose digestion is less important than initially believed (Slaytor, 1992), although symbiotic nitrogen-fixing organisms are certainly involved in increasing the nutritive value of diets of many insects (Terra, 1990).

Insect cellulases are known mainly from the lower termite *Reticulitermes speratus*, the higher termite *Nasutitermes takasagoensis*, and the woodroach *Panesthia cribrata* (Lo *et al.*, 2000; Watanabe and Tokuda, 2010). Alignments of the sequences of termite and woodroach endoglucanases from data banks showed that they belong to family 9 of glycoside hydrolases (Coutinho and Henrissat, 1999). The paradigm of this family is the endo/exocellulase from the bacteria *Thermomonospora fusca*, whose catalytic center binds a cellopentaose residue and cleaves it into cellotetraose plus glucose or cellotriose plus cellobiose, and has Asp 55 as a nucleophile and Glu 424 as a proton donor (Sakon *et al.*, 1997).

The active site groups are conserved in the termite and woodroach endoglucanases, although these enzymes lack the cellulose-binding domains that improve the binding and facilitate the activity of the catalytic domain on crystalline cellulose (Linder and Teeri, 1997). The conclusions drawn from sequence alignments were confirmed by the 3D structure resolution of the *N. takasagoensis* endoglucanase, which also revealed a Ca^{2+} -binding site near its substrate binding cleft (Khadeni *et al.*, 2002). According to Slaytor (1992), the large production of endoglucanases in termites and woodroaches would compensate for their low efficiency on crystalline cellulose.

The phytophagous beetle *Phaedon cochleariae* has cellulase activity in its midgut (Girard and Jouanin, 1999a). A cDNA that encodes one cellulase was cloned, and was shown to belong to the glycoside hydrolase family 45 and to consist only of a catalytic domain. Similar enzymes occur in the midgut of the beetles *Psacotha hilaris* (Sugimura *et al.*, 2003) and *Apriona germari* (Wei *et al.*, 2006). The cellulase from the cricket *Teleogryllus emma*, however, is from family 9 (Kim *et al.*, 2008).

11.4.3.2. Laminarinases and licheninases Licheninases (EC 3.2.1.73) digest only β -1,3;1,4-glucans, whereas laminarinases may hydrolyze β -1,3;1,4-glucans and also β -1,3-glucans (EC 3.2.1.6), or only the last polymer (EC 3.2.1.39). In spite of laminarinase activities being

widespread among insects (Terra and Ferreira, 1994), few of these enzymes were purified and characterized. Three laminarinases produced by the salivary glands were isolated from *Periplaneta americana* (LAM, LIC1 and LIC2; Genta *et al.*, 2003). LIC1 and LIC2 hydrolyze laminarin and lichenin, whereas LAM is active only against laminarin. *Abracris flavolineata* (Genta *et al.*, 2007), *T. molitor* (Genta *et al.*, 2009), and *Spodoptera frugiperda* (Bragatto *et al.*, 2010) have only one major laminarinase (ALAM, TLAM, and SLAM, respectively) produced by the midgut. ALAM and SLAM have only laminarin as substrate, and TLAM hydrolyzes, besides laminarin, yeast β -1,3- β -1,6-glucan. Except for SLAM and LIC2 (not determined) all the mentioned enzymes lyse *Saccharomyces cerevisiae* cells. The enzymes have pH optima from 5.0 to 6.5, except for SLAM (9.0), K_m values for laminarin hydrolysis from 0.074 to 0.36%, and molecular masses from 23.4 to 46.2 kDa.

Some laminarinases are processive – that is, they perform multiple rounds of catalysis when the enzyme remains attached to the substrate. The exo- β -1,3-glucanase of *A. flavolineata* has a high-affinity accessory site that, on substrate binding, causes active site exposure, followed by the transference of the substrate to the active site. Processivity results in this case from consecutive transferences of substrate between accessory and active site (Genta *et al.*, 2007).

Insect laminarinases belong to glycoside hydrolase family 16, and are evolutionarily related and strikingly similar in sequence to Gram-negative bacteria-binding proteins (GNBPs) and other glucan-binding proteins that are active in the insect innate immune system. Because of this, many true laminarinases had been wrongly annotated as glucan-binding proteins in sequence databases.

Laminarinases and GNBPs are derived from the laminarinase of the ancestor of mollusks and arthropods. The insect laminarinases lost an extended N-terminal region of the ancestral laminarinase, whereas the β -glucan binding proteins lost the catalytical residues (Figure 6) (Bragatto *et al.*, 2010).

The role of laminarinases in the insect gut is not yet clear. Insects eating contaminated food, such as *T. molitor* and *P. americana*, may use β -1,3 glucanases to digest fungi from their diet. In Lepidoptera that eat a less contaminated food, β -1,3- glucanase may be important to digest callose. Since the larvae eats on and in the same plant, callose deposition can impair nutrient digestibility in the absence of a β -1,3- glucanase. Pauchet *et al.* (2009a) correlate the increase in β -1,3-glucanase activity in *Helicoverpa armigera* after fungi or bacteria ingestion with a role of the enzyme in immunity. It cannot be ruled out, however, that the enzyme activity increase may be due to a higher substrate concentration, and not because of a role in immunity. Further studies are necessary to settle the subject.

11.4.4. Xylanases and Pectinases

Xylans constitute the major non-cellulosic polysaccharides (hemicelluloses) of primary walls of grasses and secondary walls of all angiosperms, accounting for one-third of all renewable organic carbon available on earth (Bacic *et al.*, 1988). Chemically, xylans are a family of linear β -1,4-xylans with a few branches. Endo- β -1,4-xylanase activities (EC 3.2.1.8) were found in several insects (Terra and Ferreira, 1994). One of these enzymes was cloned from a beetle and shown to correspond to a protein of 22 kDa, with high sequence identity to fungal xylanases, and conserving the usual two catalytic regions (Girard and Jouanin, 1999a). An exo- β -1,4-xylanase (EC 3.2.1.37) was partially purified from termites (Matoub and Rouland, 1995), and was thought to act synergistically with an endo- β -1,4-xylanase originating from fungus ingested by the termites. Recent work showed that xylanase genes were expressed in the hindguts of termites by symbiotic flagellates (Arakawa *et al.*, 2009). Much more work is needed on this class of enzymes that may be important mainly for detritivorous insects.

Pectin is a linear chain of a D-galacturonic acid units with α -1,4-linkages in which varying proportions of the acid groups are present as methyl esters. It is the main component of the rhamnogalacturonan backbone of the structure formed by the pectin polysaccharides. Pectin is hydrolyzed by pectinases (polygalacturonases, EC 3.2.1.15) described in many insects (Terra and Ferreira, 1994).

Pectinases are thought to be important for hemipterans, as they would facilitate penetration of their stylets through plant tissues into sap-conducting structures, and for insects boring plant parts. Accordingly, pectinases have been found in hemipteran saliva (Vonk and Western, 1984), and have been isolated and characterized from two weevils (Shen *et al.*, 1996; Dootsdaar *et al.*, 1997) and cloned from a phytophagous beetle (Girard and Jouanin, 1999a).

The pectinases from the weevils *S. oryzae* (Shen *et al.*, 1996) and *Diaprepes abbreviatus* (Dootsdaar *et al.*, 1997) were purified to electrophoretical homogeneity from whole-body extracts and gut homogenates, respectively. Purification of the pectinases was achieved by affinity chromatography through cross-linked pectate in addition to two ion-exchange chromatographic steps. The enzymes have molecular masses of 35–45 kDa, pH optimum 5.5, and K_m values of 1–4 mg/ml for pectic acid. The *D. abbreviatus* pectinase is inhibited by a polygalacturonase-inhibitor protein that may be associated with plant resistance to insects (Dootsdaar *et al.*, 1997). Although the weevil pectinases may originate from endosymbiotic bacteria (Campbell *et al.*, 1992), the finding that the cDNA-coding pectinase of the beetle *P. cochleariae* has a poly(A) tail (Girard and Jouanin,

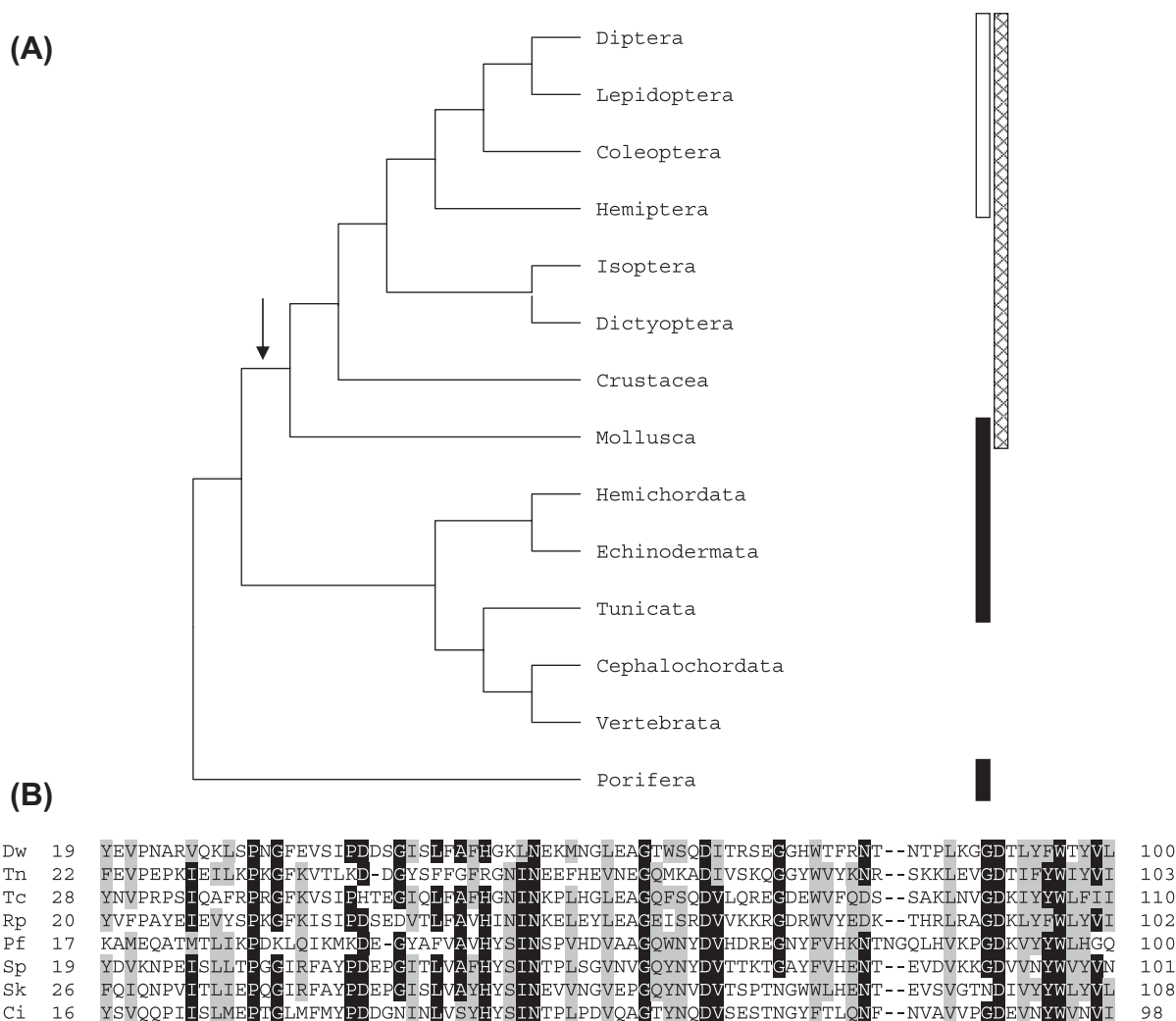


Figure 6 Occurrence of GNBPs and β -1,3-glucanases in metazoans. (A) Phylogeny tree of some metazoans according to Halanych (2004). Sidebars indicate the occurrence of insect GNBPs (white), β -1,3-glucanases lacking (crosshatched) and featuring (black) the extended N-terminal region. The arrow indicates the point where possible gene duplication occurred. (B). Partial alignment of the extended N-terminal region sequences of selected GNBPs and β -1,3-glucanases. Strongly conserved residues are shaded in black. Organisms and nucleotide GenBank Access number: Dw, *Drosophila willistoni* (XM_002061567); Tn, *Trichoplusia ni* (EU770373); Tc, *Tribolium castaneum* (XM_966587); Rp, *Rhodnius prolixus* (EF634459); Pf, *Pinctata fucata* (FJ775601); Sp, *Strongylocentrotus purpuratus* (XR_025993); Sk, *Saccoglossus kowalevskii* (XM_002740940); Ci, *Ciona intestinalis* (XM_002126654). Reprinted with permission from Bragatto, I., Genta, F.A., Ribeiro, A.F., Terra, W.R., Ferreira, C., 2010. Characterization of a β -1,3-glucanase active in the alkaline midgut of *Spodoptera frugiperda* larvae and its relation to β -glucan-binding proteins. *Insect Biochem. Mol. Biol.*, 40, 861–872.

1999a) argues against this hypothesis. The beetle pectinase belongs to family 28 of the glycoside hydrolases and is most related to fungal endopolygalacturonases, conserving the active site signature centered on the His 223 catalytic residue (Girard and Jouanin, 1999a; Markovic and Janecek, 2001).

11.4.5. Chitinases and Lysozymes

Chitin, the simplest of the glycosaminoglycans, is a β -1,4-homopolymer of N-acetylglucosamine (see Chapters 5–7 in this volume). Chitinolytic enzymes include chitinase

(EC 3.2.1.14), which catalyzes the random hydrolysis of internal bonds in chitin forming smaller oligosaccharides, and β -N-acetyl-D-glucosaminidase (EC 3.2.1.52), which liberates N-acetylglucosamine from the non-reducing end of oligosaccharides (Arakane and Muthukrishnan, 2010). Lysozyme, as described below, also has some chitinase activity, whereas chitinase rarely has lysozyme activity. These enzymes, besides being active in the ecdysial cycle, may also have a digestive role. Chitinase assays with midguts of several insects showed that there is a correlation between the presence of chitinase and a diet rich in chitin (Terra and Ferreira, 1994).

The best-known insect chitinase is the molting fluid chitinase from the lepidopteran *Manduca sexta* (Figure 5), which pertains to family 18 of glycosyl hydrolases. The enzyme has a multidomain architecture that includes a signal peptide, an N-terminal catalytic domain with the consensus sequence (F/L)DG(L/I)DLDWEYP, and a C-terminal cysteine-rich chitin-binding domain, which are connected by the interdomain serine/threonine-rich O-glycosylated linker. The residues Asp 142, Asp 144, Trp 145, and Glu 146 of the consensus sequence have been shown by site-directed mutagenesis to be involved in catalysis. Glu 146 functions as a proton donor in the hydrolysis like homologous residues in other glycoside hydrolases. Asp 144 apparently functions as an electrostatic stabilizer of the positively charged transition state, whereas Asp 142, and perhaps also Trp 145, influences the pK_a values of Asp 144 and Glu 146. The chitin-binding domains have six cysteines (with the consensus sequence CXnCXnCXnCXnCXnC, where Xn stands for a variable number of residues), and include several highly conserved aromatic residues (Tellam *et al.*, 1999). The three disulfide bonds in the domain may constrain the polypeptide to present the aromatic amino acids on the protein surface for interactions with the ring structures of sugars. Thus, the chitin-binding domains enhance activity toward the insoluble polymer, whereas the linker region facilitates secretion from the cell and helps to stabilize the enzyme in the presence of proteolytic enzymes (Lu *et al.*, 2002; Arakane *et al.*, 2003). In some cases, proteins with chitin-binding domains but lacking catalytic activity aid in the degradation of chitin (Vaaje-Kolstad *et al.*, 2005).

The *Anopheles gambiae* gut chitinase is secreted upon blood-feeding as an inactive proenzyme that is later activated by trypsin. Sequencing a cDNA coding for the gut chitinase showed that the enzyme comprises a putative catalytic domain at the N-terminus, a putative chitin-binding domain at the C-terminus, and a serine/threonine/proline-rich amino acid stretch between them (Shen and Jacobs-Lorena, 1997). The mosquito chitinase seems to modulate the thickness and permeability of the chitin-containing peritrophic membrane (see section 11.8.1). Supporting this conjecture, the authors found that the peritrophic membrane is stronger and persisted longer when the mosquitoes were fed diets containing chitinase inhibitor.

The beetle *P. cochleariae* has one group of chitinases of 40–70 kDa active at pH 5.0 and detected in guts. This enzyme has an active site centered on the catalytic residues Asp 146 and Glu 150 (*M. sexta* chitinase numbering), but lacks the C-terminal chitin-binding domain and the serine/threonine-rich interdomain (Girard and Jouanin, 1999b). *T. molitor* midgut chitinase (Genta *et al.*, 2006b) is similar to that of *P. cochleariae*, and both pertain to group IV chitinases (Arakane and Muthukrishnan, 2010) that as a rule lack a chitin-binding domain. The function

of the *T. molitor* enzyme may be digestion of the cell walls of fungi usually present in its food, without damaging the peritrophic membrane.

Lysozyme (EC 3.2.1.17) catalyzes the hydrolysis of the 1,4- β -glycosidic linkage between N-acetyl-muramic acid and N-acetylglucosamine of the peptidoglycan present in the cell wall of many bacteria, causing cell lysis. Lysozyme is part of an immune defense mechanism against bacteria, and has been described in most animals, including insects (see Chapter 14 in this volume). Lysozyme has also been implicated in the midgut digestion of bacteria by organisms which ingest large amounts of them, such as marine bivalves, or that harbor a bacterial culture in their guts (exemplified by Callewaert and Michiels, 2010).

Among insects, the capacity for digesting bacteria in the midgut seems to be an ancestral trait of Diptera Cyclorrhapha (Lemos and Terra, 1991a; Regel *et al.*, 1998), which agrees with the fact that most Diptera Cyclorrhapha larvae are saprophagous, feeding largely on bacteria (Terra, 1990). These insects have midgut lysozymes (Lemos *et al.*, 1993; Regel *et al.*, 1998) similar to those of vertebrate fermenters. Thus, these enzymes have low pI values, are more active at pH values 3–4 and when present in media with physiological ionic strengths, and are resistant to the cathepsin D-like aspartic proteinase present in midguts (Lemos *et al.*, 1993; Regel *et al.*, 1998; Fujita, 2004; Cançado *et al.*, 2008).

Sequence analyses (Daffre *et al.*, 1994; Cançado *et al.*, 2008) showed that cyclorrhaphan (*Drosophila melanogaster* and *Musca domestica*) digestive lysozymes have the same conserved residues as vertebrate lysozymes (Imoto *et al.*, 1972) (numbering according to Regel *et al.*, 1998): positions 55–61, Glu 36, and Asp 54. Glu 36 is believed to act as a general acid in catalysis, whereas Asp 54 is postulated to stabilize the resulting metastable oxocarbenium intermediate (Imoto *et al.*, 1972). More recently, Asp 54 has been implicated more strongly in catalysis of the hydrolysis of chitin-derived substrates (Matsumura and Kirsch, 1996). The ability of *D. melanogaster* and *M. domestica* purified lysozymes in hydrolyzing chitosan favors this view. The 3D structures of two *M. domestica* lysozymes were resolved (Marana *et al.*, 2006; Cançado *et al.*, 2007), and site-directed mutagenesis was performed (Cançado *et al.*, 2010). The results supported the hypothesis that the acidic pH optimum with synthetic substrates is determined by the presence of N46, S106, and T107 around the catalytic residues, which favors pK_a reduction. Furthermore, the acid pH optimum upon bacterial walls is caused by a decrease in surface positive charges.

Lysozyme is also found in the salivary glands of *Reticulitermes speratus*. This insect is a termite that feeds mainly on dead wood, which tends to lack nitrogen. Fujita *et al.* (2001) suggested, on the basis of the distribution and activity of lysozyme in this termite, that wood-feeding termites use lysozyme secreted from the salivary gland

to digest their hindgut bacteria, which are transferred by proctodeal trophallaxis. The termite lysozyme is active in neutral pH (Fujita *et al.*, 2002), thus differing from the digestive cyclorhaphan lysozymes.

11.4.6. α -Glucosidases

α -Glucosidases (EC 3.2.1.20) catalyze the hydrolysis of terminal, non-reducing α -1,4-linked glucose residues from aryl (or alkyl)-glucosides, disaccharides, or oligosaccharides. α -Glucosidases are frequently named maltases, although some of them may have weak activity on maltose. Insect α -glucosidases occur as soluble forms in the midgut lumen, or are trapped in the midgut cell glycocalyx. They are also bound to microvillar membranes (Terra and Ferreira, 1994), to perimicrovillar membranes (lipoprotein membranes ensheathing the midgut cell microvillar membranes in most hemipterans) (Silva and Terra, 1995), or to the modified perimicrovillar membranes of aphid midgut cells (Cristofolletti *et al.*, 2003). The last two membrane-bound α -glucosidases, as well as the soluble enzyme from bee midguts (Nishimoto *et al.*, 2001), were purified to electrophoretic homogeneity. *Culex pipiens* microvillar α -glucosidase is the primary target of the binary toxin of *Bacillus sphaericus*, and, although not purified, cDNA sequencing data suggest it is bound by a glycosyl phosphatidyl inositol anchor (Darboux *et al.*, 2001). α -Glucosidase is a major protein in dipteran midgut microvilli (Terra and Ferreira, 1994), and probably because of that it is the receptor of endotoxins, similar to that observed with aminopeptidase N in lepidopteran midgut cells (see section 11.5.5).

Although biochemical properties of many crude, partially or completely purified gut α -glucosidases are known, including molecular masses (range 60–80 kDa or a multiple of these values), pH optima (range 5–6.5, irrespective of the corresponding midgut pH value), pI values (range 5.0–7.2), and inhibition by tris(hydroxymethyl)aminomethane (Tris), only a few studies report on α -glucosidases specificities. These studies showed that insect α -glucosidases hydrolyze oligosaccharides up to at least maltopentaose (Terra and Ferreira, 1994), although there are exceptions. The perimicrovillar α -glucosidase from *Dysdercus peruvianus* (Silva and Terra, 1995) and *Quesada gigas* (Fonseca *et al.*, 2010) prefers oligosaccharides up to maltotetraose, and the midgut bee α -glucosidase, up to maltotriose (Nishimoto *et al.*, 2001). The purified midgut α -glucosidase of the pea aphid *Acyrtosiphon pisum* catalyzes transglycosylation reactions in the presence of excess sucrose, thus freeing glucose from sucrose without increasing the osmolarity of the medium (Cristofolletti *et al.*, 2003). This phenomenon, associated with a quick fructose absorption (Ashford *et al.*, 2000), explains why the midgut luminal osmolarity decreases as the ingested sucrose-containing phloem sap passes along

the aphid midgut. *A. pisum* α -glucosidase sequence does not contain any consensus sequences for membrane association, such as transmembrane helices or anchorage by C-terminal glycosylphosphatidyl inositol (GPI) moiety. It is argued that the enzyme may associate with the perimicrovillar membrane through its C-terminal region, which is predominantly hydrophobic (Price *et al.*, 2007).

A. pisum α -glucosidase, like other insect midgut α -glucosidases (e.g., *C. pipiens* midgut α -glucosidase, Darboux *et al.*, 2001) pertains to family 13 glycosidase. All the sequences have the invariant residues: Asp 123, His 128, Asp 206, Arg 221, Glu 271, His 296, and Asp 297 (numbers are relative to the positions in the sequence of *C. pipiens* α -glucosidases) (Darboux *et al.*, 2001) that are involved in the active site of the α -amylase family of enzymes, and the three residues Gly 69, Pro 77, and Gly 323, that have a structural role for some α -glucosidases (Janecek, 1997).

11.4.7. β -Glucosidases, β -Galactosidases, and Myrosinases

β -Glycosidases (EC 3.2.1) catalyze the hydrolysis of terminal, non-reducing β -linked monosaccharide residues from the corresponding glycoside. Depending on the monosaccharide that is removed, the β -glycosidase is named β -glucosidase (glucose), β -galactosidase (galactose), β -xylosidase (xylose), and so on. Frequently, the same β -glycosidase is able to hydrolyze several different monosaccharide residues from glycosides. In this case, β -glucosidase (EC 3.2.1.21) is used to name all enzymes that remove glucose efficiently. The active site of these enzymes is formed by subsites numbered from the glycosidic linkage to be broken, with negative (towards the non-reducing end of the substrate) or positive (towards the reducing end of the substrate) integers (Davies *et al.*, 1997). The non-reducing monosaccharide residue binds at the glycone (–1) subsite, whereas the rest of the molecule accommodates at the aglycone subsite, which actually may correspond to several monosaccharide residue-binding subsites.

Some insects have three or four digestive β -glycosidases with different substrate specificity. In others, only two of these enzymes are found, which are able to hydrolyze as many different β -glycosides as the other three or four enzymes together (Ferreira *et al.*, 1998; Azevedo *et al.*, 2003).

Insect β -glycosidases best characterized have molecular masses of 30–150 kDa, pH optima of 4.5–6.5, and pI values of 3.7–6.8, whereas K_m values, with cellobiose or p-nitrophenyl β -glucoside (Np β Glu) as substrates, are in the range of 0.2–2 mM. Although hydrolyzing several similar substances, insect digestive β -glycosidases have different specificities, preferring β -glucosides or β -galactosides as substrates, with hydrophobic or hydrophilic moieties

in the aglycone part of the substrate (Terra and Ferreira, 1994; Azevedo *et al.*, 2003).

Based on relative catalytic efficiency on several substrates, insect β -glycosidases can be divided into two classes. Class A includes the enzymes that efficiently hydrolyze substrates with hydrophilic aglycones, such as disaccharides and oligosaccharides. Class B comprises enzymes that have high activity only on substrates with hydrophobic aglycones, such as alkyl-, p-nitrophenyl-, and methylumbelliferyl-glycosides. Enzymes from class A are more abundant than β -glycosidases from class B. Class A β -glycosidases hydrolyze di- and oligosaccharides, and have four subsites for glucose binding in the active site: one in the glycone (-1) and three in the aglycone (+1, +2, +3) positions (Ferreira *et al.*, 2001, 2003; Marana *et al.*, 2001; Azevedo *et al.*, 2003). Some enzymes seem to be adapted to use disaccharides as well as oligosaccharides as substrates. Optimal hydrolysis of disaccharides relies on high affinities to glucose moieties in -1 and primarily in the +1 subsite (Ferreira *et al.*, 2003). The enzymes highly active against oligosaccharides have subsites -1, +1, and +2, with similar affinities to glucose moieties (Ferreira *et al.*, 2001, 2003).

Class A β -glycosidases are able to hydrolyze β -1,3, β -1,4, and β -1,6 glycoside bonds from di- and oligosaccharides. These enzymes are likely to be involved in the intermediate and terminal digestion of cellulose, hemicellulose, and glycoproteins present in food.

Class B β -glycosidases (or active site) with high activity against hydrophobic substrates may have the physiological role of hydrolyzing glycolipids, mainly galactolipids that are found in high amounts in vegetal tissues. The main galactolipids in plants are 2,3-diacyl β -galactoside D-glycerol (mono galactosyl diglyceride) and 2,3-diacyl 1-(α -galactosyl 1,6 β -galactosyl)-D-glycerol (digalactosyl diglyceride) (Harwood, 1980). These enzymes may act directly against the monogalactosyl diglyceride, or on digalactosyl diglyceride after the removal of one of the galactose residues by α -galactosidase. The activation by amphiphatic substances (as Triton X-100; see above) may be a mechanism to maintain high enzyme activity only in the neighborhood of plant cell membranes undergoing digestion in the insect midgut. These membranes are the source of glycolipid substrates and activating detergent-like molecules. Distant from membranes, the β -glycosidase would be less active, thus hydrolyzing plant glucosides (see below) ingested by the insect with decreased efficiency.

In agreement with the hypothesis presented above, β Gly47 from *S. frugiperda* can hydrolyze glycosylceramide, although with low activity (Marana *et al.*, 2000). In mammals, sphingosine and ceramide hydrolysis are dependent on enzyme activation by proteins called saposins (Harzer *et al.*, 2001). Given that genome sequences similar to saposins were found in *D. melanogaster*, insect

β -glycosidases active on glycolipids may also need the same kind of activators, and their absence in the assay reaction may explain why the activity against ceramides is low or not detected at all.

A few digestive β -glycosidases were purified and had their substrate specificity determined using k_{cat}/K_m values. Regarding natural substrates, two β -glycosidases from *T. molitor* (Ferreira *et al.*, 2001, 2003) and three β -glycosidases from *Diatraea saccharalis* (Azevedo *et al.*, 2003) are more active on laminaribiose (glucose β -1,3-glucose) than on cellobiose. On the other hand, the termite *Reticulitermes flavipes* β -glycosidase prefers cellobiose (glucose β -1,4-glucose) (Scharf *et al.*, 2010), and apparently the enzyme from *Neotermes koshunensis* has almost the same activity on both substrates (Tokuda *et al.*, 2002). This difference in specificity may be due to the fact that the β -glycosidases finish the digestion of the β -1,4-glucan chains of cellulose (termites, see section 11.4.3) or the β -1,3-glucan chains of hemicelluloses (lepidopterans, Terra *et al.*, 1987). It would be interesting to identify which amino acid residues are responsible for this difference in specificity. Free energy relationships (Withers and Rupitz, 1990) were used to compare the specificity of insect β -glycosidase subsites (Azevedo *et al.*, 2003). Since each of the three *D. saccharalis* β -glycosidases has a counterpart in *T. molitor*, Azevedo *et al.* (2003) speculated that insects with the same number of β -glycosidases could have similar enzymes.

The amino acid sequence was determined for a few β -glycosidases produced by the salivary glands of the termite *N. koshunensis* (Tokuda *et al.*, 2002) and *B. mori* (Byeon *et al.*, 2005), or produced by the midgut of *S. frugiperda* (Marana *et al.*, 2001), *T. molitor* (Ferreira *et al.*, 2001), and the termites *R. flavipes* (Scharf *et al.*, 2010) and *N. takasagoensis* (Tokuda *et al.*, 2009). All these enzymes pertain to family 1 of glycoside hydrolases.

The overall 3D structure of the *N. koshunensis* β -glucosidase (Jeng *et al.*, 2010) is similar to that of other glycosyl hydrolase family 1 enzymes, which have a classical (α/β)₈-TIM barrel fold. The active site, containing the catalytic residues Glu 193 and Glu 402, forms a deep slot-like cleft and is located on connecting loops at the C-terminal end of the β -sheets of the TIM barrel. In the β Gly50 from *S. frugiperda*, the pK_a of the nucleophile (Glu 399) is 4.9 and of the proton donor (Glu 187) is 7.5. In this enzyme, residue Glu 451 seems to be a key residue in determining the enzyme preference for glucosides versus galactosides. This is due to its interaction, in the glycone site, with substrate equatorial or axial hydroxyl 4, which is the only position where glucose differs from galactose. The steric hindrance of the same residue with hydroxyl 6 probably also explains why fucosides are the best substrate for many β -glycosidases (Marana *et al.*, 2002a).

Besides having a role in digestion, β -glycosidases are important in insect-plant relationships. To avoid

herbivory, plants synthesize a large number of toxic glucosides (**Figure 7**), which may be present in concentrations from 0.5% to 1% (Spencer, 1988). The presence of these glucosides in some insect diets may explain the variable number of β -glycosidases with different specificities present in their guts. Most plant glucosides have a hydrophobic aglycone and are β -linked O-glycosyl compounds. Since aglycones are usually more toxic than the glucosides themselves, intoxication may be avoided by decreasing the activity of the enzyme most active on toxic glucosides, without affecting the final digestion of hemicellulose and cellulose carried out by the other enzymes. This is exemplified by *D. saccharalis* larvae, which have three β -glycosidases in their midgut, feeding on diets containing the cyanogenic glucoside amygdalin. In this condition, the activity of the enzyme responsible for the hydrolysis of prunasin is decreased (Ferreira *et al.*, 1997). Prunasin is the saccharide resulting after the removal from amygdalin, and that forms the cyanogenic mandelonitrile upon hydrolysis (**Figure 7**). Resistance to toxic glucosides may also be achieved by the lack of enzymes able to hydrolyze toxic β -glucosides, as observed with *S. frugiperda* larvae, which have two β -glycosidases unable to efficiently hydrolyze prunasin (Marana *et al.*, 2001; S.R. Marana, personal communication). Progress in this field will require disclosing the mechanisms by which the

presence of toxic β -glucosides differentially affects the midgut β -glycosidases, and knowing the details of the active site architecture responsible for the specificity of these enzymes.

Myrosinase (EC 3.2.147) is a member of the glycosyl hydrolase family 1 that hydrolyzes glucosinolates, which are β -D-thioglycosides. The aphid *Brevicoryne brassicae* has a midgut myrosinase for which coding cDNA was cloned and sequenced (Jones *et al.*, 2002), and its 3D structure resolved (Husebye *et al.*, 2005). The enzyme has two catalytic Glu residues (plant myrosinase has only one), and its sequence is more similar to animal β -O-glucosidases than to plant myrosinases. The data suggest that myrosinase has twice arisen from β -glucosidases in plants and animals. Further structural data are necessary to clarify the features responsible for the hydrolysis of the thioglucoside bond.

11.4.8. Trehalases

Trehalase (EC 3.2.1.28) hydrolyzes α,α' -trehalose into two glucose molecules, and is one of the most widespread carbohydrases in insects, occurring in most tissues. Trehalase is very important for insect metabolism, since trehalose is the main circulating sugar in these organisms. There are many insect midgut trehalase sequences deposited in GenBank, but the single trehalase with known

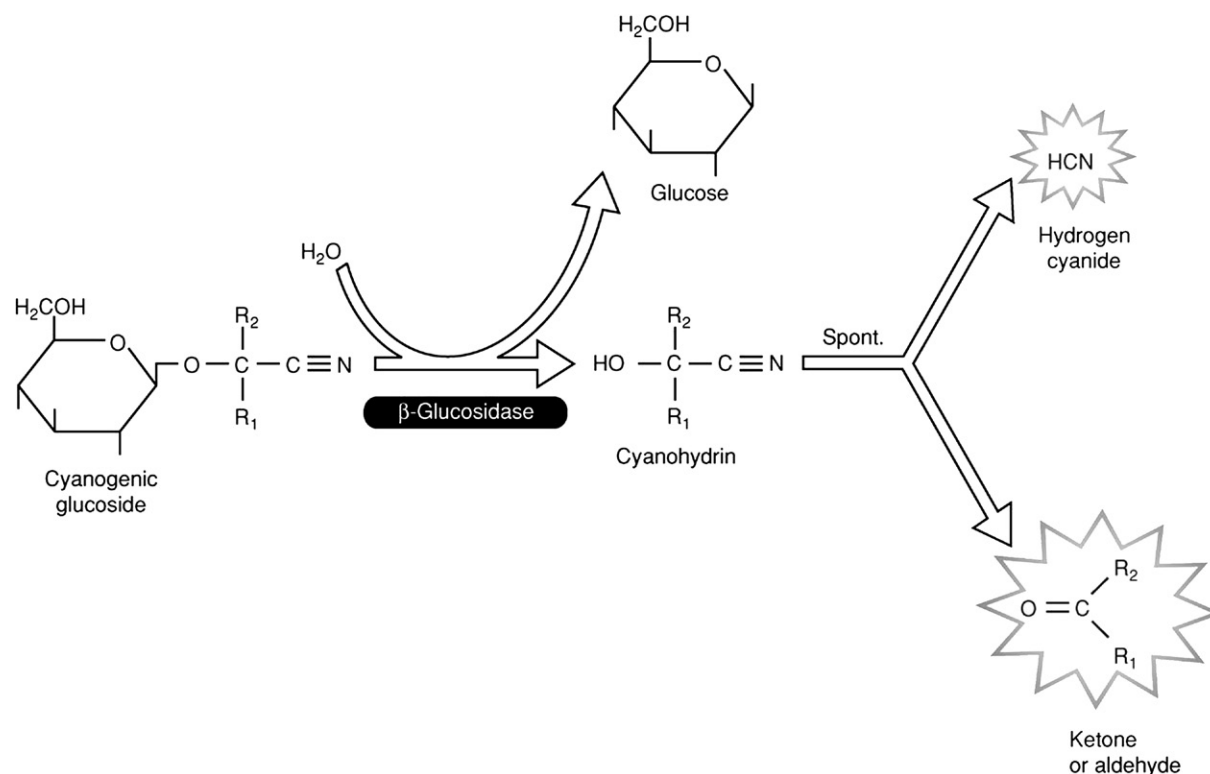


Figure 7 β -Glucosidase acting on a cyanogenic glucoside releases glucose and cyanohydrin that spontaneously decomposes, producing a ketone (or an aldehyde) and hydrogen cyanide. If $\text{R}_1 = \text{R}_2 = \text{CH}_3$, the glucoside is linamarin and the resulting ketone is acetone. If $\text{R}_1 = \text{H}$ and $\text{R}_2 = \text{phenyl}$, the glucoside is prunasin and the resulting aldehyde is benzaldehyde (see more examples in Vetter, 2000).

3D structure resolved is a periplasmic enzyme from *Escherichia coli* (Gibson *et al.*, 2007).

Apical and basal trehalases can be distinguished in insect midguts. The apical trehalase may be soluble (glycocalyx-associated or secreted into the midgut lumen) or microvillar, whereas the midgut basal trehalase is an integral protein of the basal plasma membrane. The apical midgut trehalase is a true digestive enzyme. The midgut basal trehalase probably plays a role in the midgut utilization of hemolymph trehalose (Terra and Ferreira, 1994). There is a report that localizes the soluble trehalase at the cavity of the goblet cell, and the membrane bound trehalase at the visceral muscles (Mitsumatsu *et al.*, 2005). This should be reinvestigated, because they may be artefacts (Silva *et al.*, 2009). Trehalases, partially or completely purified from insect guts, have pH optima from 4.8 to 6.0, K_m from 0.33 to 1.1 mM, pI around 4.6, and molecular masses from 60 to 138 kDa (Terra and Ferreira, 1994).

There have been a few attempts to identify important groups in the midgut trehalase active site. Terra *et al.* (1978, 1979, 1983) determined the pK_a values of the catalytical groups of the *R. americana* midgut trehalase. The pK_a value of the nucleophile was 5.0 (kinetic data) or 5.3 (carbodiimide modification results), whereas the pK_a value of the proton donor was 8.3 (kinetic data) or 7.7 (carbodiimide modification). Since there was a disagreement between the pK_a values determined for the proton donor, and taking into account that carbodiimide modification is only partially protected by trehalose, the authors suggested that the proton donor is near, but not at, the active site, and that it participates in the reaction through another amino acid residue, like histidine (Terra *et al.*, 1979). Lee *et al.* (2001), with the same approach as Terra *et al.* (1979), found pK_a values of 5.3 and 8.5 for the *Apis mellifera* trehalase. These authors and Valaitis and Bowers (1993), who worked with *Lymantria dispar* trehalase, showed that the trehalases were inactivated by diethyl pyrocarbonate (DPC), but the work did not progress further.

The active site of the *S. frugiperda* soluble midgut trehalase was modeled based on the 3D structure of *E. coli* trehalase. The model guided the choice of the trehalase residues to be mutated. Site-directed mutagenesis confirmed that D322 and E520 are the basic and acid catalysts, respectively, and showed that three Arg residues (R169, R222, and R287) are also essential for enzyme activity (Silva *et al.*, 2010). As the phenylglyoxal modified R222 has a pK_a value that is affected by a His residue in a similar way as the enzyme proton donor (Silva *et al.*, 2004), this explains the earlier implication of His residues in assisting a carboxyl group acting as a proton donor (Terra *et al.*, 1978, 1979; Lee *et al.*, 2001).

Plant toxic β -glucosides and their aglycones can inhibit, with varied efficiency, some or all trehalases from Malpighian tubules, fat body, midgut, and body wall of

P. americana, *M. domestica*, *S. frugiperda*, and *D. saccharalis* (Silva *et al.*, 2006). Toxic β -glucosides are produced by many plant species, and are present in high concentrations (see section 11.4.7). It is not known whether those glucosides or aglycones are absorbed by the insect gut and interact with trehalases in tissues other than the midgut. It would be interesting to know if this happens, and how insects resistant to toxic β -glucosides avoid the damage they can cause.

11.4.9. Acetylhexosaminidases, β -Fructosidases, and α -Galactosidases

An enzyme related to chitinolytic enzymes is β -N-acetyl-D-hexosaminidase (EC 3.2.1.52), which differs from β -N-acetyl-D-glucosaminidase in having a rather wide substrate specificity. The enzyme is found in many insects, and its presumed physiological role is the hydrolysis of N-acetylglucosamine β -linked compounds such as glycoproteins (Terra and Ferreira, 1994). Detailed studies of this digestive enzyme are lacking.

Sucrose hydrolysis is catalyzed by enzymes that are specific for the α -glucosyl (α -glucosidase, EC 3.2.1.20; see above) or for the β -fructosyl residue (β -fructosidase, EC 3.2.1.26) of the substrate. β -Fructosidase is characterized by its activity toward sucrose and raffinose, and lack of activity upon maltose and melibiose. In insect midguts, sucrose hydrolysis generally occurs by action of the conspicuous α -glucosidase rather than by β -fructosidase. However, larvae and adults of the moth *Erinnyis ello* have a midgut β -fructosidase with pH optimum 6.0, K_m 30 mM (sucrose), pI 5.2, and molecular mass of 78 kDa. The physiological role of this enzyme is to hydrolyze sucrose, the major leaf (larvae) or nectar (adults) carbohydrate, which is not efficiently digested by *E. ello* midgut α -glucosidase (Santos and Terra, 1986). After this first report, the presence of β -fructosidase was described in other Lepidoptera (Sumida *et al.*, 1994; Carneiro *et al.*, 2004). The presence of this enzyme is thought to be an adaptation to the presence of alkaloid sugars in latex that inhibits the α -glucosidase but not the β -fructosidase (Daimon *et al.*, 2008). As there seem to be exceptions for this inhibition (Hirayama *et al.*, 2007), more data should be gathered to settle the hypothesis.

The first β -fructosidase cDNA sequence was found in a proteomic survey of the larval gut lumen of *H. armigera* (Pauchet *et al.*, 2008). Later on, other genes were found in a midgut transcriptome and in lepidopteran-specific public EST data sets. The results indicated that β -fructosidase genes are widespread among Lepidoptera, and that they could be acquired from bacteria via horizontal gene transfer (Pauchet *et al.*, 2010).

α -Galactosidase (α -D-galactoside galactohydrolase, EC 3.2.1.22) catalyzes the hydrolysis of α -D-galactosidic linkages in the non-reducing end of oligosaccharides,

galactomannans, and galactolipids, and is widely distributed in nature (Dey and Pridham, 1972). Galactooligosaccharides, such as melibiose, raffinose, and stachiose, are common in plants, mainly in lipid-rich seeds (Shiroya, 1963), whereas galactolipids are widespread among leaves. The major lipids in chloroplast membranes are mono-galactosyldiglyceride and digalactosyldiglyceride (Harwood, 1980).

There have been few attempts to resolve insect midgut α -galactosidases. Gel filtration and heat inactivation suggested that there is a single α -galactosidase activity (30 kDa, pH optimum 5.0) in *D. peruvianus* midgut that is more efficient on raffinose than on melibiose and NP α Gal (Silva and Terra, 1997). There are two α -galactosidases in *A. flavolineata* midguts: the major (112 kDa, pH optimum 5.4) is active on melibiose and raffinose in addition to NP α Gal, whereas the minor (70 kDa, pH optimum 5.7) hydrolyzes only NP α Gal (Ferreira *et al.*, 1999). In the case of *Psacothia hilaris*, gel filtration gave evidence of the presence of multiple overlapping α -galactosidases more active on NP α Gal than on melibiose (Scrivener *et al.*, 1997). There are three midgut luminal α -galactosidases (TG1, TG2, and TG3) from *T. molitor* larvae that are partially resolved by ion-exchange chromatography (Grossmann and Terra, 2001). The enzymes have approximately the same pH optimum (5.0), pI value (4.6), and molecular mass (46–49 kDa). TG2 hydrolyzes α -1,6-galactosaccharides, exemplified by raffinose, whereas TG3 acts on melibiose and apparently also on digalactosyldiglyceride, the most important compound in thylacoid membranes of chloroplast, converting it into monogalactosyldiglyceride. *S. frugiperda* larvae have three midgut α -galactosidases (SG1, SG2, and SG3) partially resolved by ion-exchange chromatography (Grossmann and Terra, 2001). The enzymes have similar pH optimum (5.8), pI value (7.2), and molecular mass (46–52 kDa). SG1 and SG3 hydrolyze melibiose, and SG3 digests raffinose and, perhaps, digalactosyldiglyceride.

11.5. Digestion of Proteins

11.5.1. Initial Considerations

The initial digestion of proteins is carried out by proteinases (endopeptidases) that break internal bonds in proteins. Different proteinases are necessary to do this because the amino acid residues vary along the peptide chain. There are three subclasses of proteinases involved in digestion, classified according to their active site group (and hence by their mechanism): serine, cysteine, and aspartic proteinases. In each of the mentioned subclasses, there are several proteinases differing in substrate specificities. The oligopeptides resulting from proteinase action are attacked from the N-terminal end by aminopeptidases and from the C-terminal end by carboxypeptidases, both

enzymes liberating one amino acid residue at each catalytic step. Finally, the dipeptides formed are hydrolyzed by dipeptidases.

11.5.2. Serine Proteinases

Serine proteinases (EC 3.4.21) (MEROPS) have serine, histidine, and aspartic acid residues (called the catalytic triad) in the active site. The family of enzymes homologous to chymotrypsin (Barrett *et al.*, 1998) includes the major digestive enzymes trypsin, chymotrypsin, and elastase. These enzymes differ in structural features that are associated with their different substrate specificities, as detailed below. The numbering of residues in enzyme polypeptide chains is referred to that of bovine chymotrypsin.

11.5.2.1. Trypsins Trypsins (EC 3.4.21.4) preferentially cleave protein chains on the carboxyl side of basic L-amino acids such as arginine and lysine. Most insect trypsins have molecular masses in the range 20–35 kDa, pI values 4–5, and pH optima 8–10. These enzymes occur in the majority of insects, with the remarkable exception of hemipteran species and some taxa belonging to the series Cucujiformia of Coleoptera like Curculionidae (Terra and Ferreira, 1994). Nevertheless, some heteropteran Hemiptera have trypsin in the salivary glands (Zeng *et al.*, 2002).

Trypsin is usually identified in insect midgut homogenates using benzoyl-arginine p-nitroanilide (B-R-pNA, often referred to as BApNA) or benzoylarginine 7-amino-4-methyl coumarin (B-R-MCA) as substrates, and with N- α -tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF), or diisopropyl-fluorophosphate (DFP) as inactivating compounds. The substrates of choice for assaying insect trypsins are shown in **Figure 8**. Trypsins from Orthoptera, Dictyoptera, and Coleoptera are usually purified by ion-exchange chromatography, and those from Diptera and Lepidoptera by affinity chromatography – either in benzamidine-agarose (elution with benzamidine or by change in pH) or in soybean trypsin inhibitor (SBTI)-Sephacrose (elution by change in pH). Due to significant autolysis, lepidopteran trypsins are more frequently purified by chromatography on benzamidine-Agarose with elution with benzamidine.

There are a great number of insect trypsin sequences registered in GenBank. Examples may be found among Hemiptera (Zeng *et al.*, 2002), Coleoptera (Zhu and Baker, 1999), Diptera (Ramalho-Ortigão *et al.*, 2003), Siphonaptera (Gaines *et al.*, 1999), and Lepidoptera (Peterson *et al.*, 1994; Zhu *et al.*, 2000). The complete sequences have signal and activation peptides, and the features typical of trypsin-like enzymes, including the conserved N-terminal residues IVGG, the catalytic amino acid triad of serine proteinase active sites (His 57, Asp 102, and Ser 195), three pairs of conserved cysteine residues for

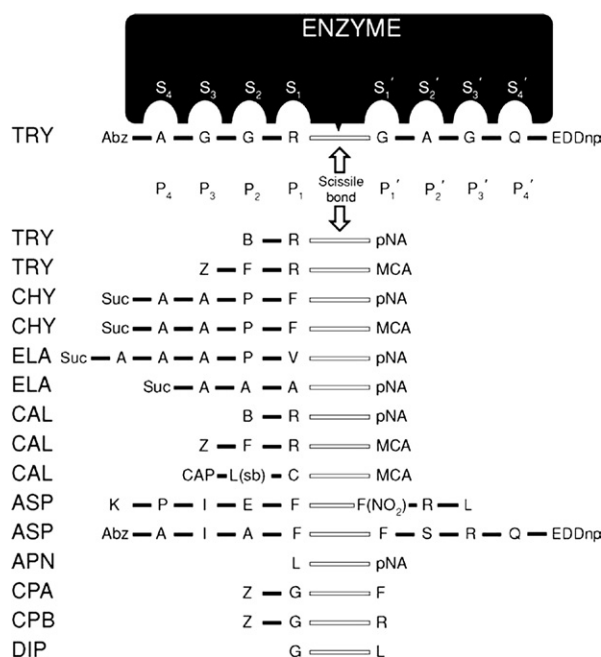


Figure 8 Substrates used in the assay of enzymes involved in protein digestion. Sn are subsites in the enzymes, and Pn are amino acid residues in substrates. The arrows point to bonds cleaved by the different enzymes. Abz-Xn-EDDnp is a class of peptides with quenching (EDDnp) and fluorescent (Abz) groups at the C- and N-terminal ends, respectively, so that after hydrolysis the peptides become fluorescent. Substrates with C-terminal MCA are fluorescent, and those with pNA are colorimetric. Contrary to GL, LG is also hydrolyzed by APN in addition to dipeptidase. For further details, see text. TRY, trypsin; CHY, chymotrypsin; ELA, elastase; CAL, cathepsin L-like enzyme; ASP, aspartic proteinase; APN, aminopeptidase N; CPA, carboxypeptidase A; CPB, carboxypeptidase B; DIP, dipeptidase.

disulfide bonds, and the residue Asp 189 that determines specificity in trypsin-like enzymes (see **Figure 5**). In spite of having structural features resembling vertebrate trypsins, insect trypsins differ from these because they are not activated or stabilized by calcium ions, and frequently are unstable in acidic pH (Terra and Ferreira, 1994). Finally, other differences between vertebrate and insect trypsins include their substrate specificities and their interaction with protein inhibitors.

Amino acyl residues in proteinase substrates are numbered from the hydrolyzed peptide bonds as P1, P2, P3, ..., Pn in the direction of the N-terminus, and P'1, P'2, P'3, ..., P'n in the direction of the peptide C-terminus, whereas the corresponding enzyme subsites are numbered S1, S2, S3, ..., Sn and S'1, S'2, S'3, ..., S'n (Schechter and Berger, 1967) (**Figure 8**). Mammalian trypsin preferably cleaves substrates having arginine rather than lysine at P1 (primary specificity) (Craik *et al.*, 1985). The same primary specificity was found for insect trypsins, except those from lepidopterans, which prefer lysine at P1 (Lopes *et al.*, 2004). This will be discussed below in relation to

trypsin insensitivity to protein inhibitors. In order to characterize the trypsin specificity at subsites other than S1, quenched fluorescent substrates such as o-aminobenzoyl-GGRGAGQ-2,4-dinitrophenyl-ethylene diamine (where R stands for arginine at P1 position) were synthesized with 15 amino acid replacements at each of the positions P'1, P'2, P'3, and P'4. The results suggested that trypsin subsites are more hydrophobic in trypsins from the more evolved insects (Lopes *et al.*, 2006). Trypsins from different insects also differ in the strength with which their subsites bind the substrate or the transition state (high-energy intermediate of the reaction). In other words, trypsin subsites differ in how they favor substrate binding or catalysis (Marana *et al.*, 2002b).

Plants have protein inhibitors (PIs) of insect midgut serine proteinases that affect insect development (Ryan, 1990). Insects may adapt to the presence of PIs in the diet by overexpressing proteinases (Bown *et al.*, 1997; Broadway, 1997; Gatehouse *et al.*, 1997), by proteolytical inactivation of PIs mediated by the insect's own proteinases (Giri *et al.*, 1998), or by expressing new proteinases that are resistant to the inhibitor (Mazumdar-Leighton and Broadway, 2001a, 2001b). Current research is investigating the molecular basis of the difference between sensitive and inhibitor-insensitive trypsins, as well as the regulation of these enzymes.

PIs produced by plants have a region, named the reactive site, that interacts with the active site of their target enzymes. The reactive sites of many PIs are hydrophilic loops with a lysine residue at P1 (Lopes *et al.*, 2004). As lepidopteran trypsins have hydrophobic subsites and prefer lysine rather than arginine at P1 (see above), they are usually more resistant to PIs than the other insect trypsins. In this respect, it is interesting to note that PI-insensitive trypsins from *Heliothis virescens* bind more tightly to a hydrophobic chromatographic column than do sensitive trypsins (Brito *et al.*, 2001). These observations led to the hypothesis that the molecular differences between sensitive and insensitive trypsins must rely on the interactions of PIs with residues in and around the enzyme active site.

An interesting approach to studying insect-PI interactions was introduced by Volpicella *et al.* (2003), who compared the sequence of a sensitive trypsin from *Helicoverpa armigera* with the insensitive trypsin from the closely related species *Helicoverpa zea*. The 57 different amino acids observed between the two enzymes were superimposed on the porcine trypsin crystal structure, where the residues known to be in contact with a Kunitz-type inhibitor (Song and Suh, 1998) were identified. The residues at positions (chymotrypsin numbering) 41, 57, 60, 95, 99, 151, 175, 213, 217, and 220 were considered by Volpicella *et al.* (2003) to be important in *H. zea* trypsin-PI interaction. However, some of the interacting residues may have been misidentified, because trypsins from different species were compared. In a similar approach,

Lopes *et al.* (2004) aligned all available trypsin sequences characterized as sensitive or insensitive to Kunitz-type inhibitor (Bown *et al.*, 1997; Mazumdar-Leighton and Broadway, 2001a) with porcine trypsin. After discounting conserved positions and positions not typical of sensitive or insensitive trypsin, the remaining positions that agreed with those involved in porcine trypsin-PI (Bowman-Birk type, Lin *et al.*, 1993; Kunitz type, Song and Suh, 1998) or substrate (Koepke *et al.*, 2000) interactions were: 60, 94, 97, 98, 99, 188, 190, 213, 215, 217, 219, 228. These positions support the tree branches in a neighbor-joining analysis of sensitive (I, III) and insensitive (II) trypsin sequences (Lopes *et al.*, 2004). Site-directed mutagenesis of trypsin, followed by the determination of the binding constants of mutated trypsin with PIs, may help to resolve the discrepancy.

The mechanism by which PIs in the diet induce the synthesis of insensitive trypsin in responsive insects remains unknown, although it was found that the first step in the process is the expression of the whole set of midgut trypsin (Brioschi *et al.*, 2007). The evolutionary “arms race” between plants and insects regarding evolving new digestive proteinases and new PIs are reviewed in Christeller (2005).

11.5.2.2. Chymotrypsins Chymotrypsin (EC 3.4.21.1) preferentially cleaves protein chains at the carboxyl side of aromatic amino acids. Insect chymotrypsins usually have molecular masses of 20–30 kDa and pH optima of 8–11, and they differ from their vertebrate counterparts in their instability at acidic pH, inhibition pattern with SBTI (Terra and Ferreira, 1994), and, finally, in reacting with N- α -tosyl-L-phenylalanine chloromethyl ketone (TPCK) (see below). The distribution of chymotrypsin among insect taxa is similar to that of trypsin (Terra and Ferreira, 1994), including the occurrence in the salivary glands of some heteropteran bugs (Colebatch *et al.*, 2002). The earlier failure to detect chymotrypsin activity in insect midguts was a consequence of using mammalian chymotrypsin substrates, such as benzoyl-tyrosine p-nitroanilide (B-Y-pNA) or benzoyl-tyrosine ethyl ester (B-Y-ee), in the assays. Insect chymotrypsins prefer Phe at P₁, and are almost inactive if Tyr is at that position (Lopes *et al.*, 2009). Furthermore, insect chymotrypsins have an extended active site (as in mammalian chymotrypsin; see Lopes *et al.*, 2009) and larger substrates, like succinyl-AAPF-p-nitroanilide (Suc-AAPF-pNA), are usually necessary for their detection (Lee and Anstee, 1995; Lopes *et al.*, 2009) (Figure 8). Insect chymotrypsins are usually purified by affinity chromatography in phenyl butylamina-Sepharose (elution with phenyl butylamina) or in SBTI-Sepharose (elution with benzamidine) for enzymes from lepidopterans, and by ion-exchange chromatography for those from dictyopterans, orthopterans, hymenopterans, and dipterans. They have been purified from several

sources: Hemiptera (Colebatch *et al.*, 2002), Coleoptera (Oliveira-Neto *et al.*, 2004; Elpidina *et al.*, 2005), Hymenoptera (Whitworth *et al.*, 1998), Siphonaptera (Gaines *et al.*, 1999), Diptera (de Almeida *et al.*, 2003; Ramalho-Ortigão *et al.*, 2003), and Lepidoptera (Peterson *et al.*, 1995; Volpicella *et al.*, 2006). A large number of sequences is also available for insect chymotrypsins at GenBank. All the sequences have a signal peptide, an activation peptide (ending with an arginine residue), the catalytic triad (His 57, Asp 102, and Ser 195), three pairs of conserved cysteine residues, conserved N-terminal sequence IVGG, and Ser/Gly/Tyr 189, which confers specificity to chymotrypsin-like enzymes (Figure 5).

The substrate preferences of chymotrypsins from insects of three different orders were studied with quenched fluorescent substrates. The result showed that although substrate preferences vary among the different chymotrypsins, no evolutionary trend as described for trypsin was observed. In spite of those differences, the data suggested that in lepidopteran chymotrypsins S₂ and S₁' bind the substrate ground state, whereas only S₁' binds the transition state, supporting aspects of the present accepted mechanism of catalysis (Sato *et al.*, 2008).

The insect digestive chymotrypsin that has been most thoroughly studied is that of *M. sexta* (Lepidoptera: Sphingidae) (Peterson *et al.*, 1995). In this enzyme, the activation peptide is longer and has a net charge different from that of bovine chymotrypsinogen, leading the authors to suggest that the insect enzyme is activated by a peculiar mechanism. The mammalian chymotrypsin has a pH optimum around 8, with two catalytic important pK_as of 6.8 and 9.5, corresponding to the active-site histidine and N-terminal leucine, respectively. In contrast, the *M. sexta* chymotrypsin has pH optimum 10.5–11, and a single kinetically significant pK_a at pH 9.2. This pK_a may represent the active-site histidine in an appropriate environment, although several other hypotheses were discussed (Peterson *et al.*, 1995). It is not clear whether the insect chymotrypsin active-site changes associated with TPCK resistance (see below) may also be the cause of the putative histidine pK_a displacement.

The resolution of the 3D structure of the fire-ant digestive chymotrypsin led to the conclusion that it is strikingly similar to mammalian chymotrypsin, but has differences beyond those found among homologs from different mammalian systems (Botos *et al.*, 2000). The similarities include a conserved backbone scaffold and structural domains. Differences include the activation mechanism and substitutions in the subsite S₁ and mainly in the other subsites (S₄–S'₄) that suggest different substrate specificities and interactions with PIs. In agreement with this, different insect chymotrypsins are sensitive to distinct PIs and, like trypsin, PI-insensitive chymotrypsins may be induced in insects ingesting PI-containing diets (Bown *et al.*, 1997; Mazumdar-Leighton and Broadway, 2001b).

The molecular mechanism of chymotrypsin PI inhibition was investigated. Two chymotrypsins were purified from the midgut of *Helicoverpa punctigera*, one PI-sensitive and the other PI-insensitive. After their corresponding cDNAs were cloned and sequenced, molecular modeling revealed that a Phe→Leu substitution at position 37 in the chymotrypsin results in the loss of important contacts with the PI. This was confirmed by site-directed mutagenesis of chymotrypsin molecules, followed by inhibition tests (Dunse *et al.*, 2010). Chymotrypsins from insects that routinely ingest ketone-releasing compounds (like several plant glycosides) (see **Figures 7 and 9**) are not affected much by these compounds and others that react with His 57. Thus, in comparison with bovine chymotrypsin, the chymotrypsin from polyphagous lepidopteran insects reacts slowly with chloromethyl ketones, whereas those of oligophagous pyralid insects react rapidly (Lopes *et al.*, 2009). Modeling *Spodoptera frugiperda* (Noctuidae) chymotrypsin, based on its sequence and on crystallographic data of bovine chymotrypsin, showed that the neighborhood of His 57 differs from bovine chymotrypsin, thus affecting His reactivity (Lopes *et al.*, 2009). These adaptations are new examples of the interplay between insects and plants during their evolutionary arms race, and deserve more attention through site-directed mutagenesis of recombinant chymotrypsins.

11.5.2.3. Elastases Since Christeller *et al.* (1990) described an elastase (EC 3.4.21.36)-like enzyme in the cricket *Teleogryllus commodus*, this enzyme has been described in many other insects, including in homogeneous form (Terra and Ferreira, 1994; Whitworth *et al.*, 1998). Usually elastase is identified with the substrate Suc-AAPL-pNA (**Figure 8**), combined with the observation of lack of activity on B-Y-pNA or B-Y-ee and resistance to TPCK. Since the mentioned substrate may also be hydrolyzed by chymotrypsin, and lack of activity on B-Y-pNA and/or resistance to TPCK are usual among chymotrypsins (see **section 11.5.2.2**), most described elastases may actually be chymotrypsins. True elastases

were isolated from gypsy moth midguts (Valaitis, 1995) and from whole larvae of *Solenopsis invicta* (Whitworth *et al.*, 1998). The last-mentioned enzymes hydrolyze Suc-APA-pNA, but not substrates with phenylalanine at P₁. Although the specific substrate for elastase (Suc-AAA-pNA) (Bieth *et al.*, 1974) was not tested, the hydrolysis of Suc-AAAPV-pNA and the lack of hydrolysis of substrates with phenylalanine in P₁ discount a chymotrypsin. One of the *S. invicta* elastases (E2) was cloned, sequenced, and shown to be more similar to chymotrypsin than to elastase (Whitworth *et al.*, 1999). This work confirms the occurrence of elastase in insect midgut. Further work is necessary to evaluate the extent of this enzyme in insect midguts, and its importance in digestion.

11.5.3. Cysteine Proteinases

Cysteine proteinase is usually assayed in insect midgut contents or midgut homogenates at pH 5–6 with B-R-pNA, B-R-NA, casein, or hemoglobin as substrate. Activation by sulfhydryl agents (dithiothreitol (DTT) or cysteine) and inhibition by trans-epoxysuccinyl-L-leucyl-amido (4-guanidinobutane) (E-64) are usually indicative of the presence of the enzyme. The observation of inhibition of hydrolytic activity on any of the mentioned substrates by E-64 is insufficient for a positive identification of cysteine proteinase. Trypsin hydrolyzes the same substrates, and may be reversibly inhibited by E-64 (Novillo *et al.*, 1997). The identification of cysteine proteinase was made easier with the substrate e-amino-caproyl-leucyl-(S-benzyl)-cysteinyl-MCA, which is hydrolyzed by cysteine proteinase but not by serine proteinases (Alves *et al.*, 1996). Using such criteria, cysteine proteinases were described in Hemiptera Heteroptera and in species belonging to the series Cucujiformia of Coleoptera (Terra and Ferreira, 1994). Exceptions to this rule are the identification of cysteine proteinase in Hemiptera Sternorrhyncha (aphids) (Cristofolletti *et al.*, 2003; Deraison *et al.*, 2004), and the lack of this enzyme in cucujiform cerambycid beetles (Johnson and Rabosky, 2000).

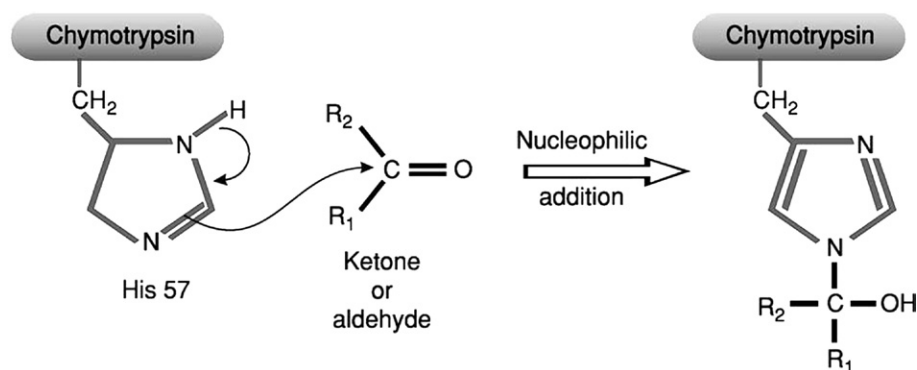


Figure 9 Ketones or aldehydes formed after the action of β -glucosidases on cyanogenic glucosides (**Figure 7**) may react with His 57 of chymotrypsin, inactivating it.

Insect midgut cysteine proteinases were at first denoted as cathepsin B (EC 3.4.22.1)-like enzymes, because cathepsin B was the first animal cysteine proteinase described. Later on it became known that cathepsin B is more important as a peptidyl dipeptidase, rather than as an endopeptidase, because of the existence of an extended loop that forms a cap to the active-site cleft, and carries a pair of histidine residues that are thought to bind to the C-terminal carboxylate of the substrate (Barrett *et al.*, 1998). Cathepsin L (EC 3.4.22.15) is a true endopeptidase that preferentially cleaves peptide bonds with hydrophobic amino acid residues in P₂ (cathepsin B prefers arginine at the same position) (Barrett *et al.*, 1998). Thus, by using substrates like carbobenzoxy (Z)-FR-MCA and Z-RR-MCA it is possible to distinguish between the two enzymes (see Figure 8). Current research has revealed that cathepsin L-like enzymes are the only insect midgut cysteine proteinase that is quantitatively important. Much more difficult to ascertain is that a cathepsin L-like enzyme assayed in insect midguts has been secreted into midgut contents, and hence may be considered as a truly digestive enzyme. As in other animals (Barrett *et al.*, 1998), cathepsin L-like enzymes in insects are expected to occur in lysosomes and never leave the cells. The same difficulties arise in trying to relate digestion to cathepsin L-like enzymes encoded by cDNAs cloned from midgut cells.

The problems that may arise during cathepsin L-like enzyme characterization are well illustrated in a study with *T. molitor* larvae. Three cathepsin L-like sequences were recognized in a cDNA library prepared from *T. molitor* midguts. One sequence, after being expressed and used to raise antibodies, was found to correspond to a lysosomal cathepsin L immunolocalized mainly at hemocytes and fat body cells. The second sequence was not related yet with any enzyme active in midgut. Finally, the third sequence corresponds to a cathepsin L-like enzyme purified from midgut contents (Cristofaletti *et al.*, 2005).

Digestive cathepsin L-like enzymes have been purified to homogeneity only from *Diabrotica virgifera* (Coleoptera: Cucujiformia) (Koiwa *et al.*, 2000), *Acyrtosiphon pisum* (Hemiptera: Sternorrhyncha) (Cristofaletti *et al.*, 2003), and *T. molitor* (Coleoptera: Cucujiformia) (Cristofaletti *et al.*, 2005). The *A. pisum* enzyme is cell membrane-bound and faces the luminal contents, whereas those in *D. virgifera* and *T. molitor* are soluble enzymes secreted into midgut contents. The complete purification of these enzymes was achieved by affinity chromatography with soyacystatin as a ligand, or by a combination of ion-exchange chromatographies. The enzymes have pH optima of 5–6, molecular masses of 20–40 kDa, prefer Z-FR-MCA over Z-RR-MCA, are inhibited by E-64, and are activated by cysteine or DTT. At least, the *A. pisum* enzyme is also inhibited by chymostatin (completely) and elastinal (partly) (Cristofaletti *et al.*, 2003).

There are many cathepsin L-like sequences corresponding to coleopterans and hemipterans (those known to have digestive cathepsins) registered in GenBank. The sequences demonstrate the features characteristic of family 1 of cysteine proteinases (Barrett *et al.*, 1998): an N-terminal propeptide that must be removed to activate the enzyme and the catalytic triad, Cys 25, His 169, and Asn 175 (papain numbering), and the ERFNIN motif (Figure 5). The sequences form a monophyletic grouping, and a polyphyletic array seems to correspond to lysosomal and digestive enzymes, respectively (Cristofaletti *et al.*, 2005). The data suggest that the Hemiptera digestive cathepsin is close to the lysosomal one from *T. molitor* (CAL 1), whereas those from the coleopterans (except from *Sitophilis oryzae*) are similar to the *T. molitor* digestive enzyme (CAL 3). It is probable that the *S. oryzae* enzymes are not true digestive but lysosomal, like *T. molitor* CAL (Cristofaletti *et al.*, 2005). More work is needed to clarify the role of cathepsin L-like enzymes in insect digestion.

11.5.4. Aspartic Proteinases

Aspartic proteinases are active at acid pH, hydrolyze internal peptide bonds in proteins, and attack some synthetic substrates – either chromophoric (Dunn *et al.*, 1986) or internally quenched fluorescent substrates (Pimenta *et al.*, 2001) (Figure 8). Mammalian cathepsin D has a substrate-binding cleft that can accommodate up to seven amino acids, and prefers to cleave between two hydrophobic residues (Barrett *et al.*, 1998).

The first report of aspartic proteinases in insects was made by Greenberg and Paretsky (1955), who found a strong proteolytic activity at pH 2.5–3.0 in homogenates of whole bodies of *Musca domestica*. Lemos and Terra (1991b) showed that the enzyme occurs in midguts, and is cathepsin D-like. An aspartic proteinase similar to cathepsin D was found in families of Hemiptera and Heteroptera, and in several families belonging to the cucujiform series of Coleoptera (Terra and Ferreira, 1994). Thus, it is possible that aspartic proteinases occur together with cysteine proteinase in Hemiptera and in most Coleoptera. The aspartic proteinase isolated from *Callosobruchus maculatus* (pH optimum 3.3, 62 kDa) (Silva and Xavier-Filho, 1991) and *Tribolium castaneum* (pH optimum 3.0, 22 kDa) (Blanco-Labra *et al.*, 1996) were partially purified and shown to be similar to cathepsin D.

M. domestica midguts express 3 cathepsin D-like enzymes (CAD1, CAD2, and CAD3). All CADs have catalytic Asp 33 (together with T34 and G35) and Asp 229 (together with T230 and G231) (bovine cathepsin D numbering), and the conserved substrate binding pockets (Padilha *et al.*, 2009). CAD3 is a luminal digestive enzyme, and lacks the proline loop (as defined by the motif: DxPxPx (G/A)P, thus resembling vertebrate pepsin). CAD2 also lacks the proline loop and may be

another digestive enzyme. CAD1 is expressed in all *M. domestica* tissues (not only in midgut as CAD2 and CAD3), and shows the proline loop as the vertebrate lysosomal cathepsin D (Padilha *et al.*, 2009). The data suggest that on adapting to deal with a bacteria-rich food in an acid midgut region, *M. domestica* CAD resulted from the same archetypical gene as the intracellular cathepsin D, paralleling what happened with vertebrates. The lack of the proline loop may be somehow associated with the extracellular role of both pepsin and digestive CAD3. Further work is necessary to clarify this point.

11.5.5. Aminopeptidases

Aminopeptidases sequentially remove amino acids from the N-terminus of peptides, and are classified on the basis of their dependence on metal ions (usually Zn^{2+} or Mn^{2+}) and substrate specificity. Aminopeptidase N (EC 3.4.11.2) has a broad specificity, although it preferentially removes alanine and leucine residues from peptides, whereas aminopeptidase A (EC 3.4.11.7) prefers aspartyl (or glutamyl) peptides as substrates. Both are metalloenzymes (Norén *et al.*, 1986).

In insect midguts, major amounts of soluble aminopeptidases are found in less evolved insects (e.g., Orthoptera, Hemiptera, Coleoptera Adephaga), whereas in more evolved insects (e.g., Coleoptera Polyphaga, Diptera, and Lepidoptera) aminopeptidase is found mainly bound to the microvillar membranes of midgut cells (Terra and Ferreira, 1994). Insect midgut aminopeptidases are metalloenzymes (ethylenediaminetetraacetic acid (EDTA) inhibition) and have pH optima of 7.2–9.0, irrespective of the pH of the midgut lumen from the different species, K_m values (L-pNA) of 0.13–0.78 mM, and molecular masses of 90–130 kDa. With a single exception (see below), all known insect aminopeptidases have a broad specificity, hydrolyzing a variety of amino acyl β -naphthylamides (except acidic amino acyl β -naphthylamides), indicating they are aminopeptidases N (Terra and Ferreira, 1994) (Figure 8). The exception is a soluble glycocalyx-associated midgut aminopeptidase from *R. americana*. This enzyme is an aminopeptidase removing N-terminal aspartic acid or glutamic acid residues from peptides that are not efficiently attacked by the other aminopeptidases (Klinkowstrom *et al.*, 1994).

In addition to a midgut aminopeptidase A, the dipteran *R. americana* has three midgut aminopeptidase Ns (one soluble and two membrane-bound). The soluble aminopeptidase N (115.7 kDa) prefers tetrapeptides over tripeptides (Ferreira and Terra, 1984), like the minor 107-kDa membrane-bound enzyme, whereas the contrary is true for the major 169-kDa membrane-bound aminopeptidase (Ferreira and Terra, 1985, 1986a, 1986b). The single midgut aminopeptidase N of the coleopterans *Attagenus megatoma* (Baker and Woo, 1981) and *T. molitor* (Cristofolletti and Terra, 1999) resemble the 115.7-kDa

and 107-kDa aminopeptidases of *R. americana*. Approximately the same substrate specificity was observed with the two midgut aminopeptidases of the lepidopteran *Tineola bisselliella* (Ward, 1975a, 1975b), that from the aphid *A. pisum* (Cristofolletti *et al.*, 2006), and that of the cerambycid beetle *Morimus funereus* (Bozic *et al.*, 2008). The data suggest that panorpoid insects (Diptera and Lepidoptera) present multiple aminopeptidases with different substrate specificities, in contrast with the single aminopeptidase of coleopterans. However, considerably more data are needed to support this hypothesis.

There have been few attempts to characterize the active site of insect midgut aminopeptidases. Using multiple inhibition analysis and observing the protection against EDTA inactivation that different competitive inhibitors conferred to the enzyme, two subsites were proposed to occur in the active center of *R. americana* microvillar aminopeptidase: a hydrophobic subsite, to which isoamyl alcohol binds, exposing the metal ion, and a polar subsite, to which hydroxylamine binds. Exposure of the metal ion after isoamyl alcohol binding may be analogous to the situation that results when part of the substrate occupies the hydrophobic subsite, causing conformational changes associated with the catalytic step (Ferreira and Terra, 1986b). The effect of pH at different temperatures on kinetic parameters of *T. molitor* midgut aminopeptidase and its inactivation by different compounds were studied (Cristofolletti and Terra, 2000). The data showed that *T. molitor* aminopeptidase catalysis depends on a metal ion, a carboxylate, and a protonated imidazole group, and is somehow influenced by an arginine residue in the neighborhood of the active site. The catalytic metal binding depends on at least a deprotonated imidazole. In addition to the above-mentioned groups involved in catalysis, at least one phenol group and one carboxylate are associated with substrate binding. Thus, *T. molitor* aminopeptidase shares common features with those of other zinc metallopeptidases, especially with mammalian aminopeptidase N, but it differs in some details. An imidazole group seems to be involved in catalysis in *T. molitor* aminopeptidase; this is not observed in mammalian aminopeptidase N, which has an imidazole group participating in substrate binding.

Sequences of aminopeptidase N from insect midgut are available from different insects. Some examples are *Trichoplusia ni* (Wang *et al.*, 2005), *A. pisum* (Cristofolletti *et al.*, 2006), *Aedes aegypti* (Chen *et al.*, 2009), *H. armigera* (Angelucci *et al.*, 2008), *Ostrinia nubilalis*, and *B. mori* (Crava *et al.*, 2010). The sequences have a signal peptide, a conserved RLP motif near the N-terminal, a zinc binding/gluzincin motif HEXXH₁₈E, a GAMEN conserved motif, and a long hydrophobic C-terminal containing a glycosyl phosphatidyl inositol anchor (Figure 5). Based on the crystal structure of leukotriene A₄ hydrolase, the two histidine residues and the distant glutamic acid residue of the gluzincin motif are zinc ligands, the glutamic acid

residue between the histidine residues is involved in catalysis (Hooper, 1994; Rawlings and Barrett, 1995), and the glutamic acid residue of the GAMEN motif binds the substrate N-terminal amino acid (Luciani *et al.*, 1998). In contrast to the situation in mammals, insect aminopeptidase N is membrane bound at the C-terminal. No soluble insect aminopeptidase N has been sequenced.

Dendrograms derived from alignments of lepidopteran midgut aminopeptidases suggest that there are at least four major groups of lepidopteran aminopeptidases, with the isoforms of the same animal distributed among the groups (Wang *et al.*, 2005; Cristofolletti *et al.*, 2006; Crava *et al.*, 2010). The existence of a number of different aminopeptidases in lepidopterans could be explained by the need for enzymes with different substrate specificities (as shown above for *R. americana*) or different susceptibilities to inhibitors, similar to serine proteinases (see section 11.5.2).

Probably associated with the fact that aminopeptidases are major proteins in some microvillar membranes (55% of *T. molitor* midgut microvillar proteins) (Cristofolletti and Terra, 1999), they are targets of insecticidal *Bacillus thuringiensis* crystal δ -endotoxins. These toxins, after binding to aminopeptidases and receptor molecules called cadherins, form channels through which cell contents leak, leading to the death of the insect (Knight *et al.*, 1995). Although data on substrate specificity for lepidopteran aminopeptidase isoforms are lacking, there is evidence that the isoforms may have differences in toxin binding (Nakanishi *et al.*, 2002; Rajagopal *et al.*, 2003).

Cloning and sequencing dipteran aminopeptidases, for which differences in substrate specificity are known, and a study of substrate specificities of lepidopteran aminopeptidases, may clarify the selective advantages of the evolution of aminopeptidase groups. Furthermore, this study may support the hypothesis that aminopeptidase gene duplications have occurred in the panorpoid ancestor, before differentiation between dipterans and lepidopterans.

11.5.6. Carboxypeptidases and Dipeptidases

Carboxypeptidases hydrolyze single amino acids from the C-terminus of the peptide chain, and are divided into classes on the basis of their catalytic mechanism. There are two digestive metallocarboxypeptidases in mammals: carboxypeptidase A (EC 3.4.17.1), which hydrolyzes (in alkaline medium) C-terminal amino acids, except arginine, lysine, and proline; and carboxypeptidase B (EC 3.4.17.2), which releases (in alkaline conditions) C-terminal lysine and arginine preferentially. Insect digestive carboxypeptidases have been classified as carboxypeptidase A or B depending on activity in alkaline medium against Z-GF (or hippuryl β -phenyllactic acid) or Z-GR (or hippuryl-L-arginine), respectively (Figure 8). Digestive insect carboxypeptidase A-like enzymes are widespread among insects, and most of them have pH optima

of 7.5–9.0 and molecular masses of 20–50 kDa (Terra and Ferreira, 1994). They have been cloned and sequenced from Diptera (Ramos *et al.*, 1993; Edwards *et al.*, 1997) and Lepidoptera (Bown *et al.*, 1998), and the enzyme from the lepidopteran *H. armigera* was also submitted to crystallographic studies (Estébanez-Perpiña *et al.*, 2001). The sequences have signal and activation peptides, and the features typical of carboxypeptidase As, including the residues His 69, Glu 72, and His 196, which bind the catalytic zinc ion; Arg 71, Asn 144, Arg 145, and Tyr 248, responsible for substrate binding; and Arg 127 and Glu 270, responsible for catalysis. In spite of the overall similarity of *H. armigera* procarboxypeptidase with human procarboxypeptidase A2, there are differences in the loops between the conserved secondary structures, including the loop where the activation processing occurs. Another important difference is the residue 255 (bottom of the S'1 pocket) that defines the enzyme specificity. In mammalian sequences, Asp 255 is found in carboxypeptidase B and Ile 255 in carboxypeptidase A. In insect carboxypeptidase As, this residue varies (but never is an acid residue) (Figure 5).

Carboxypeptidase B-like enzymes have been detected in insect midguts (Terra and Ferreira, 1994), and the enzyme from *H. zea* (CPBz) has been described in 3D detail (Bayés *et al.*, 2005). CPBhz has Glu 255 in the specificity pocket and differs from other carboxypeptidase Bs in some loops, which renders it insensitive to the potato carboxypeptidase inhibitor. Furthermore, the enzyme is unable to hydrolyze substrates ending in Arg because of its bulky size, whereas it cleaves quite well substrates ending in Lys (Bayés *et al.*, 2005).

Dipeptidases hydrolyze dipeptides, and are classified according to their substrate specificities. Dipeptidases comprise the least known of the insect peptide hydrolases. There have been few studies in which dipeptidase assays were performed, and even fewer attempts to characterize the enzymes (Terra and Ferreira, 1994). The larval midgut of *R. americana* has three dipeptidases (two soluble, of 63 kDa and 73 kDa, respectively, and one membrane-bound) that hydrolyze Gly-Leu, resembling dipeptide hydrolase (dipeptidase, E.C. 3.4.13.18), although, in contrast to the mammalian enzyme, they are very active upon Pro-Gly (Figure 8). *R. americana* also seems to have an amino acyl-histidine dipeptidase (carnosinase, EC 3.4.13.3) (Klinkowstrom *et al.*, 1995). More work on insect digestive dipeptidases is urgently needed.

11.6. Digestion of Lipids and Phosphates

11.6.1. Overview

Lipids that contain fatty acids comprise storage lipids and membrane lipids. Storage lipids, such as oils present in seeds, and fats in adipose tissue of animals, are

triacylglycerols (triglycerides), and are hydrolyzed by lipases. Membrane lipids include phospholipids and glycolipids, such as mono- and digalactosyldiglycerides (see section 11.4.7). Phospholipids are digested by phospholipases. A combination of α - and β -galactosidases may remove galactose residues from mono- and digalactosyldiglyceride to leave a diacylglycerol which may be hydrolyzed by a triacylglycerol lipase.

Phosphate moieties need to be removed from phosphorylated compounds prior to absorption. This is accomplished by non-specific phosphatases. The phosphatases may be active in an alkaline (alkaline phosphatase, EC 3.1.3.1) or acid (acid phosphatase, EC 3.1.3.2) medium.

11.6.2. Lipases

Triacylglycerol lipases (EC 3.1.1.3) are enzymes that preferentially hydrolyze the outer links of triacylglycerols and act only on the water-lipid interface. Activity of the lipase is increased as the interface becomes larger due to lipid emulsification caused by emulsifiers (surfactants). Insects lack emulsifiers comparable to the bile salts of vertebrates, but surfactant phospholipids, including lysolecithin, occur in their midguts in sufficient concentration to alter the surface tension of midgut contents (De Veau and Schultz, 1992). Lysolecithin, and other surfactants, may be formed by the action of phospholipase A on ingested phospholipids (see below, and Figure 2).

Insect midgut triacylglycerol lipases have been studied in few insects, and only in crude preparations. The data suggest that the enzyme preferentially releases fatty acids from the α -positions, prefers unsaturated fatty acids, and is activated by calcium ions, thus resembling the action of mammalian pancreatic lipase. The resulting 2-monoacylglycerol may be absorbed or hydrolyzed (Terra *et al.*, 1996). Hydrolysis of 2-monoacylglycerol may be accomplished by triacylglycerol lipase, following migration of the fatty acid to the 1-position, which seems to be favored by the alkaline midgut pH, at least in *M. sexta* (Terra *et al.*, 1996).

Current research on insect midgut lipases is focused on classifying the different enzymes in the families already described for mammals. Thus, a large group of proteins is identified as neutral (or pancreatic) lipase. They have the catalytic triad (Ser 152, Asp 176, His 263, human pancreatic lipase numbering) and two important features that are associated with triacylglycerol hydrolysis; the β 9 loop and lid, which cover the active site and are implicated in substrate recognition (Horne *et al.*, 2009). The C-terminal domain is absent in insect neutral lipases, which is consistent with the lack of a colipase, the protein associated with the vertebrate pancreatic lipases. Also differing from the pancreatic lipases, insect neutral lipases may have the lid structure severely reduced and the β 9 loop partially deleted, resembling phospholipases (Christeller

et al., 2010). In accordance with this, the major triacylglycerol lipase from *Manduca sexta* fat body is also an active phospholipase A1 (Arrese *et al.*, 2006).

Another large group of lipases correspond to the acid (gastric) lipases. They have conserved catalytic triad and lid similar to those of the neutral lipases (Horne *et al.*, 2009). Alignment of the sequences of insect midgut lipases with those of mammalian lipases, for which substrate specificity information is available, suggests that the lepidopteran neutral lipases are galactolipases and phospholipases *in vivo*, whereas the lepidopteran acid lipases are triacylglycerol lipases (Christeller *et al.*, 2010).

Insect midgut lipases may have a primary role in the acquisition of dietary lipid, but may also have strong antiviral activity by disrupting viral envelopes, as observed in *B. mori* larvae (Ponnuvel *et al.*, 2003).

Esterases, which are usually named the carboxylesterases (ali-esterases, EC 3.1.1.1) catalyze the hydrolysis of carboxyl ester into alcohol and carboxylate. This enzyme, in contrast to lipases, attacks molecules that are completely dissolved in water. It also hydrolyzes water-insoluble long-chain fatty acid esters in the presence of surfactants, but at a rate much slower than that of triacylglycerol lipase. A role for esterases in digestion is unclear, and because of this they are not reviewed in detail here.

In spite of the fact that a requirement for essential fatty acids is probably universal in insects, progress has been limited in the study of lipid digestion. Presumably, the lack of comparatively simple, sensitive assays, and the complexities of digestion related to lipid solubilization, have hindered work in this area. Another reason to study enzymes associated with lipid digestion is that they might be important in limiting the development of pathogens and parasites. Hydrolysis of membrane lipids might cause cellular lysis, and fatty acid products of digestion may possess antibiotic effects.

11.6.3. Phospholipases

Phospholipase A2 (EC 3.1.1.4) and phospholipase A1 (EC 3.1.1.32) remove the fatty acids from phosphatides attached to the 2- and 1-positions, respectively, resulting in a lysophosphatide (Figure 2). Lysophosphatide is more stable in micellar aggregates than on membranes. Thus, the action of phospholipase A on the membrane phosphatides causes the solubilization of cell membranes, rendering the cell contents free to be acted upon by the appropriate digestive enzymes. Phospholipase is widespread among insects (Terra *et al.*, 1996). Phospholipase A2 partially purified from the midgut of adult beetle *Circindella circumscripta* has a molecular mass of 22 kDa and pH optimum 9.0, is calcium dependent, and is inhibited by the site-specific inhibitor oleyoxyethyl phosphorylcholine. Unfed beetles did not express the phospholipase in the midgut contents (Uscian *et al.*, 1995).

Since no ESTs characteristic of classical phospholipases have been reported from lepidopteran midgut, it is likely the phospholipase A₂ activities that have been characterized from the midgut and salivary gland of *M. sexta* (Rana and Stanley, 1999; Tunaz and Stanley, 2004) are actually neutral lipases.

Although lysophosphatide may be further hydrolyzed by a lysophospholipase (phospholipase B, EC 3.1.1.5), evidence suggests it is absorbed intact by insects (Terra *et al.*, 1996). Phosphatides may also be hydrolyzed by phospholipase C (EC 3.1.4.3), yielding the phosphoryl base moiety and diacylglycerol, or by phospholipase D (EC 3.1.4.4), resulting in phosphatide and the base (Figure 2). Both enzymes have been found in insect midgut (Terra *et al.*, 1996), but have not been studied in detail.

11.6.4. Phosphatases

Alkaline phosphatase is usually a midgut microvillar membrane marker in dipteran and lepidopteran species, although it may also occur in midgut basolateral membranes, and even as a secretory enzyme. Acid phosphatase is usually soluble in the cytosol of midgut cells in many insects, and may also appear in midgut contents or be found membrane-bound in midgut cells (Terra and Ferreira, 1994).

The best-known alkaline phosphatases are those from *B. mori* (Lepidoptera: Bombycidae) larval midgut. The major membrane-bound and the minor soluble alkaline phosphatases were purified and shown to be monomeric enzymes with the following properties: (1) soluble enzyme, molecular mass of 61 kDa, pH optimum 9.8; (2) membrane-bound enzyme, molecular mass of 58 kDa, pH optimum 10.9. Both enzymes have wide substrate specificity and are inhibited by cysteine. The membrane-bound alkaline phosphatase occurs in the microvillar membranes of columnar cells, whereas the soluble enzyme is loosely attached to the goblet cell apical membrane facing the cell cavity (Eguchi, 1995). The determination of the complete sequence of the membrane-bound alkaline phosphatase led to the finding of putative regions for phosphatidylinositol anchoring and zinc-binding site, but not for N-glycosylation, despite the fact that the enzyme contains N-linked oligosaccharides (Itoh *et al.*, 1991). The sequence of the soluble alkaline phosphatase was also determined, and has high identity with the membrane-bound enzyme (Itoh *et al.*, 1999).

Acid phosphatases have been characterized in some detail only in *Rhodnius prolixus* (Hemiptera: Reduviidae). The major enzyme activity is soluble, and has the following properties: wide specificity, a molecular mass of 82 kDa, K_m for p-nitrophenyl phosphate 0.7 mM, and is inhibited by fluoride, tartrate, and molybdate. The minor enzyme activity is membrane-bound, and is resolved into

two enzymes (123 and 164 kDa) that are resistant to fluoride and tartrate (Terra *et al.*, 1988).

11.7. Microvillar Membranes

11.7.1. Isolation, Chemistry, and Enzymology

Midgut cells are associated with one another by junctions that separate the plasma cell membranes into an apical and a basolateral domain. The apical domain is usually modified into finger-like projections, the microvilli. The insect midgut cell microvillus is homologous to that described in vertebrates and reviewed by Bement and Mooseker (1996). Thus, a bundle of parallel actin filaments cross-linked by actin-bundling proteins like fimbrin and villin form the core of a microvillus. Lateral side arms (composed of myosin I and calmodulin) connect the sides of the actin bundle to the overlying plasma membrane.

It has been known for a long time that insect midgut cell apices are involved in the transport of water (Wigglesworth, 1933) and organic compounds (Treherne, 1959). Nevertheless, only since 1980 has it been recognized that insect midgut apical cell membrane plays a role in digestive events. Before 1980 all insect digestive enzymes were considered to be secreted into the lumen, as with mammals, where, in 1961, Miller and Crane had provided cell fractionation data showing that disaccharidases are firmly bound to cell membrane covering the enterocyte microvilli.

Insect midgut microvilli were isolated for the first time by Ferreira and Terra (1980) from *R. americana* (a lower dipteran) larvae using a differential calcium (magnesium) precipitation technique (Schmitz *et al.*, 1973) developed for mammals. Digestive enzymes performing final digestion were found associated with microvilli. A few months later, Hanozet *et al.* (1980) used the same technique to isolate microvilli from the columnar (principal) cell of the midgut of lepidopteran larvae, in order to study *in vitro* amino acid transport. After this paper, and complementary data (Terra *et al.*, 2006), differential precipitation became the method of choice to prepare microvilli from columnar cells of lepidopteran midguts. In addition to lower Diptera and Lepidoptera, differential precipitation has been used to isolate microvilli from midgut cells from other insect taxa, such as Dictyoptera (Parenti *et al.* 1986), Coleoptera (Ferreira *et al.*, 1990; Reuveni *et al.*, 1983), and higher Diptera (Lemos and Terra, 1992).

Microvilli prepared by differential precipitation methods are free from contaminants from other cells (such as goblet cells in the case of lepidopterans), but still contain most of the microvillus skeleton. A successful procedure was developed for cytoskeleton removal from insect midgut microvilli by treatment with hyperosmotic Tris, followed by pelleting the purified membranes with negligible amounts of cytoskeleton and slight contamination

by basolateral membranes (Coleoptera and Diptera, see Jordão *et al.*, 1995; Lepidoptera, see Capella *et al.*, 1997).

Insect midgut microvillar membrane densities vary widely, with insects appearing later in evolution (more derived insects) having denser membranes (and hence a higher protein content) than insects appearing earlier in evolution (less derived insects) (Terra *et al.*, 2006). Although higher protein content does not necessarily mean a richer variety of proteins, there is evidence supporting this. SDS-PAGE of midgut microvillar proteins of a coleopteran (membrane density 1.08–1.10) resolves fewer clearly visible bands than in the case of a lepidopteran or a dipteran (membrane density 1.14–1.16) (Jordão *et al.*, 1995; Capella *et al.*, 1997). The observed range of protein–lipid mass ratio of insect microvillar membranes is 1.41–3.13, which is wider than that found among mammalian enterocytes (Terra *et al.*, 2006).

Apparently there is an inverse relationship between the protein–lipid mass ratio (or membrane density) and the cholesterol and carbohydrate content in insect microvillar membranes. Thus, protein–lipid mass ratio, carbohydrate ($\mu\text{g}/\text{mg}$ protein) and cholesterol ($\mu\text{g}/\text{mg}$ protein) contents are, respectively: 1.4–1.7, 400–700, and 110–140 for Coleoptera; 2.0–2.6, 240–410, and 40–59 for higher Diptera; and 2.6–3.8, 0–80, 17–28 for Lepidoptera (Jordão *et al.*, 1995; Capella *et al.*, 1997). A detailed study of the chemical composition of microvilli (microvillar membranes plus contaminant cytoskeleton) from *B. mori* midgut cells (Leonardi *et al.*, 2001) confirms previous data. Thus, the protein–lipid ratio is smaller (1.85) in anterior plus middle in comparison to posterior midgut (2.3). Phospholipids account for 77% (phosphatides make up 62%) of total lipids, with glycolipids summing 8%.

11.7.2. Microvillar Proteins

The physiological role of midgut microvillar membranes may change along the midgut and among insect taxa, and should include surface (terminal) digestion, absorption, ion homeostasis, signaling, and unique digestive enzyme secretion mechanisms. At first, microvilli preparations revealed that microvillar integral digestive enzymes vary among different taxa. Most frequently, they are aminopeptidase, alkaline phosphatase, carboxypeptidase, dipeptidase, and α -glucosidase (Terra and Ferreira, 1994). Many of those proteins have been characterized as detailed before in the corresponding headings.

To date, the comprehensive studies of microvillar proteins are performed with microvilli preparations according to two approaches. The proteomics approach is based on the resolution of the microvillar proteins and mass spectrometry for identification. The resolution of the proteins usually uses two-dimensional gel electrophoresis (2-D PAGE), with electrofocusing in the first-dimension, followed by SDS-PAGE in the second dimension

(Candas *et al.*, 2003; McNall and Adang, 2003; Krishnamoorthy *et al.*, 2007; Bayyareddy *et al.*, 2009). Another variant of the proteomics approach is the shotgun method of Popova-Butler and Dean (2009). In this method, a microvilli preparation is subjected to trypsin and chymotrypsin digestion. The combined digesta is then submitted to two-dimensional liquid chromatography coupled with tandem spectrometry. Pauchet *et al.* (2009b) used two different separation procedures: separation of microvillar Triton X-100 (or digitonin)-solubilized microvilli proteins by anion-exchange chromatography or native electrophoresis in one dimension, followed by SDS-PAGE in the second dimension.

The immunoscreening approach to identify microvillar proteins is based on immunoscreening a midgut-specific cDNA library using antibodies raised against cytoskeleton-free microvillar proteins, followed by sequencing the positive clones and searching for similarities in databases (Ferreira *et al.*, 2007). The proteomics approach is limited by solubility problems affecting many proteins, by occasional failures of protein bands in originating useful mass spectra, and by the quality of peptide mass fingerprints obtained when the sequences of the specific organism under study are not abundant in the databases (frequent among insects). Furthermore, all the reports based on the proteomic approach use microvilli preparations instead of cytoskeleton-free microvillar membranes, resulting in the recovery of proteins deriving from cytoskeleton, mitochondria, and cytosol among the microvillar proteins. The immunoscreening method also has some possible sources of errors: (1) undetected proteins because of a lack of reacting antibodies caused by extremely low amounts of antigens, or because they were not immunogenic enough; (2) contaminants detected because they are highly immunogenic; (3) non-microvillar proteins detected because they share epitopes or were accidentally associated with microvillar proteins; (4) failure of inserted-cDNA-phage expression.

In spite of the limitations discussed above, both methods allowed the characterization of a substantial number of midgut microvillar proteins of different taxa. Thus, three groups of predicted microvillar proteins were recognized in *T. molitor* (Coleoptera) (Ferreira *et al.*, 2007): digestive enzymes (aminopeptidase and α -mannosidase), putative peritrophic membrane ancillary protein (PMAP)-like proteins; and peritrophic membrane proteins (peritrophins). The aminopeptidase corresponds to that described previously (Cristofaletti and Terra, 1999, 2000). PMAP may be involved in PM formation, and is homologous to proteins having insect-allergen repeat-related domains (Ferreira *et al.*, 2008) (see section 11.8.1).

Lepidopteran midgut microvillar proteins are grouped into six classes (McNall and Adang, 2003; Ferreira *et al.*, 2007; Krishnamoorthy *et al.*, 2007; Pauchet *et al.*, 2009b): (1) digestive enzymes (aminopeptidases with GPI-anchors,

carboxypeptidase, alkaline phosphatase, astacin-like protein, dipeptidyl peptidase A, maltase-like protein; (2) midgut protection (thioredoxin peroxidase; protein disulfide isomerase; aldehyde dehydrogenase, serpin); (3) peritrophic membrane formation (peritrophins); (4) membrane-tight-bound cytoskeleton proteins (fimbrin, actin, afadin, desmocollin, cadherin-like proteins); (5) proteins associated with microapocrine secretion (annexin, calmodulin, gelsolin); and (6) others (V-ATPase, chlorophyllide A binding protein, ABC transporters). Microapocrine secretion is discussed in [section 11.10](#). V-ATPase is thought to be a contamination by goblet cells.

Larval mosquito midgut microvillar proteins fall into four categories ([Bayyareddy et al., 2009](#); [Popova-Butler and Dean, 2009](#)): (1) digestive enzymes (alkaline phosphatase, trypsin, serine proteinase, zinc-metalloprotease, α -amylase); (2) midgut protection (protein disulfide isomerase, aldehyde dehydrogenase, peroxiredoxin); (3) membrane-tightly-bound cytoskeleton proteins (fotillins, prohibitin, actin); and (4) others (V-ATPase, calmodulin).

Lepidopteran and dipteran midgut microvillar membranes are denser and show a greater variety of proteins than those of coleopterans, because there are a host of microvillar proteins in lepidopterans and dipterans that assist the larvae in dealing quickly with huge amounts of food derived from a variety of plants. There are proteins involved in counteracting plant chemical defenses, in protecting the midgut surface against the larval serine proteinases, and in promoting peculiar secretory mechanisms. In addition to those proteins, there are still others that lepidopterans and dipterans share with coleopterans; namely, those forming the peritrophic membrane, a few digestive enzymes, and those not found among predicted proteins, like receptors and ion and organic compound transporters. Coleopterans attack a variety of plants, but they usually deal with their food more slowly than lepidopterans and dipterans. Perhaps because of this, their digestive physiology strategy seems to rely less on midgut microvillar proteins than is the case in lepidopterans and dipterans.

11.8. The Peritrophic Membrane

11.8.1. The Origin, Structure, and Formation of the Peritrophic Membrane

There is a film surrounding the food bolus in most insects that is occasionally fluid (peritrophic gel), but is more frequently membranous (peritrophic membrane, PM). The PM is made up of a matrix of proteins (mainly peritrophins) and chitin to which other components (e.g., enzymes, food molecules) may associate. This anatomical structure is sometimes called the peritrophic matrix, but this term should be avoided because it does not convey the idea of a film and rather suggests that it is the

fundamental substance of some structure, usually filling a space, as in the mitochondrial matrix. The argument that “membrane” means a lipid bilayer is not valid, because the PM is not a cell part but an anatomical structure, like the nictitating membrane of birds and reptiles.

PM proteins have been classified by [Tellam et al. \(1999\)](#) according to the ease with which they can be extracted: class 1 proteins can be removed with physiological buffers, class 2 with mild detergents, and class 3 with strong denaturants (e.g., urea), while class 4 proteins are the remaining residue. Class 1 proteins are thought to be digestive enzymes and food proteins loosely adsorbed at the PM surface. Class 2 proteins, at least in lepidopteran PMs, may correspond to proteins enclosed in membrane vesicles entrapped between PM sheets. These membrane vesicles bud off from the cell microvilli ([Ferreira et al., 1994](#); [Bolognesi et al., 2001](#); see also 11.8.2.2.7). The numerous hydrolases found in the PM proteomes ([Campbell et al., 2008](#); [Dinglasan et al., 2009](#)) may correspond to those entrapped enzymes. The same might be true for the so-called “GNBP-like” proteins (β -glucan binding proteins) ([Campbell et al., 2008](#)), which might correspond to active laminarinases (see 11.4.3.2).

Class 3 proteins are the integral proteins of PM. Class 4 proteins are likely the same as those in class 3, since [Campbell et al. \(2008\)](#) were able to completely solubilise PM with anhydrous trifluoromethanesulfonic acid, and found the same proteins previously described as class 3. The major class 3 proteins are named peritrophins, are characterized by the presence of chitin-binding domains (CBDs) (called peritrophin, pfam 01607, CBM_14) (CDART database) ([Geer et al., 2002](#)), and may also have mucin-like domains ([Tellam et al., 1999](#)).

Peritrophins are made of several domains. The major domain (peritrophin A-domain) is a cysteine-rich domain with chitin-binding properties having the consensus sequence CX_{13–20}CX_{5–6}CX_{9–19}CX_{10–14}CX_{4–14}C (where X is any amino acid except cysteine), which includes several conserved aromatic amino acids. Variations of this chitin-binding domain are the peritrophin-B and peritrophin-C domains, with consensus sequences CX_{12–13}CX_{20–21}CX₁₀CX₁₂CX₂CX₈CX_{7–12}C and CX_{8–9}CX_{17–21}CX_{10–11}CX_{12–13}CX₁₁C, respectively. These variations may be absent from many insects, exemplified by the predicted sequences of *T. castaneum* proteins ([Jasrapuria et al., 2010](#)). The mucin-like domains occurring in peritrophins are proline/threonine-rich domains that are heavily glycosylated and similar to mucins ([Lang et al., 2007](#)). Peritrophins may have one (e.g., Cb-peritrophin-15 from *Lucilia cuprina*) to several (e.g., peritrophin-44 from *L. cuprina*) chitin-binding domains or chitin-binding domains with small (e.g., Ag-AperI from *Anopheles gambiae*) or very large mucin-like domains (e.g., IIM from *Trichoplusia ni*) ([Wang and Granados, 1997](#); [Shen and Jacobs-Lorena, 1998](#); [Tellam et al., 1999, 2003](#)).

The availability of genome data permitted the description of the complete sets of peritrophins of *Ae. aegypti* (65 peritrophins), *T. castaneum* (25 peritrophins), and *D. melanogaster* (65 peritrophins) (Venancio *et al.*, 2009). It is not necessary for all the peritrophins to be true components of PM. The presence of those peritrophins in PM needs to be experimentally confirmed in the light that peritrophin-like proteins may have functions other than to form PM (Jasrapuria *et al.*, 2010).

The 3D structure of PM is thought to result from chitin fibrils being interlocked with the chitin-binding domains of peritrophins. Mucin-like domains of peritrophins are thought to face the ectoperitrophic and endoperitrophic sides of the PM. As these domains are highly hydrated, they lubricate the surface of the PM, easing the movement of food inside the PM and of the ectoperitrophic fluid outside the PM. Furthermore, the glucan chains associated with peritrophin mucin-like domains may ensure high proteinase resistance to PM (see **Figure 9** in Schorderet *et al.*, 1998; **Figure 5** in Wang and Granados, 2001; **Figure 2** in Hegedus *et al.*, 2008).

The structure of peritrophins prompted Terra (2001) to develop a speculative model of the origin and evolution of the PM. As mucins have a very early origin among animals (confirmed by Lang *et al.*, 2007), Terra (2001) proposed that PM derived from the ancestral mucus. According to this hypothesis, the peritrophins, the major PM proteins, evolved from mucins by acquiring chitin-binding domains. The concomitant evolution of chitin secretion by midgut cells permitted the formation of the chitin–protein network characteristic of the PM structure, described above. Later in evolution, some peritrophins lost their mucin-like domains. If the hypothesis that the PM is derived from the gastrointestinal mucus is correct, it should have originally been synthesized by midgut cells along the whole midgut, and should have had the properties of the mucus. Later in evolution, insect species would have appeared with a chitin–protein network, resulting in PM formation. Therefore, the formation of the PM by the whole midgut epithelium is the ancestral condition, whereas the restriction of PM production to midgut sections, or the lack of a PM and its replacement by the peritrophic gel, are derived conditions.

PMs are classified into two types (Peters, 1992). Type I PM is found in cockroaches (Dictyoptera), grasshoppers (Orthoptera), beetles (Coleoptera), bees, wasps, and ants (Hymenoptera), moths and butterflies (Lepidoptera), and in hematophagous adult mosquitoes (Diptera). Type II PM occurs in larval and adult (except hematophagous ones) mosquitoes and flies (Diptera), and in a few adult Lepidoptera.

Other PM integral proteins are chitin deacetylases (CDA) (Dixit *et al.*, 2008). This kind of protein was first found in the PM of *Trichoplusia ni* by Guo *et al.* (2005). The authors did not detect any chitin deacetylase activity,

and suggested that binding might be the only interaction of those proteins with chitin. The interaction was suggested by its extraction from PM with calcofluor. Taking into account that some CDA-like proteins found associated with PM are active in producing chitosan from chitin (e.g., in *Mamestra configurata* PM; Toprak *et al.*, 2008), Campbell *et al.* (2008) suggested several functions for chitosan: (1) it might be present to modify the flexibility of chitin fibers; (2) it may regulate redox condition, as it has antioxidant properties; (3) it may have antimicrobial properties; and, finally, (4) modification of chitin may be required for the binding of other proteins.

Type I PM is formed either by the whole midgut epithelium, or by part of it (anterior or posterior regions). The formation of these PMs is frequently induced by the distension of the gut caused by food ingestion. PM produced by the whole or anterior midgut epithelium envelops the food along the whole midgut. When PM is produced only by the posterior part, the anterior midgut epithelium is usually covered with a viscous material, the peritrophic gel, as observed in carabid beetles and bees. This gel is also observed in the anteriorly placed midgut ceca of some insects, and along the whole midgut of others (Terra, 2001).

During formation of type I PM, chitin is synthesized outside the cells by a chitin synthase bound to microvillar membranes using precursors formed inside the cells (Zimoch and Merzendorfer, 2002; Arakane *et al.*, 2005). Once chitin is self-organized in chitin fibers (Hegedus *et al.*, 2008), it interlocks with peritrophin molecules that are released by exocytosis (Bolognesi *et al.*, 2001). The micrographs of Harper and Hopkins (1997) show that, during the formation of Type I PM, a fibrous material appears first at the tips of the microvilli of anterior midgut cells and then is rapidly included in a thin PM surrounding the food bolus. Thus, the crucial events in PM formation appear to take place among microvilli.

Peritrophins are soluble proteins that are extensively immobilized at the surface of *T. molitor* cells due to cell glycocalyx association (Ferreira *et al.*, 2007). This arguably facilitates chitin–peritrophin association, and it is conceivable that ancillary proteins help this process. *T. molitor* PMAP may be one of those ancillary proteins. PMAP is homologous to proteins having insect allergen-related repeat domains like AEG12 (Shao *et al.*, 2005). The role of PMAP is based on circumstantial evidence: (1) PMAP is a soluble protein that, like peritrophins, occurs partially immobilized at the cell surface; and (2) PMAP is found associated with PM, but, in contrast with peritrophins, it seems to be removed from the PM as it moves along the midgut. A search of EST databanks led to the suggestion that PMAP-like proteins concur in the formation of type I, but not type II, PM. Nevertheless, lepidopterans do not have PMAP-like proteins, and may have other ancillary proteins.

Type II PM is secreted by a few rows of cells at the entrance of the midgut (cardia), and is usually found in insects irrespective of food ingestion. Peritrophins are secreted by exocytosis (Eisemann *et al.*, 2001).

A comparison of the complete sets of peritrophins from PM from larval *Ae. aegypti* and *D. melanogaster* (type II PM) showed they have more complex domain structures than from adult *Ae. aegypti* and *T. castaneum* (type I PM). Furthermore, mucin-like domains of peritrophins from *T. castaneum* (feeding on rough food) are lengthier than those of adult *Ae. aegypti* (blood-feeding). This suggested that type I and type II PMs may have variable architectures determined by different peritrophins and/or ancillary proteins, which may be partly modulated by diet (Venâncio *et al.*, 2009).

Although a PM is found in most insects, it does not occur in Hemiptera and Thysanoptera, which have perimicrovillar membranes in their cells (see below). The other insects that apparently do not have a PM are adult Lepidoptera, Phthiraptera, Psocoptera, Zoraptera, Strepsiptera, Raphidioptera, Megaloptera, adult Siphonaptera, bruchid beetles, and some adult ants (Hymenoptera) (Peters, 1992). These insects may have a peritrophic gel instead of PM, one example being bruchid beetles (Terra, 2001), or may have had their PMs overlooked because the insects were unfed.

Another possibility is that minute hematophagous insects (like Siphonaptera and Phthiraptera) have lost their PM because the blood clot ensures countercurrent flows (see section 11.8.2.2.3), and their small size makes for easy and efficient diffusion of digestion products up to the midgut surface.

The PM may have a wide range of pore sizes, with some being small or very large, but most of them in the middle range. The average pore sizes of PM may be determined by comparing molecular masses of enzymes restricted to the ectoperitrophic fluid (Figure 1) with those of enzymes present inside PM. This method of pore size estimation is probably the most accurate, since it reflects *in vivo* conditions. Pore sizes have also been determined by feeding insects with colloidal gold particles or fluorescent dextran molecules of known molecular masses, and recording their passage through the PM *in vivo*, or using PM mounted as a sac and measuring diffusing rates. Determinations performed with these techniques by different authors found pores in the range 7–9 nm for insects pertaining to different orders. Other authors described pores in the range 17–36 nm (Terra, 2001). Pore sizes in the range 17–36 nm were obtained with fluorescent dextran molecules in conditions able to detect very small amounts of substances traversing the PM. Those pores probably correspond to the large pores occurring at low frequency in PMs. Although these large pores are supposed to be of no importance regarding digestive events, they set the size limits for an infecting particle to successfully pass through the PM.

As a consequence of its small pores, the PM hinders the free movement of molecules, dividing the midgut lumen into two compartments (Figure 1) with different molecules. The functions of this structure include those of the mucus (protection against food abrasion and invasion by microorganisms), and several roles associated with the compartmentalization of the midgut. These roles result in improvements in digestive physiology efficiency, thereby leading to decreased digestive enzyme excretion, and restrict the production of the final products of digestion close to their transporters, thus facilitating absorption. These roles will be detailed below (see section 11.8.2).

Major points needing clarification are how the chemical nature of peritrophic gel and PM define their strength, elasticity, and porosity, and how these structures are self-assembled in the midgut lumen.

11.8.2. The Physiological Role of the Peritrophic Membrane

11.8.2.1. Protection against food abrasion and invasion by microorganisms. As mentioned before, gut cells in most animals are covered with a gel-like coating of mucus, which has been most thoroughly studied in mammals (Lang *et al.*, 2007). In these animals, the mucus is supposed to lubricate the mucosa, protecting it from mechanical damage, and to trap bacteria and parasites. Since the insect midgut epithelium lacks a mucus coating, PM functions were supposed to be analogous to that of mucus. Thus, insects deprived of PM may have the midgut cells damaged by coarse food, and may be liable to microorganism invasion (see Peters, 1992; Tellam, 1996; Lehane, 1997).

The PM as a barrier against invasion by microorganisms has particular relevance in insects that transmit viruses and parasites to human beings, as these microorganisms may have specific developmental phases in insect tissues (Tellam, 1996; Lehane, 1997). Microorganisms invade the insect midgut cells after disrupting the PM with the use of chitinase (Shahabuddin, 1995), or by using a proteinase such as enhancin that specifically affects the peritrophins (Peng *et al.*, 1999; Ivanova *et al.*, 2003).

A barrier against microorganism invasion is probably less important for the majority of insects that feed on plants, as exemplified by observations carried out with the moth *T. ni*. Larvae of this insect deprived of PM by Calcofluor treatment show high mortality. Examination of dead larvae showed no signs of microbial infection or cell damage by Calcofluor, although these larvae were more susceptible to experimental infection (Wang and Granados, 2000). The results may be interpreted as Calcofluor killing larvae by affecting PM functions in digestion.

Larvae deprived of PM by Calcofluor treatment show a five-fold decrease in the growth rate (GR) that cannot be explained by only a two-fold reduction in the consumption

rate (CR). The GR decrease should be a consequence of the increase in the metabolic costs associated with the conversion of food into body mass, rather than the reduction of consumption. One of the presumed causes of the increase in metabolic costs is the increase in digestive enzyme excretion (see section 11.8.2.2.3) (Bolognesi *et al.*, 2008).

Similarly, it has been observed that some plants respond to herbivorous insect attack by producing a unique 33-kDa cysteine proteinase with chitin-binding activity. This proteinase damages the PM, resulting in significant reduction in caterpillar growth caused by impaired nutrient utilization (Pechan *et al.*, 2002).

11.8.2.2. Enhancing digestive efficiency

11.8.2.2.1. Overview The proposal of roles for the PM in digestion has benefited from studies on the organization of the digestive process. These studies (for reviews, see Terra, 1990; Terra and Ferreira, 1994, 2009) revealed that in most insects, initial digestion occurs in the endoperitrophic space (Figure 10), intermediate digestion in the ectoperitrophic space, and final digestion at the surface of midgut cells. Such studies led to the formulation of the hypothesis of the endo–ectoperitrophic circulation of digestive enzymes. It was suggested that there is a recycling mechanism (Figure 10) whereby food flows inside the PM from the anterior midgut to the posterior, whereas in the ectoperitrophic space water flows from the posterior midgut to the ceca. When the polymeric food molecules become sufficiently small to pass through the PM (with the accompanying polymer hydrolases), the flow patterns result in carriage towards the ceca or the anterior midgut, where intermediate and final digestion occurs.

Terra and colleagues (for reviews, see Terra and Ferreira, 1994; Terra, 2001) hypothesized that, as a consequence of the compartmentalization of digestive events, there is an increase in the efficiency of digestion of polymeric food by allowing the removal of the oligomeric molecules from the endoperitrophic space, which is powered by the recycling mechanism associated with the midgut fluxes. Because oligomers may be substrates or inhibitors for some polymer hydrolases, their presence should decrease the rate of polymer degradation. Fast polymer degradation ensures that polymers are not excreted, and hence increases their digestibility. Another possible consequence of compartmentalization is an increase in the efficiency of oligomeric food hydrolysis due to the transference of oligomeric molecules to the ectoperitrophic space and restriction of oligomer hydrolases to this compartment. In these conditions, oligomer hydrolysis occurs in the absence of probable partial inhibition (because of non-productive binding) by polymer food and presumed non-specific binding by non-dispersed undigested food. This process should lead to the production of food monomers

in the vicinity of midgut cell surface, causing an increase in the concentration of the final products of digestion close to their transporters, thus facilitating absorption. Experimental evidence supporting the adaptations for increasing digestive efficiency proposals are discussed in the following sections.

11.8.2.2.2. Prevention of non-specific binding A model system was used to test the hypothesis that the PM prevents non-specific binding of undigested material onto midgut cell surface, with beneficial results (Terra, 2001). For this, purified microvillar membranes were isolated and added to peritrophic membrane contents. The activity of the microvillar enzymes decreased in these conditions, thus supporting the hypothesis (Bolognesi *et al.*, 2008).

11.8.2.2.3. Prevention of enzyme excretion This function was at first proposed based on results obtained with dipteran larvae (for reviews, see Terra and Ferreira, 1994; Terra, 2001). Both *R. americana* and *M. domestica* present a decreasing trypsin gradient along midgut contents (putatively generated by the recycling mechanism), and excreted less than 15% of midgut luminal trypsin after each gut emptying. The findings have now been extended to include Lepidoptera (Borhegyi *et al.*, 1999; Bolognesi *et al.*, 2001, 2008), Coleoptera (Ferreira *et al.*, 2002; Caldeira *et al.*, 2007), and Orthoptera (Biagio *et al.*, 2009). Furthermore, a theoretical model has been developed that is able to calculate the distribution of digestive enzymes along the midgut contents, given the site of water secretion and absorption, or to identify accurately the enzyme secretory site, given the other variables (Bolognesi *et al.*, 2008).

11.8.2.2.4. Increase in the efficiency of digestion of polymeric food favored by oligomer removal A model system was used to test this hypothesis (Bolognesi *et al.*, 2008). Midgut contents from *S. frugiperda* larvae were placed in dialysis bags suspended in stirred and unstirred media. Trypsin activities in stirred and unstirred bags were 210% and 160%, respectively, over the activities of similar samples maintained in a test tube (Bolognesi *et al.*, 2008).

11.8.2.2.5. Increase in the efficiency of oligomeric food hydrolysis The hypothesis that the efficiency of oligomer digestion (intermediate digestion) increases if separated from the initial digestion (Terra, 2001) was confirmed by the experiments of Bolognesi *et al.* (2008). These authors collected ectoperitrophic fluid from the large midgut ceca of *R. americana*, and assayed several digestive enzymes restricted to the fluid. When those enzymes were put in the presence of PM contents, their activities decreased in relation to controls. These decreases in activity probably result from oligomer hydrolase competitive inhibition by luminal polymers.

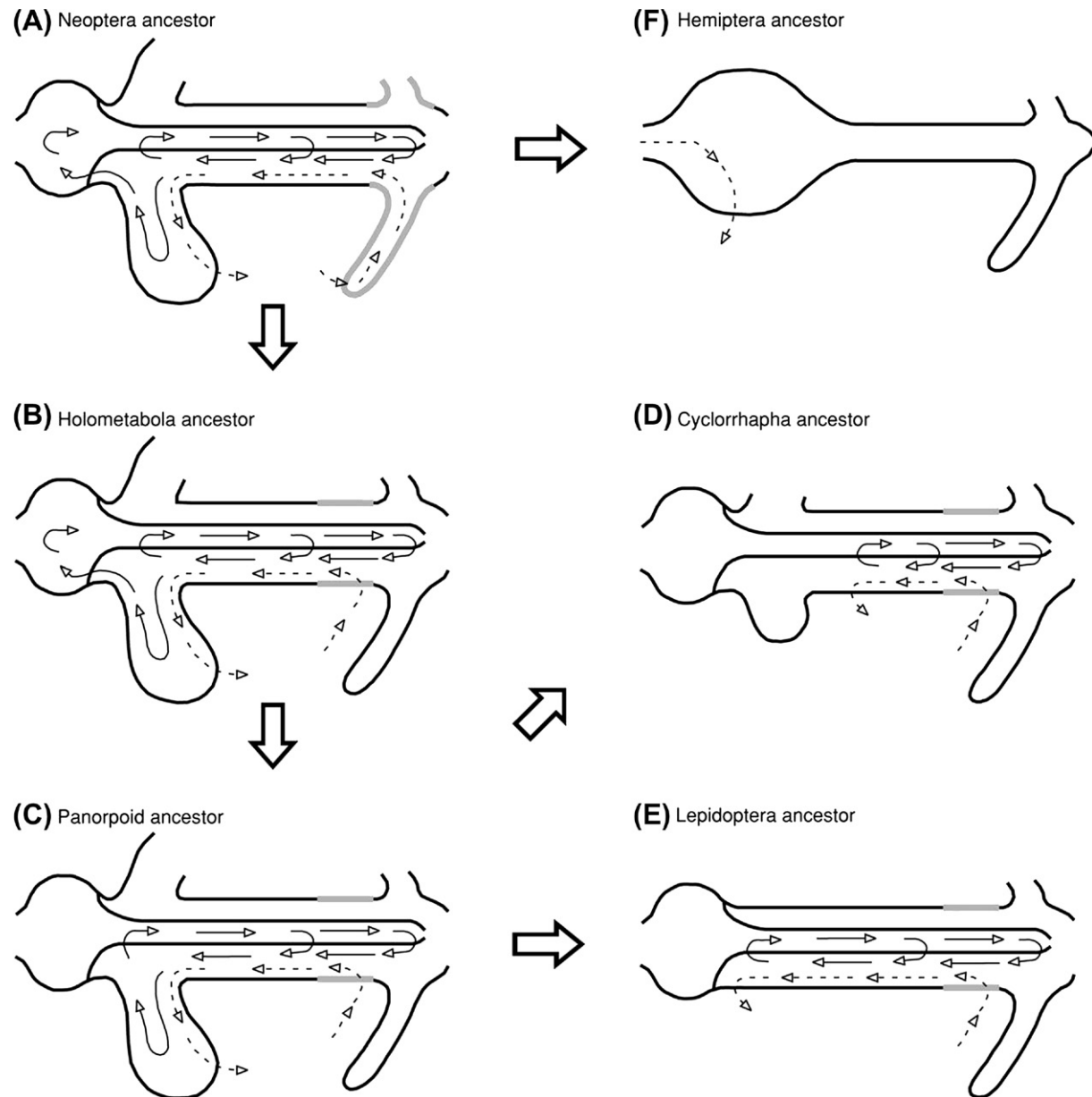


Figure 10 Diagrammatic representation of water fluxes (dotted arrows) and of the circulation of digestive enzymes (solid arrows) in putative insect ancestors that correspond to the major basic gut plans.

In Neoptera ancestors (A), midgut digestive enzymes pass into the crop. Countercurrent fluxes depend on the secretion of fluid by the Malpighian tubules and its absorption by the ceca. Enzymes involved in initial, intermediate, and final digestion circulate freely among gut compartments. Holometabola ancestors (B) are similar except that secretion of fluid occurs in posterior ventriculus. The ancestors of hymenopteran and panorpid (Lepidoptera and Diptera assemblage) insects (C) display countercurrent fluxes like Holometabola ancestors, midgut enzymes are not found in the crop, and only the enzymes involved in initial digestion pass through the peritrophic membrane. Enzymes involved in intermediate digestion are restricted to the ectoperitrophic space and those responsible for terminal digestion are immobilized at the surface of midgut cells. Cyclorrhapha ancestors (D) have a reduction in ceca, absorption of fluid in middle midgut, and anterior midgut playing a storage role. Lepidoptera ancestors (E) are similar to panorpid ancestors, except that the anterior midgut replaces the ceca in fluid absorption. Hemiptera ancestors (F) have lost crop, ceca, and fluid-secreting regions. Fluid is absorbed in anterior midgut. Reprinted with permission from Terra, W.R., Ferreira, C., 2009. Digestive system. In: Resh, V.H., Cardé, R.T. (eds), *Encyclopedia of Insects*, 2nd edn. Academic Press, San Diego, CA, pp. 273–281. ©Elsevier.

11.8.2.2.6. Restriction of food monomer production at the cell surface

This is a consequence of restricting oligomer hydrolases to the ectoperitrophic space (see section 11.8.2.2.5), and causes an increase in the concentration of the final products of digestion close to the carriers responsible for their absorption. A model system should be developed to test this hypothesis.

11.8.2.2.7. Enzyme immobilization Midgut luminal enzymes, in addition to occurring in the endoperitrophic and ectoperitrophic spaces, may be associated with the PM. For example, results obtained with *S. frugiperda* larvae showed that PM may contain up to 13% and 18% of the midgut luminal activity of amylase and trypsin, respectively (Ferreira *et al.*, 1994). Hence, enzyme immobilization may play a role in digestion, although a minor one. The attachment mechanism of enzymes in PM is not well known. Nevertheless, there is evidence, at least in *S. frugiperda*, that trypsin, amylase, and microvillar enzymes are incorporated into the jelly-like substance associated with PM when the enzymes, still bound to membranes, are released from midgut cells by a microapocrine process (Jordão *et al.*, 1999; Bolognesi *et al.*, 2001).

11.8.2.2.8. Toxin binding Although potentially toxic dietary tannins are attached to and excreted with *Schistocerca gregaria* PM (Bernays and Chamberlain, 1980), toxin binding by the PM seems to be a less widespread phenomenon than previously suggested. Thus, tannins in *M. sexta* (Barbehenn and Martin, 1998) and lipophilic and amphiphilic noxious substances in *Melanoplus sanguinipes* (Barbehenn, 1999) are maintained in the endoperitrophic space because they form high molecular weight complexes, not because of PM binding. However there is evidence that PM may bind heme in blood-feeding insects (Devenport *et al.*, 2006).

11.8.2.2.9. Peritrophic membrane functions and insect phylogeny Current data detailed below suggest that PMs of all insects have functions (see sections 11.8.2.1, 11.8.2.2.2–11.8.2.2.4), whereas functions (see sections 11.8.2.2.5 and 11.8.2.2.6) are demonstrable only in PMs of Panorpodea (the taxon that includes Diptera and Lepidoptera) and of the hymenopterans sawflies. PM function (see section 11.8.2.2.7) may occur in all insects, but this needs further confirmation. Function (see section 11.8.2.2.8), although it may be important for some insects, should be viewed as opportunistic. In other words, the PM probably evolved from a protective role (see section 11.8.2.1) to more sophisticated functions (see sections 11.8.2.2.2–11.8.2.2.7) under selective pressures and, due to the chemical properties of their constituents, the PM also developed the ability to bind different compounds including toxins.

11.9. Organization of the Digestive Process

11.9.1. Evolutionary Trends of Insect Digestive Systems

After studying the spatial organization of the digestive events in insects of different taxa and diets, it was realized that insects may be grouped relative to their digestive physiology, assuming they have common ancestors. Those putative ancestors correspond to basic gut plans from which groups of insects may have evolved by adapting to different diets (Terra and Ferreira, 1994, 2009).

The basic plan of digestive physiology for most winged insects (Neoptera ancestors) is summarized in Figure 10. In these ancestors, the major part of digestion is carried out in the crop by digestive enzymes propelled by antiperistalsis forward from the midgut. Saliva plays a variable role in carbohydrate digestion. Shortly after ingestion, the crop contracts, transferring digestive enzymes and partly digested food into the ventriculus. The anterior ventriculus is acid and has high carbohydrase activity, whereas the posterior ventriculus is alkaline and has high proteinase activity. This differentiation along the midgut may be an adaptation to the instability of ancestral carbohydrases in the presence of proteinases. The food bolus moves backward in the midgut of the insect by peristalsis. As soon as the polymeric food molecules are digested and become sufficiently small to pass through the peritrophic membrane, they diffuse with the digestive enzymes into the ectoperitrophic space (Figure 1). The enzymes and nutrients are then displaced toward the ceca with a countercurrent flux caused by secretion of fluid at the Malpighian tubules and its absorption back by cells at the ceca (Figure 10), where final digestion is completed and nutrient absorption occurs. When the insect starts a new meal, the ceca contents are moved into the crop. As a consequence of the countercurrent flux, digestive enzymes occur at a decreasing gradient in the midgut, and their excretion is lowered.

The Neoptera basic plan gave origin to that of the Polyneoptera orders, which include Dictyoptera (cockroaches, termites and mantids), Orthoptera and Phasmatodea, and evolved to the basic plans of Paraneoptera and Holometabola. The characteristics of the Paraneoptera ancestors cannot be inferred, because midgut function data are available only for Hemiptera.

The basic gut plan of the Holometabola (Figure 10B) (which include Coleoptera, Megaloptera, Hymenoptera, Diptera, and Lepidoptera) is similar to that of Neoptera, except that fluid secretion occurs by the posterior ventriculus, instead of by the Malpighian tubules. Because the posterior midgut fluid does not contain wastes, as is the case for Malpighian tubular fluid, the accumulation of wastes in the ceca is decreased. Ceca loss probably further decreases the accumulation of noxious substances in

the midgut, which would be more serious in insects that have high relative food consumption rates, as is common among Holometabola.

The basic plan of Coleoptera did not evolve dramatically from the holometabolous ancestor, whereas the basic plan of the Hymenoptera, Diptera, and Lepidoptera ancestor (hymenopteran–panorpid ancestor, **Figure 10C**) presents important differences. Thus, hymenopteran–panorpid ancestors have countercurrent fluxes like holometabolous ancestors, but differ from them in the lack of crop digestion, midgut differentiation in luminal pH, and in which compartment is responsible for each phase of digestion. In holometabolous ancestors all phases of digestion occur in the endoperitrophic space (**Figure 1**), whereas in hymenopteran–panorpid ancestors only initial digestion occurs in that region. In the latter ancestors, intermediate digestion is carried out by free enzymes in the ectoperitrophic space, and final digestion occurs at the midgut cell surface by immobilized enzymes. The free digestive enzymes do not pass through the PM, because they are larger than the PM's pores. As a consequence of the compartmentalization of digestive events in hymenopteran and panorpid insects, there is an increase in the efficiency of digestion of polymeric food, as discussed previously.

The evolution of insect digestive systems summarized above and in **Figure 10** was proposed, as discussed above, from studies carried out in 12 species pertaining to 4 insect orders. To give further support to the hypothesis that the characteristics of gut function and morphology depend more on phylogeny than on diet, another approach was used. A total of 29 gut morphology and digestive physiology characteristics (e.g., luminal pH, ratio of gut section volumes, type of peritrophic membrane, presence of special gut cells, distribution of digestive enzymes along the gut, major proteinase) were identified in 23 species from 8 different insect orders. Making use of these characteristics, a cladogram was constructed, putting together the data from studied species (**Figure 11**). The data confirmed that the morphological and functional traits associated with the digestive system are more dependent on taxon than on dietary habits of the different insects (Dias, Vanin, Marques, and Terra, unpublished data). There are two insect species that do not apparently fit the model: *Anopheles* spp. and *Themos malaisei*. *Anopheles* spp. is an adult, whereas the other Diptera is larval. *T. malaisei* is an unexpected finding that will be discussed below (see section 11.9.2.7).

11.9.2. Digestion in the Major Insect Orders

The organization of the digestive process in the different insect orders has been reviewed several times (Terra, 1988, 1990; Terra and Ferreira, 1994, 2009). The following section is therefore an abridged version of those texts, highlighting new findings and trying to identify points that

deserve more research, especially in relation to molecular aspects. Only key references before 2000 are cited, and the reader will find more references in the above-mentioned reviews.

11.9.2.1. Dictyoptera Dictyoptera comprises two suborders: Blattaria (cockroaches) and Mantodea (mantids). 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 family (Termitidae) that is sister to that of the woodroach *Cryptocercus* (Cryptocercidae). The branch Cryptocercidae–Termitidae is a sister of Blattidae, forming Blattodea that is a sister of Blaberoidea (Blattellidae plus Blaberidae), which in addition to Polyphagoidea form the Blattaria. Cockroaches are usually omnivorous. It is thought that digestion in cockroaches occurs as described for the Neoptera ancestor (**Figure 10A**), except that part of the final digestion of proteins occurs on the surface of midgut cells (Terra and Ferreira, 1994). This was confirmed by the finding in *P. americana* that most trypsin, maltase, and amylase is found in the crop, whereas aminopeptidase predominates in the microvillar membranes of posterior midgut. There is a decreasing gradient of trypsin, maltase, and amylase along

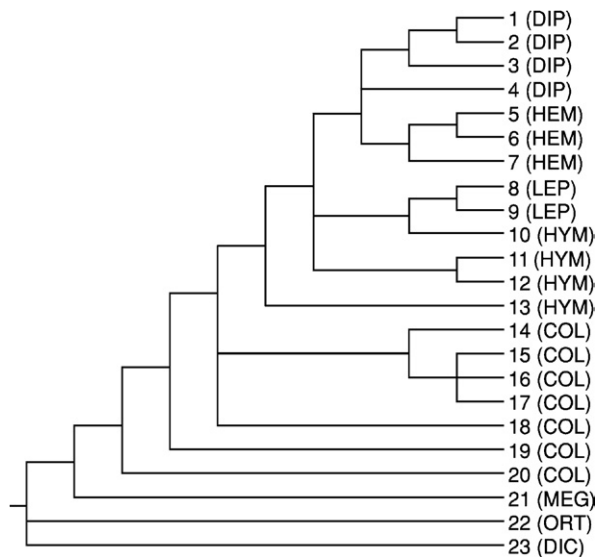


Figure 11 Cladogram of representative insects based on 29 gut morphology and digestive physiology characteristics. Insects: 1, *Trichosia pubescens*; 2, *Rhynchosciara americana*; 3, *Musca domestica*; 4, *Anopheles* spp.; 5, *Rhodnius prolixus*; 6, *Dysdercus peruvianus*; 7, *Acyrtosiphon pisum*; 8, *Erinnyis ello*; 9, *Spodoptera frugiperda*; 10, *Themos malaisei*; 11, *Camponotus rufipes*; 12, *Scaptotrigona bipunctata*; 13, *Bracon hebetor*; 14, *Tenebrio molitor*; 15, *Migdolus fryanus*; 16, *Sphenophorus levis*; 17, *Cyrtomon solana*; 18, *Dermestes maculatus*; 19, *Pyrearinus termitilluminans*; 20, *Pheropsophus aequinoctialis*; 21, *Corydalus* spp.; 22, *Abracris flavolineata*; 23, *Periplaneta americana*. Courtesy of A.B. Dias.

the midgut contents, and less than 5% of trypsin and maltase (amylase, 27%) is excreted during each midgut emptying. This suggests the existence of midgut digestive enzyme recycling, with amylase excretion increased probably due to excess dietary starch. The recycling mechanism is thought to be powered by water fluxes, as in the Neoptera ancestor, although there are no data supporting this. Major digestive proteinases are trypsin and chymotrypsin (Dias and Terra, unpublished data).

The differentiation of pH along the midgut (acid anterior midgut and alkaline posterior midgut) is not conserved among some cockroaches, like *P. americana*, (Blattidae) and *Blattella germanica* (Blattellidae), but is maintained in others, exemplified by the blaberid *Nauphoeta cinerea* (Elpidina *et al.*, 2001a; Vinokurov *et al.*, 2007). The organization of digestion in this cockroach seems similar to that in *P. americana*, although data on enzyme excretion are lacking. At least, blaberoid cockroaches possess proteinase inhibitory proteins active in the anterior midgut (Elpidina *et al.*, 2001b; Vinokurov *et al.*, 2007). These inhibitors are thought to be a primitive device to decrease the proteolytic inactivation of the animal's own carbohydrases, which are thus expected to be more active in the anterior midgut. The digestive carbohydrases from more evolved insects are stable in the presence of their own proteinases (Terra, 1988). Recently, several proteinase inhibitors have been partially purified from *N. cinerea* (Elpidina *et al.*, 2001b).

Another difference between cockroaches and the Neoptera ancestor is the enlargement of hindgut structures, noted mainly in wood-feeding cockroaches. These hindgut structures harbor bacteria producing acetate and butyrate from ingested wood or other cellulose-containing materials. Acetate and butyrate are absorbed by the hindgut of all cockroaches, but this is more remarkable with woodroaches (Terra and Ferreira, 1994). Cellulose digestion may be partly accomplished by bacteria in the hindgut of *P. americana* or protozoa in *Cryptocercus punctulatus*. Nevertheless, now it is clear that *P. americana* saliva contains two cellulases and three laminarinases that may open plant cells and lyse fungal cells (Genta *et al.*, 2003). This agrees with the omnivorous detritus-feeding habit of the insect. The woodroach *Panestria cribrata* also has its own cellulase (Scrivener *et al.*, 1989).

Termites may be seen as insects derived from woodroaches, and more adapted to dealing with refractory material such as wood and humus. Associated with this specialization, they lost the crop and midgut ceca, and enlarged their hindgut structures. Both lower and higher termites digest cellulose with their own cellulase, despite the occurrence of cellulose-producing protozoa in the paunch – an enlarged region of the anterior hindgut in lower termites. The products of cellulose digestion pass from the midgut into the hindgut, where they are converted into acetate and butyrate by hindgut bacteria, as

in woodroaches. Symbiotic bacteria are also responsible for nitrogen fixation in the hindgut, resulting in bacterial protein. This is incorporated into the termite body mass after being expelled in feces by one individual, and being ingested and digested by another. This explains the ability of termites to develop successfully on diets very poor in protein. Both lower- and higher-feeding termites seem to have an endo-ectoperitrophic circulation of digestive enzymes (Terra and Ferreira, 1994; Nakashima *et al.*, 2002; see also section 11.4.3.1).

11.9.2.2. Orthoptera Grasshoppers feed mainly on grasses, and their digestive physiology has clearly evolved from the neopteran ancestor. Carbohydrate digestion occurs mainly in the crop, under the action of midgut enzymes, whereas protein digestion and final carbohydrate digestion take place at the anterior midgut ceca. The abundant saliva (devoid of significant enzymes) produced by grasshoppers saturates the absorbing sites in the midgut ceca, thus hindering the countercurrent flux of fluid. This probably avoids excessive accumulation of noxious wastes, coming from Malpighian tubule secretion, in the ceca, and makes possible the high relative food consumption observed among locusts in their migratory phases. Starving grasshoppers present midgut countercurrent fluxes. Cellulase found in some grasshoppers is believed to facilitate the access of digestive enzymes to the plant cells ingested by the insects, by degrading the cellulose framework of cell walls (Dow, 1986; Terra and Ferreira, 1994; Marana *et al.*, 1997).

Crickets are omnivorous or predatory insects with initial starch digestion occurring in their capacious crop and ending in ceca lumina. Regarding protein, initial trypsin digestion occurs mostly in ceca lumina, whereas final aminopeptidase digestion takes place in ceca and ventriculus. The emptying of ceca in some crickets is propelled by peristalsis, whereas in others it depends on the relative pressure produced by proventriculus and ceca. Differing from grasshoppers, the final digestion of both protein and carbohydrates depends on membrane-bound enzymes in addition to soluble ones. Both starving and feeding crickets present midgut countercurrent fluxes (Woodring and Lorenz, 2007; Biagio *et al.*, 2009).

11.9.2.3. Phasmatodea The stick and leaf insects are remarkable mimics of the stems and leaves on which they feed (Grimaldi and Engel, 2005). The Phasmatodea ancestors derived from the Neoptera ancestors. The stick insect lacks ceca, which were replaced in *Phibalosoma phyllinum* (Phasmatidae) by the anterior midgut as the site of fluid absorption. Countercurrent flux of fluid resembles that in grasshoppers in being detected only in starving animals. Initial digestion of protein and carbohydrates takes place in the crop. The final digestion of proteins and carbohydrates occurs along the ventriculus, under

the action of a microvillar aminopeptidase and a soluble-glycocalyx-associated maltase, respectively (Monteiro, Tamaki, Terra, Ribeiro, unpublished data).

11.9.2.4. Hemiptera The Hemiptera comprise insects of the major suborders Auchenorrhyncha (cicadas, spittlebugs, leafhoppers, and planthoppers) and Sternorrhyncha (aphids and white flies), which feed almost exclusively on plant sap, and Heteroptera (e.g., assassin bugs, plant bugs, stink bugs, and lygaeid bugs), which are adapted to different diets.

The ancestor of the entire order Hemiptera is supposed to have been a cell-feeder similar to the present day phloem-feeder Auchenorrhyncha. Evolutionary shifts to phloem feeding or predation were not associated with a marked increase in body size. In contrast, xylem feeders are large to be able to extract xylem fluid from a host plant (Novotny and Wilson, 1997). Phloem and xylem sap have a very low protein content (with the exception of a few phloem saps; see below) and carbohydrate polymer content, and are relatively poor in free essential amino acids. In contrast to xylem sap, phloem sap is very rich in sucrose (Douglas, 2006). Thus, except for dimer (sucrose) hydrolysis, no food digestion is usually necessary in sap-suckers. Upon adapting to dilute phloem and/or xylem sap, hemipteran ancestors would lose the enzymes involved in initial and intermediate digestion, and lose the peritrophic membrane (Figure 10F). These changes are associated with the lack of luminal digestion.

The major problem facing a sap-sucking insect (especially on dilute phloem or xylem sap) is to absorb nutrients, such as essential amino acids, that are present in very low concentrations in sap. Whichever mechanism is used, xylem feeders may absorb as much as 99% of dietary amino acids and carbohydrate (Andersen *et al.*, 1989). Amino acids may be absorbed according to a hypothesized mechanism that depends on perimicrovillar membranes, which are membranes ensheathing the midgut microvilli with a dead end (Figure 12). A role in midgut amino acid absorption depends on the presence of amino acid-K⁺ symports on the surface of the perimicrovillar membranes, and of amino acid carriers and potassium pumps on the microvillar membranes. Although amino acid carriers have been found in the microvillar membranes of several insects (Wolfersberger, 2000), no attempts have been made to study the other postulated proteins. Thus, in spite of the model providing an explanation for the occurrence of these peculiar cell structures in Hemiptera, it is supported only by: (1) evidence that amino acids are absorbed with potassium ions in *D. peruvianus* (Silva and Terra, 1994); and (2) occurrence of particles studying the cytoplasmic face of the midgut microvillar membranes of *D. peruvianus*. These might be ion pumps responsible for the putative potassium ion transport, like similar structures in several epithelia (Silva *et al.*, 1995).

Another problem that deserves more attention regarding perimicrovillar membranes is their origin. Immunolocalization of the perimicrovillar enzyme marker, α -glucosidase, suggests that these membranes are formed when double membrane vesicles fuse their outer membranes with the microvillar membranes and their inner membranes with the perimicrovillar membranes. A double-membrane Golgi cisterna (on budding) forms the double-membrane vesicles (Silva *et al.*, 1995).

Organic compounds in xylem sap need to be concentrated before they can be absorbed by the perimicrovillar system. This occurs in the filter chamber of Cicadoidea and Cercopoidea, which concentrates xylem sap 10-fold. The filter chamber consists of a thin-walled, dilated anterior midgut in close contact with the posterior midgut and the proximal ends of the Malpighian tubules. This arrangement enables water to pass directly from the anterior midgut to the Malpighian tubules, concentrating food in the midgut and eliminating excess water. The high permeability of the filter chamber membrane to water results from the occurrence of specific channels formed by proteins named aquaporins. These were characterized as 15- to 26-kDa membrane proteins, and were immunolocalized in the filter chamber of several xylem sap feeders (Le Cahérec *et al.*, 1997).

Sternorrhyncha, as exemplified by aphids, may suck, more or less continuously, phloem sap of sucrose concentration up to 1.0 M and osmolarity up to three times that of the insect hemolymph. This results in a considerable hydrostatic pressure caused by the tendency of water to move from the hemolymph into midgut lumen. To withstand these high hydrostatic pressures, aphids have developed several adaptations. Midgut stretching resistance is helped by the existence of links between apical lamellae (replacing usual midgut cell microvilli) that become less conspicuous along the midgut. As a consequence of the links between the lamellae, the perimicrovillar membranes could no longer exist and were replaced by membranes seen associated with the tips of the lamellae, the modified perimicrovillar membranes (Ponsen, 1991; Cristofolletti *et al.*, 2003). A modified perimicrovillar membrane-associated α -glucosidase frees fructose from sucrose without increasing the osmolarity by promoting transglycosylations. As the fructose is quickly absorbed the osmolarity decreases, resulting in a honeydew iso-osmotic with hemolymph (Ashford *et al.*, 2000; Cristofolletti *et al.*, 2003). Another interesting adaptation is observed in whiteflies, where a trehalulose synthase forms trehalulose from sucrose, thus making available less substrate for an α -glucosidase that otherwise would increase the osmolarity of ingested fluid on hydrolyzing sucrose (Salvucci, 2003).

A cathepsin L (see section 11.5.3) bound to the modified perimicrovillar membranes of *A. pisum* (Cristofolletti *et al.*, 2003) may explain the capacity of some phloem sap feeders to rely on protein found in some phloem saps

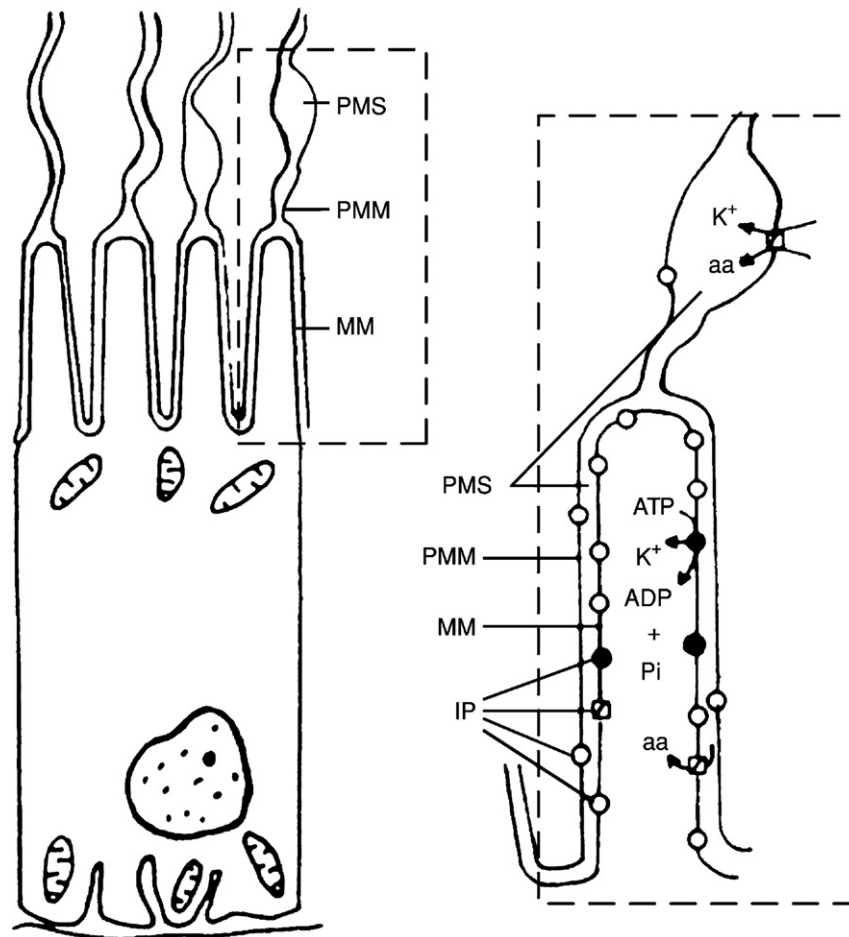


Figure 12 Model for the structure and physiological role of the microvillar border of midgut cells from Hemiptera. The left figure is a diagrammatic representation of a typical Hemiptera midgut cell, and the right figure details its apex. The microvillar membrane (MM) is ensheathed by the perimicrovillar membrane (PMM), which extends toward the luminal compartment with a dead end. The microvillar and perimicrovillar membranes delimit a closed compartment, i.e., the perimicrovillar space (PMS). The microvillar membrane is rich, and the perimicrovillar membrane poor, in integral proteins (IP). Microvillar membranes actively transport potassium ions (the most important ion in sap) from PMS into the midgut cells, generating a concentration gradient between the gut luminal sap and the PMS. This concentration gradient may be a driving force for the active absorption of organic compounds (amino acids, for example) by appropriate carriers present in the PMM. Organic compounds, once in the PMS, may diffuse up to specific carriers on the microvillar surface. This movement is probably enhanced by a transfer of water from midgut lumen to midgut cells, following (as solvation water) the transmembrane transport of compounds and ions by the putative carriers. Reprinted with permission from Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol. B* 109, 1–62; ©Elsevier.

(Salvucci *et al.*, 1998), and the failure of other authors to find an active proteinase in sap feeders. These other authors worked with homogenate supernatants or supernatants of Triton X-100-treated samples, under which conditions the cathepsin L would remain in the pellet.

An aminopeptidase, also bound to the modified perimicrovillar membranes, is the major binding site of the lectin Concanavalin A. On binding, the lectin impairs aphid development, in spite of the fact that the lectin does not affect aminopeptidase activity. It is thought that the aminopeptidase is located near the amino acid carriers responsible for amino acid absorption, and that these are inhibited when the lectin binds to the aminopeptidase (Cristofolletti *et al.*, 2006).

Amino acid absorption in *A. pisum* midguts is influenced by the presence of the bacteria *Buchnera* in the mycetocytes of the mycetomes occurring in the aphid hemocoel (Douglas, 2006). The molecular mechanisms underlying this phenomenon are not known, in spite of the fact that there is strong evidence showing that *Buchnera* uses the non-essential amino acids absorbed by the host in the synthesis of essential amino acids (Shigenobu *et al.*, 2000; Douglas, 2006).

The evolution of Heteroptera was associated with regaining the ability to digest polymers. Because the appropriate digestive enzymes were lost, they instead used enzymes derived from lysosomes. Lysosomes are cell organelles involved in intracellular digestion carried

out by special proteinases referred to as cathepsins. Compartmentalization of digestion was maintained by the perimicrovillar membranes, as a substitute for the absent peritrophic membrane. Digestion in the two major Heteroptera taxa, Cimicomorpha (exemplified by the blood-feeder *R. prolixus*), and Pentatomorpha (exemplified by the seed-sucker *D. peruvianus*), are similar. The dilated anterior midgut stores food and absorbs water, and, at least in *D. peruvianus*, also absorbs glucose, which is transported with the aid of a uniporter (GLUT) and a K⁺-glucose symporter (SGLT) (Bifano *et al.*, 2010). Digestion of proteins and absorption of amino acids occurs in the posterior ventriculus. Most protein digestion occurs in the lumen with the aid of a cysteine proteinase, and ends in the perimicrovillar space under the action of aminopeptidases and dipeptidases (Terra and Ferreira, 1994). Symbiotic bacteria may occur in blood-feeders, putatively to provide vitamins (see section 11.2.5). At least in *R. prolixus*, the neuroendocrine system has factors important for maintaining the ultrastructural organization of the midgut epithelial cells (Gonzales *et al.*, 1998).

11.9.2.5. Megaloptera The Megaloptera includes alderflies and dobsonflies, and is often considered to be the most primitive group of insects with complete metamorphosis. All their larvae are aquatic predators feeding on invertebrates (Theischinger, 1991). Megaloptera ancestors are like Holometabola ancestors, except for the anterior midgut ceca, which were lost and replaced in function by the anterior midgut. Thus, in *Corydalus* spp. larvae, most digestion occurs in the crop under the action of soluble amylase, maltase, aminopeptidase, and trypsin (major proteinase). Digestive enzyme recycling should occur, as less than 3.3% of midgut amylase, maltase, and aminopeptidase are lost at each midgut emptying. The higher excretory rate of trypsin (27%) probably results from excess dietary protein (Dias and Terra, unpublished data).

11.9.2.6. Coleoptera Coleoptera ancestors are like those of Megaloptera. Nevertheless, there are evolutionary trends leading to a great reduction or loss of the crop, and, as in the panorpoid orders, occurrence of at least final digestion of proteins at the surface of midgut cells. Thus, in predatory Carabidae most of the digestive phases occur in the crop by means of midgut enzymes, whereas in predatory larvae of Elateridae initial digestion occurs extra-orally by the action of enzymes regurgitated onto their prey. The preliquified material is then ingested by the larvae, and its digestion is finished at the surface of midgut cells (Terra and Ferreira, 1994).

Initial digestion of glycogen and proteins occurs in the dermestid larval endoperitrophic space, which is limited by a peritrophic gel in the anterior midgut and a peritrophic membrane in the posterior midgut. Final digestion takes place at the midgut cell surface, in the anterior

and posterior midgut in the case of glycogen and proteins, respectively. There is a decreasing gradient along the midgut of amylase and trypsin (major proteinase), suggesting the occurrence of digestive enzyme recycling (Terra and Ferreira, 1994; Caldeira *et al.*, 2007). Thus, dermestid beetles digest keratin with serine proteinases. Keratins are the major protein components of wool, hair, horn, and feathers, and have peptide chains cross-linked by disulfide bonds to give a very stable structure. These bonds are reduced only in a very reducing (−100 to −300 mV) environment, characteristic of the midgut of these insects (Vonk and Western, 1984).

Like dermestid beetles, the larva of *Sphenophorus levis* (Curculionidae) has a peritrophic gel and a peritrophic membrane in the anterior and posterior midgut, respectively, and a decreasing gradient of amylase, maltase, and proteinase (cathepsin L) along the midgut. A microvillar protein carries out the final digestion of proteins at the posterior midgut (Soares-Costa *et al.*, 2011). The spatial organization of digestion in the larvae of *Migdolus fryanus* (Cerambycidae) is similar to that of *S. levis*, except that their major digestive proteinase is trypsin (Dias and Terra, unpublished data). Tenebrionid larvae also have aminopeptidase as a microvillar enzyme, and the distribution of enzymes in gut regions of adults is similar to that in the larvae (Terra and Ferreira, 1994). This suggests that the overall pattern of digestion in larvae and adults of Coleoptera is similar, despite the fact that, in contrast to adults, beetle larvae usually lack a crop.

Insects of the series Cucujiformia (which includes Tenebrionidae, Chrysomelidae, Bruchidae, and Curculionidae) have cysteine proteinases (see section 11.5.3) in addition to (or in place of) serine proteinases as digestive enzymes, suggesting that the ancestors of the whole taxon were insects adapted to feed on seeds rich in serine proteinase inhibitors (Terra and Ferreira, 1994). The occurrence of trypsin as the major proteinase in *M. fryanus* (Dias and Terra, unpublished data) confirmed the preliminary work (Murdock *et al.*, 1987), according to which cerambycid larvae reacquired digestive serine proteinases.

Scarabaeidae and several related families are relatively isolated in the series Elateriformia, and evolved considerably from the Coleoptera ancestor. Scarabid larvae, exemplified by dung beetles, usually feed on cellulose materials undergoing degradation by a fungus-rich flora. Digestion occurs in the midgut, which has three rows of ceca, with a ventral groove between the middle and posterior row. The alkalinity of gut contents increases to almost pH 12 along the midgut ventral groove. This high pH probably enhances cellulose digestion, which occurs mainly in the hindgut fermentation chamber, through the action of endogenous and bacterial cell-bound enzymes. The final product of cellulose degradation is mainly acetic acid, which is absorbed through the hindgut wall (Terra and Ferreira, 1994; Huang *et al.*, 2010). There is controversy as

to whether scarabid larvae ingest feces to obtain nitrogen compounds, as described above for termites. Nevertheless, this is highly probable on the grounds that the microbial biomass in the fermentation chamber is incorporated into the larval biomass (Li and Brune, 2005).

Keratin beetles, as *Trox* sp. (Scarabaeoidea: Trogidae), digest keratin with serine proteinases (Hughes and Vogler, 2006) like dermestids.

11.9.2.7. Hymenoptera Organization of the digestive process is variable among hymenopterans, and to understand its peculiarities it is necessary to review briefly their evolution. The hymenopteran basal lineages are phytophagous as larvae, feeding both ecto- and endophytically, and include several superfamilies such as Xyeloidea and Tenthredinoidea, all known as sawflies. Close to these are the Siricoidea (wood wasps), which are adapted to ingest fungus-infected wood. Wood wasp-like ancestors gave rise to the Apocrita (wasp-waisted Hymenoptera), which are parasitoids of insects. They use their ovipositor to injure or kill their host, which represents a single meal for their complete development. A taxon sister of Ichneumonoidea in Apocrita gave rise to Aculeata (bees, ants, and wasps with thin waists) (Quicke, 2003).

The digestive systems of Hymenoptera ancestors are like the panorpoid ancestors (Figure 10C). However, there are evolutionary trends leading to the loss of midgut ceca (replaced in function by the anterior midgut) and changes in midgut enzyme compartmentalization. These trends appear to be associated with the development of parasitoid habits, and were maintained in Aculeata, as described below.

The sawfly *T. malaisei* (Tenthredinoidea: Argidae) larva has a midgut with a ring of anterior ceca that forms a U at the ventral side. Luminal pH is above 9.5 in the first two-thirds of the midgut. Trypsin (major proteinase) and amylase have a decreasing activity along the endoperitrophic space, suggesting enzyme recycling. Maltase predominates in the anterior midgut tissue as a soluble glycocalyx-associated enzyme, whereas aminopeptidase is a microvillar enzyme in the posterior midgut (Dias, Ribeiro, and Terra, unpublished data). These characteristics (except the presence of ceca) are similar to those of lepidopteran larvae (see section 11.9.2.10), and explain the fact that this insect is close to the lepidopterans in Figure 11. Otherwise, Aculeata, with their less sophisticated midgut (see below) branches closer to coleopterans (Figure 11).

Wood wasp larvae of the genus *Sirex* are believed to be able to digest and assimilate wood constituents by acquiring cellulase and xylanase, and possibly other enzymes, from fungi present in the wood on which they feed (Martin, 1987). The larvae of Apocrita present a midgut which is closed at its rear end, and which remains unconnected with the hindgut until the time of pupation. It is probable that this condition evolved as an adaptation of

endoparasitoid Apocrita ancestors to avoid the release of toxic compounds into the host in which they lived (Terra, 1988).

In larval bees, most digestion occurs in the endoperitrophic space. Countercurrent fluxes seem to occur, but there is no midgut luminal pH gradient. Adult bees ingest nectar and pollen. Sucrose from nectar is hydrolyzed in the crop by the action of a sucrase from the hypopharyngeal glands. After ingestion, pollen grains extrude their protoplasm in the ventriculus, where digestion occurs. As in larvae, there is also evidence of an endo-ectoperitrophic circulation of digestive enzymes (Terra and Ferreira, 1994).

Although many authors favor the view that pollen grains are digested in bees after their extrusion by osmotic shock, this subject is controversial not only in bees, but also among pollen-feeder beetles (Human and Nicholson, 2003).

Digestion in larval ants is similar to that in larval bees (Erthal et al., 2007), whereas worker ants feed on nectar, honeydew, and plant exudates, acquiring the necessary amino acids for growth with the aid of microsymbionts (Cook and Davidson, 2006). Worker ants may also feed on partly digested food regurgitated by their larva. Thus, they have frequently been said to lack digestive enzymes or display only those enzymes associated with intermediate and (or) final digestion (Terra and Ferreira, 1994). Although this seems true for leaf-cutting ants (Erthal et al., 2007), which appear to rely only on monosaccharides produced by fungal enzymes acting on plant polysaccharides (Silva et al., 2003), it is not widespread. Thus, adult *Camponotus rufipes* (Formicinae) have soluble amylase, trypsin (major proteinase), maltase, and aminopeptidase enclosed in a type I PM in their midguts. As only 14% of amylase and less than 7% of the other digestive enzymes are excreted during the midgut emptying, these insects may have a digestive enzyme recycling mechanism (Dias and Terra, unpublished data).

11.9.2.8. Diptera The Diptera evolved along two major lines: an assemblage (early Nematocera) of suborders corresponding to the mosquitoes, including the basal Diptera, and the suborder Brachycera, which includes the most evolved flies (Cyclorrhapha). The dipteran ancestor is similar to the panorpoid ancestor (Figure 10C) in having the enzymes involved in intermediate digestion free in the ectoperitrophic fluid (mainly in the large ceca), whereas the enzymes of terminal digestion are membrane bound at the midgut cell microvilli (Terra and Ferreira, 1994). These characteristics correspond to those of a nematoceran sciarid larvae, *R. americana* (Terra and Ferreira, 1994). As expected, the digestive process in larval mosquitoes follows a similar pattern, according to a microarray-based analysis of *A. gambiae* midgut transcripts (Oviedo et al., 2008).

Non-hematophagous adults store liquid food (nectar or decay products) in their crops. Digestion occurs in their midgut, as in larvae. Nectar ingested by mosquitoes (males and females) is stored in the crop, and is digested and absorbed at the anterior midgut. Blood, which is ingested only by females, passes to the posterior midgut, where it is digested and absorbed (Billingsley, 1990; Terra and Ferreira, 1994).

The adult *Ae. aegypti* midgut surface is covered, in large part, by tubular bilayers with a diameter four-fold smaller than the microvilli. They fuse and branch, forming bundles that seem to originate in the intercellular crypts and appear to be fused with the microvillar surface (Ziegler *et al.*, 2000). These structures are not related with the perimicrovillar membranes of Hemiptera. The latter envelop the microvilli before extending into the lumen in structures that may resemble the tubular membrane bilayers of *Ae. aegypti* (see section 11.9.2.4 and Figure 12). The puzzling structures of *Ae. aegypti* should be further studied to discover their relationships with digestion.

Gall midges (Cecidomyiidae) are interesting because they manipulate plant growth, arguably with small secretory proteins that lack matches in gene sequence databases (Zhang *et al.*, 2010).

The Cyclorrhapha ancestor (Figure 10D) evolved dramatically from the panorpoid ancestor (Figure 10C), apparently as a result of adaptations to a diet consisting mainly of bacteria. Digestive events in Cyclorrhapha larvae are exemplified by larvae of the housefly *M. domestica*. These ingest food rich in bacteria. In the anterior midgut there is a decrease in the starch content of the food bolus, facilitating bacterial death. The bolus now passes into the middle midgut, where bacteria are killed by the combined action of low pH, a special lysozyme (see section 11.4.5), and a cathepsin D-like proteinase (see section 11.5.4). Finally, the material released by bacteria is digested in the posterior midgut, as is observed in the whole midgut of insects of other taxa. Countercurrent fluxes occur in the posterior midgut, powered by secretion of fluid in the distal part of the posterior midgut and its absorption back into the middle midgut. The middle midgut has specialized cells for buffering the luminal contents in the acidic zone (Figure 4), in addition to those functioning in fluid absorption. Cyclorrhaphan adults, except for a few blood-suckers, feed mainly on liquids associated with decaying material (rich in bacteria), in a way similar to housefly *M. domestica* adults. These salivate (or regurgitate their crop contents) onto their food. After the dispersed material is ingested, starch digestion is accomplished primarily in the crop by the action of salivary amylase. Digestion follows in the midgut, essentially as described for larvae (Terra and Ferreira, 1994).

The stable fly *Stomoxys calcitrans* stores and concentrates the blood meal in the anterior midgut, and gradually passes it to the posterior midgut, where digestion takes place, resembling what occurs in larvae. These adults lack the characteristic cyclorrhaphan middle midgut and

the associated luminal low pH. Stable flies occasionally take nectar (Jordão *et al.*, 1996a).

11.9.2.9. Lepidoptera Lepidopteran ancestors (Figure 10E) differ from panorpoid ancestors because they lack midgut ceca, have all their digestive enzymes (except those of initial digestion) immobilized at the midgut cell surface, and present long-necked goblet cells and stalked goblet-cells in the anterior and posterior larval midgut regions, respectively. Goblet cells excrete K⁺ ions that are absorbed from leaves ingested by larvae. Goblet cells also seem to assist anterior columnar cells in water absorption, and posterior columnar cells in water secretion (Terra and Ferreira, 1994; Ortego *et al.*, 1996).

Although most lepidopteran larvae have a common pattern of digestion, species that feed on unique diets generally display some adaptations. *Tineola bisselliella* (Tineidae) larvae feed on wool, and display a highly reducing midgut for cleaving the disulfide bonds in keratin to facilitate proteolytic hydrolysis of this otherwise insoluble protein (Terra and Ferreira, 1994). Similar results were obtained with *Hofmannophila pseudospretella* (Christeller, 1996). Wax moths (*Galleria mellonella*) infest beehives, and digest and absorb wax. The participation of symbiotic bacteria in this process is controversial. Another adaptation has apparently occurred in lepidopteran adults, which feed solely on nectar. Digestion of nectar only requires the action of an α -glucosidase (or a β -fructosidase) to hydrolyze sucrose, the major component present. Many nectar-feeding lepidopteran adults have amylase in salivary glands, and several glycosidases and peptidases in the midgut (Terra and Ferreira, 1994).

Woods and Kingsolver (1999) developed a chemical reactor model of the caterpillar midgut, and used the model as a framework for generating hypotheses about the relationship between feeding responses to variable dietary proteins, and the physical and biochemical events in the midgut and body. They concluded that absorption (or post-absorptive processes) was limiting in a caterpillar maintained on artificial diets. Caterpillars eating leaves may not have the same limiting step, and this deserves a similar detailed study. Another interesting study would be the development of a model for the beetle midgut. This would determine whether beetles have digestion or consumption as the limiting step to compensate for their less sophisticated midguts.

11.10. Digestive Enzyme Secretion Mechanisms

Digestive enzyme secretory mechanisms and control are probably the least understood areas in insect digestion. Studies of secretory mechanisms have only described major differences, which seem to include unique aspects not seen in other animals.

Insects are continuous (e.g., Lepidoptera and Diptera larvae) or discontinuous (e.g., predators and many hematophagous insects) feeders. The synthesis and secretion of digestive enzymes in continuous feeders seem to be constitutive – that is, they occur continuously (at least between molts) – whereas in discontinuous feeders they are regulated (Lehane *et al.*, 1996). Digestive enzymes, as with all animal proteins, are synthesized in the rough endoplasmic reticulum and processed in the Golgi complex, and are packed into secretory vesicles (Figure 13). There are several mechanisms by which the contents of the secretory vesicles are freed in the midgut lumen. In holocrine secretion, secretory vesicles are stored in the cytoplasm until they are released, at which time the whole secretory cell is lost to the extracellular space. During exocytic secretion, secretory vesicles fuse with the midgut cell apical membrane, emptying their contents without any loss of cytoplasm (Figure 13A). In contrast, apocrine secretion involves the loss of at least 10% of the apical cytoplasm following the release of secretory vesicles (Figure 13B). These have previously undergone fusions, leading to larger vesicles that, after release, eventually free their contents by solubilization (Figure 13B). When the loss of cytoplasm is very small, the secretory mechanism is called microapocrine. Microapocrine secretion consists

in releasing budding double-membrane vesicles (Figure 13C) or, at least in insect midguts, pinched-off vesicles that may contain a single or several secretory vesicles (Figure 13D). In both cases, the secretory vesicle contents are released by membrane fusion and/or by membrane solubilization caused by high pH contents or by luminal detergents.

The secretory mechanisms of insect midgut cells reviewed below are based on immunocytochemical data, or on data combining biochemical procedures and electron micrographs. Studies based only on traditional cytology have been reviewed elsewhere (Terra and Ferreira, 1994; Lehane *et al.*, 1996).

Holocrine secretion is usually described on histological grounds mainly in midgut of insects other than higher Holometabola. These insects have large numbers of regenerative cells in their midguts. Thus, it is probable that cell renewal in these insects is being misinterpreted as holocrine secretion (Terra and Ferreira, 1994). In spite of this, immunocytochemical data showed that trypsin-containing vesicles along with cell organelles are discharged by opaque zone cells of adult stable flies, suggesting holocrine secretion (Jordão *et al.*, 1996a).

Exocytic, apocrine, and microapocrine secretory mechanisms depend largely on midgut regions. Digestive

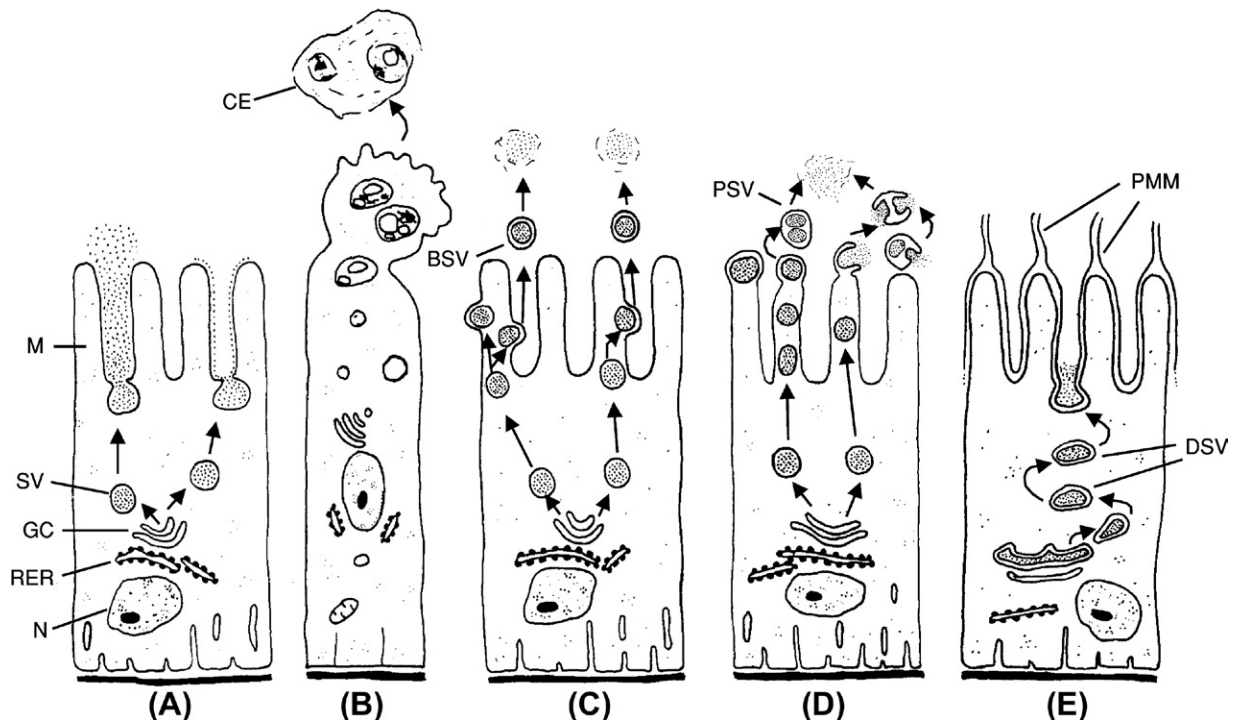


Figure 13 Models for secretory processes of insect digestive enzymes. (A) Exocytic secretion; (B) apocrine secretion; (C) microapocrine secretion with budding vesicles; (D) microapocrine secretion with pinched-off vesicles; (E) modified exocytic secretion in hemipteran midgut cell. BSV, budding secretory vesicle; CE, cellular extrusion; DSV, double-membrane secretory vesicle; GC, Golgi complex; M, microvilli; N, nucleus; PMM, perimicrovillar membrane; PSV, pinched-off secretory vesicle; RER, rough endoplasmic reticulum; SV, secretory vesicle. Reprinted with permission from Terra, W.R., Ferreira, C., 2009. Digestive system. In: Resh, V.H., Cardé, R.T. (eds.), *Encyclopedia of Insects*, 2nd edn. Academic Press, San Diego, CA, pp. 273–281; ©Elsevier.

enzymes are usually secreted by exocytosis in the posterior midgut, whereas alternate mechanisms may be observed in anterior midgut. Thus, trypsin is secreted by the posterior midgut of adult mosquitoes (Graf *et al.*, 1986), larval flies (Jordão *et al.*, 1996b), and caterpillars (Jordão *et al.*, 1999) by exocytosis, as well as β -glycosidase by *T. molitor* middle midguts (Ferreira *et al.*, 2002). Trypsin is secreted by the anterior midgut of caterpillars using a microapocrine route (Santos *et al.*, 1986; Jordão *et al.*, 1999), whereas in the anterior midgut of *T. molitor* amylase secretion occurs by an apocrine mechanism (Cristofolletti *et al.*, 2001). Based only on morphological evidence, it appears that, in addition to *E. ello* and *Spodoptera frugiperda*, microapocrine secretion occurs in other lepidopteran species, such as *Manduca sexta*, whereas apocrine secretion is observed in some Orthoptera and in many coleopteran species other than *T. molitor* (Terra and Ferreira, 1994).

Immunocytolocalization data (Silva *et al.*, 1995) showed that secretion by hemipteran midgut cells displays special features, as the cells have perimicrovillar membranes in addition to microvillar ones (Figure 13E). In this case, double-membrane vesicles bud from modified (double membrane) Golgi structures (Figure 13E). The double-membrane vesicles move to the cell apex, their outer membranes fuse with the microvillar membrane, and their inner membranes fuse with the perimicrovillar membranes, emptying their contents (Figure 13E).

The molecular mechanisms underlying the insect midgut secretory processes are unknown. Nevertheless, there is suggestive evidence involving calmodulin, annexin, and midgut-specific gelsolin in the unique microapocrine process (Ferreira *et al.*, 2007). This area of research deserves more effort, because it may provide insights regarding new insect control procedures.

11.11. Concluding Remarks

In spite of numerous gaps demanding further research, already indicated in this chapter, it is clear that insect digestive biochemistry is becoming a developed science and that its methods are powerful enough to lead to steady progress. It is conceivable that, in the next few decades, knowledge of the structural biology and function of digestive enzymes, and of the control of expression of alternate digestive enzymes and their secretory mechanisms, as well as on microvillar biochemistry, will support the development of more effective and specific methods of insect control. According to a Brazilian proverb, “*Quem viver, verá*” – “Whoever is alive, will see.”

Acknowledgments

Our work was supported by Brazilian research agencies Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) (Temático program) and CNPq. The authors

are staff members of the Biochemistry Department, research fellows of CNPq, and members of the INCT-Entomologia molecular.

References

- Alves, L. C., Almeida, P. C., Franzoni, L., Juliano, L., & Juliano, M. A. (1996). Synthesis of N- α -protected aminoacyl 7-amino-4-methyl-coumarin amide by phosphorus oxychloride and preparation of specific fluorogenic substrates for papain. *Peptide Res.*, 9, 92–96.
- Andersen, P. C., Brodbeck, B. V., & Mizell, R. F. (1989). Metabolism of aminoacids, organic acids and sugars extracted from the xylem fluid of four host plants by adult *Homalodisca coagulata*. *Entomol. Exp. Appl.*, 50, 149–159.
- Angelucci, C., Barrett-Wilt, G. A., Hunt, D. F., Akhurst, R. J., East, P. D., Gordon, K. H. J., & Campbell, P. M. (2008). Diversity of aminopeptidases derived from four lepidopteran gene duplications, and polycalins expressed in the midgut of *Helicoverpa armigera*: Identification of proteins binding the δ -endotoxin, Cry 1AC of *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol.*, 38, 685–696.
- Appel, H. M. (1994). The chewing herbivore gut lumen: Physicochemical conditions and their impact on plant nutrients, allelochemicals, and insect pathogens. In E. A. Bernays (Ed.), *Insect-Plant Interactions* (Vol. 5, pp. 203–223). Boca Raton, FL: CRC Press.
- Appel, H. M., & Martin, M. M. (1990). Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.*, 16, 3277–3290.
- Applebaum, S. W. (1985). Biochemistry of digestion. In G. A. Kerkut, & L. I. Gilbert (Eds.), *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* (Vol. 4, pp. 279–312). Oxford, UK: Pergamon.
- Arakane, Y., & Muthukrishnan, S. (2010). Insect chitinase and chitinase-like proteins. *Cell. Mol. Life Sci.*, 67, 201–216.
- Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S., & Kramer, K. J. (2003). Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem. Mol. Biol.*, 33, 631–648.
- Arakane, Y., Muthukrishnan, S., Kramer, K. J., Specht, C. A., Tomoyasu, Y., et al. (2005). The *Tribolium* chitin synthase genes TcCHS1 and TcCHS2 are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. *Insect Molec. Biol.*, 14, 453–463.
- Arakawa, G., Watanabe, H., Yamasaki, H. Y., Meekawa, H., & Tokuda, G. (2009). Purification and molecular cloning of xylanases from the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Biosci. Biotechnol. Biochem.*, 73, 710–718.
- Arrese, E. L., Patel, R. T., & Soulages, J. L. (2006). The main triglyceride-lipase from the insect fat body is an active phospholipase A1: Identification and characterization. *J. Lipid Res.*, 47, 2656–2667.
- Ashford, D. A., Smith, W. A., & Douglas, A. E. (2000). Living on a high sugar diet: The fate of sucrose ingested by a phloem-feeding insect, the pea aphid *Acyrtosiphon pisum*. *J. Insect Physiol.*, 46, 335–341.

- Azevedo, T. M., Terra, W. R., & Ferreira, C. (2003). Purification and characterization of three β -glycosidases from midgut of the sugar cane borer, *Diathraea saccharalis*. *Insect Biochem. Mol. Biol.*, 33, 81–92.
- Bacic, A., Harris, P. J., & Stone, B. A. (1988). Structure and function of plant cell walls. In P. K. Stumpf, & E. E. Conn (Eds.), *Biochemistry of Plants* (Vol. 14, pp. 297–371). New York, NY: Academic Press.
- Baker, J. E., & Woo, S. M. (1981). Properties and specificities of a digestive aminopeptidase from larvae of *Attageus megatoma* (Coleoptera: Dermestidae). *Comp. Biochem. Physiol. B*, 69, 189–193.
- Barbehenn, R. V. (1999). Non-absorption of ingested lipophilic and amphiphilic allelochemicals by generalist grasshoppers: The role of extractive ultrafiltration by the peritrophic envelope. *Arch. Insect Biochem. Physiol.*, 42, 130–137.
- Barbehenn, R. V., & Martin, M. M. (1998). Formation of insoluble and colloiddally dispersed tannic acid complexes in the midgut fluid of *Manduca sexta* (Lepidoptera: Sphingidae): An explanation for the failure of tannic acid to cross the peritrophic envelopes of Lepidopteran larvae. *Arch. Insect Biochem. Physiol.*, 39, 109–117.
- Barbehenn, R. V., Poopat, U., & Spencer, B. (2003). Semiquinone and ascorbyl radicals in the gut fluids of caterpillars with EPR spectrometry. *Insect Biochem. Mol. Biol.*, 33, 125–130.
- Barbehenn, R. V., Jaros, A., Lee, G., Mozola, C., Weir, Q., & Salminen, J. -P. (2009). Hydrolyzable tannins as “qualitative defences”: Limited impact against *Lymantria dispar* caterpillars on hybrid poplar. *J. Insect Physiol.*, 55, 297–304.
- Barrett, A. J., Rawlings, N. D., & Wasner, J. F. (1998). *Handbook of Proteolytic Enzymes*. London, UK: Academic Press.
- Bayés, A., Comellas-Bigler, M., de la Vega, M. R., Maskos, K., Bode, W., et al. (2005). Structural basis of the resistance of an insect carboxypeptidase to plant protease inhibitors. *Proc. Natl. Acad. Sci., USA*, 102, 16602–16607.
- Bayyareddy, K., Andacht, T. M., Abdullah, M. A., & Adang, M. J. (2009). Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes (Stegomyia) aegypti* Linnaeus (Diptera: Culicidae) larvae. *Insect Biochem. Mol. Biol.*, 39, 279–286.
- Bement, W. M., & Mooseker, M. S. (1996). The cytoskeleton of the intestinal epithelium: Components, assembly, and dynamic rearrangements. In J. E. Hesketh, & J. F. Pryme (Eds.), *The Cytoskeleton: A Multi-volume Treatise* (3, pp. 359–404). Greenwich, CT: JAI Press.
- Berenbaum, M. (1980). Adaptive significance of midgut pH in larval Lepidoptera. *Am. Natural.*, 115, 138–146.
- Bernays, E. A., & Chamberlain, D. J. (1980). A study of tolerance of ingested tannin in *Schistocerca gregaria*. *J. Insect Physiol.*, 26, 415–420.
- Bernays, E. A., Chamberlain, D. J., & Leather, E. M. (1981). Tolerance of acridids to ingested condensed tannin. *J. Chem. Ecol.*, 7, 247–256.
- Biagio, F. P., Tawaki, F. K., Terra, W. R., & Ribeiro, A. F. (2009). Digestive morphophysiology of *Gryllodes sigillatus* (Orthoptera: Gryllidae). *J. Insect Physiol.*, 55, 1125–1133.
- Bieth, J., Spiess, B., & Wermuth, C. G. (1974). The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem. Med.*, 11, 350–357.
- Bifano, T. D., Alegria, T. G.P., & Terra, W. R. (2010). Transporters involved in glucose absorption in the *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) anterior midgut. *Comp. Biochem. Physiol.*, 157 B, 1–9.
- Billingsley, P. F. (1990). The midgut ultrastructure of hemaphagous insects. *Annu. Rev. Entomol.*, 35, 219–248.
- Blake, J. D., Murphy, P. T., & Richards, G. N. (1971). Isolation and A/B classification of hemicelluloses. *Carbohydr. Res.*, 16, 49–57.
- Blanco-Labra, A., Martinez-Gallardo, N. A., Sandoval-Caroso, L., & Delano-Frier, J. (1996). Purification and characterization of a digestive cathepsin D proteinase isolated from *Tribolium castaneum* larvae (Herbst). *Insect Biochem. Mol. Biol.*, 26, 95–100.
- Bolognesi, R., Ribeiro, A. F., Terra, W. R., & Ferreira, C. (2001). The peritrophic membrane of *Spodoptera frugiperda*: Secretion of peritrophins and role in immobilization and recycling digestive enzymes. *Arch. Insect Biochem. Physiol.*, 47, 62–75.
- Bolognesi, R., Terra, W. R., & Ferreira, C. (2008). Peritrophic membrane role in enhancing digestive efficiency. Theoretical and experimental models. *J. Insect Physiol.*, 54, 1413–1422.
- Borhegyi, N. H., Molnár, K., Csikós, G., & Sass, M. (1999). Isolation and characterization of an apically sorted 41-kDa protein from the midgut of tobacco hornworm (*Manduca sexta*). *Cell Tissue Res.*, 297, 513–525.
- Botos, I., Meyer, E., Nguyen, M., Swanson, S. M., Koomen, J. M., et al. (2000). The structure of an insect chymotrypsin. *J. Mol. Biol.*, 298, 895–901.
- Bown, D. P., Wilkinson, H. S., & Gatehouse, J. A. (1997). Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. *Insect Biochem. Mol. Biol.*, 27, 625–638.
- Bown, D. P., Wilkinson, H. S., & Gatehouse, J. A. (1998). Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae: Enzyme characterization, cDNA cloning and expression. *Insect Biochem. Mol. Biol.*, 28, 739–749.
- Bozic, N., Ivanovic, J., Nenadovic, V., Berdstrom, J., Larsson, T., & Vujcic, Z. (2008). Purification and properties of a major leucyl aminopeptidase of *Morimus funereus* (Coleoptera: Cerambycidae) larvae. *Comp. Biochem. Physiol. B*, 149, 454–462.
- Bragatto, I., Genta, F. A., Ribeiro, A. F., Terra, W. R., & Ferreira, C. (2010). Characterization of a β -1,3-glucanase active in the alkaline midgut of *Spodoptera frugiperda* larvae and its relation to β -glucan-binding proteins. *Insect Biochem. Mol. Biol.*, 40, 861–872.
- Brioschi, D., Nadalini, L. D., Bengtson, M. H., Sogayar, M. C., Moura, D. S., & Silva-Filho, M. C. (2007). General upregulation of *Spodoptera frugiperda* trypsin and chymotrypsins allows its adaptation to soybean proteinase inhibitor. *Insect Biochem. Mol. Biol.*, 37, 1283–1290.
- Brito, L. O., Lopes, A. R., Parra, J. R.P., Terra, W. R., & Silva-Filho, M. C. (2001). Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitor may be mediated by the synthesis of new proteinases. *Comp. Biochem. Physiol. B*, 128, 365–375.

- Broadway, P. (1997). Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. *J. Insect Physiol.*, 43, 855–874.
- Brune, A. (1998). Termite guts: The world's smallest bioreactors. *Trends Biotechnol.*, 16, 16–21.
- Byeon, M. G., Lee, K. S., Gui, Z. Z., Kim, I., Kamg, P. D., et al. (2005). A digestive β -glucosidase from the silkworm, *Bombyx mori*: cDNA cloning, expression and enzymatic characterization. *Comp. Biochem. Physiol. B*, 418–427.
- Caldeira, W., Dias, A. B., Terra, W. R., & Ribeiro, A. F. (2007). Digestive enzyme compartmentalization and recycling and sites of absorption and secretion along the midgut of *Dermestes maculatus* (Coleoptera) larvae. *Arch. Insect Biochem. Physiol.*, 64, 1–18.
- Callegaert, L., & Michiels, C. W. (2010). Lysozymes in the animal kingdom. *J. Biosci.*, 35, 127–160.
- Campbell, B. C., Bragg, T. S., & Turner, C. E. (1992). Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16S ribosomal DNA. *Insect Biochem. Mol. Biol.*, 22, 415–421.
- Campbell, P. M., Cao, A. T., Hines, E. R., East, P. D., & Gordon, K. H.J. (2008). Proteomic analysis of the peritrophic matrix from the gut of the caterpillar, *Helicoverpa armigera*. *Insect Biochem. Mol. Biol.*, 38, 950–958.
- Cançado, F. C., Valério, A. A., Marana, S. R., & Barbosa, J. A. (2007). The crystal structure of a lysozyme c from housefly *Musca domestica*, the first structure of a digestive lysozyme. *J. Struct. Biol.*, 160, 83–92.
- Cançado, F. C., Chimoy Effio, P., Terra, W. R., & Marana, S. R. (2008). Cloning, purification and comparative characterization of two digestive lysozymes from *Musca domestica* larvae. *Braz. J. Med. Biol. Res.*, 41, 969–977.
- Cançado, F. C., Barbosa, J. A. G., & Marana, S. R. (2010). Role of the triad N46, S106 and T107 and the surface changes in the determination of the acidic pH optimum of the digestive lysozymes from *Musca domestica*. *Comp. Biochem. Physiol.*, 155B, 387–395.
- Candas, M., Loseva, O., Oppert, B., Kosaraju, P., & Bulla, L. A., Jr. (2003). Insect resistance to *Bacillus thuringiensis*: Alterations in the Indian-meal moth larval gut proteome. *Molec. Cell Proteomics.*, 2, 19–28.
- Capella, A. N., Terra, W. R., Ribeiro, A. F., & Ferreira, C. (1997). Cytoskeleton removal and characterization of the microvillar membranes isolated from two midgut regions of *Spodoptera frugiperda* (Lepidoptera). *Insect Biochem. Mol. Biol.*, 27, 793–801.
- Carneiro, C. N., Isejima, E. M., Samuels, R. I., & Silva, C. P. (2004). Sucrose hydrolases from the midgut of the sugarcane stalk borer *Diatraea saccharalis*. *J. Insect Physiol.*, 50, 1093–1101.
- Charlab, R., Valenzuela, J. G., Rowton, E. D., & Ribeiro, J. M. (1999). Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly *Lutzomyia longipalpis*. *Proc. Natl. Acad. Sci. USA*, 96, 15155–15160.
- Chen, J., Aimanova, K. G., Pan, S., & Gill, S. S. (2009). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem. Mol. Biol.*, 39, 688–696.
- Christeller, J. T. (1996). Degradation of wool by *Hofmannophila pseudospretella* (Lepidoptera: Oecophoridae) larval midgut extracts under conditions simulating the midgut environment. *Arch. Insect Biochem. Physiol.*, 33, 99–119.
- Christeller, J. T. (2005). Evolutionary mechanisms acting on proteinase inhibitor variability. *FEBS J.*, 272, 5710–5722.
- Christeller, J. T., Laing, W. A., Shaw, B. D., & Burgess, E. P.J. (1990). Characterization and partial purification of the digestive proteases of the black field cricket, *Teleogryllus commodus* (Walker): Elastase is a major component. *Insect Biochem.*, 20, 157–164.
- Christeller, J. T., Poulton, J., Markwick, N. M., & Simpson, R. M. (2010). The effect of diet on the expression of lipase genes in the midgut of the lightbrown apple moth (*Epiphyas postvittana* Walker; Tortricidae). *Insect Mol. Biol.*, 19, 9–25.
- Clark, T. M. (1999). Evolution and adaptive significance of larval midgut alkalization in the insect superorder Mecoptera. *J. Chem. Ecol.*, 25, 1945–1960.
- Colebatch, G., Cooper, P., & East, P. (2002). cDNA cloning of a salivary chymotrypsin-like protease and the identification of six additional cDNAs encoding putative digestive proteases from the green mirid, *Creontiades dilutus* (Hemiptera: Miridae). *Insect Biochem. Mol. Biol.*, 32, 1065–1075.
- Cook, S. C., & Davidson, D. W. (2006). Nutritional and functional biology of exudate-feeding ants. *Ent. Exp. Appl.*, 118, 1–10.
- Coutinho, P.M., Henrissat, B., 1999. Carbohydrate-active enzymes server. <http://www.afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>.
- Craik, C. S., Largman, C., Fletcher, T., Roczmia, S., Barr, P. J., et al. (1985). Redesigning trypsin: Alteration of substrate specificity. *Science*, 228, 291–297.
- Crava, C. M., Bel, Y., Lee, S. F., Manachini, B., Heckel, D. G., & Esviche, B. (2010). Study of the aminopeptidase N gene family in the lepidopterans *Ostrinia nubilalis* (Hubner) and *Bombyx mori* (L.): Sequence mapping and expression. *Insect Biochem. Mol. Biol.*, 40, 506–515.
- Cristofolletti, P. T., & Terra, W. R. (1999). Specificity, anchoring, and subsites in the active center of a microvillar aminopeptidase purified from *Tenebrio molitor* (Coleoptera) midgut cells. *Insect Biochem. Mol. Biol.*, 29, 807–819.
- Cristofolletti, P. T., & Terra, W. R. (2000). The role of amino acid residues in the active site of a midgut microvillar aminopeptidase from the beetle *Tenebrio molitor*. *Biochim. Biophys. Acta.*, 1479, 185–195.
- Cristofolletti, P. T., Ribeiro, A. F., & Terra, W. R. (2001). Apocrine secretion of amylase and exocytosis of trypsin along the midgut of *Tenebrio molitor*. *J. Insect Physiol.*, 47, 143–155.
- Cristofolletti, P. T., Ribeiro, A. F., Deraison, C., Rahbé, Y., & Terra, W. R. (2003). Midgut adaptation and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrtosiphon pisum*. *J. Insect Physiol.*, 49, 11–24.
- Cristofolletti, P. T., Ribeiro, A. F., & Terra, W. R. (2005). The cathepsin L-like proteinases from the midgut of *Tenebrio molitor* larvae: Sequence, properties, immunocytochemical localization and function. *Insect Biochem. Mol. Biol.*, 35, 883–901.

- Cristofolletti, P. T., Mendonça-de-Sousa, F. A., Rahbé, Y., & Terra, W. R. (2006). Characterization of a membrane-bound aminopeptidase purified from *Acyrtosiphon pisum* midgut cells. A major binding site for toxic mannose lectins. *FEBS J.*, 273, 5574–5588.
- Daffre, S., Kylsten, P., Samakovlis, C., & Hultmark, D. (1994). The lysozyme locus in *Drosophila melanogaster*: An expanded gene family adapted for expression in the digestive tract. *Mol. Gen. Genet.*, 242, 152–162.
- Daimon, T., Taguchi, T., Meng, Y., Katsuma, S., Mita, K., & Shimoda, T. (2008). Beta-fructofuranosidase genes of the silkworm, *Bombyx mori*: Insights into enzymatic adaptation of *B. mori* to toxic alkaloids in mulberry latex. *J. Biol. Chem.*, 283, 15271–15279.
- Da Lage, J. L., Von Wormhoudt, A., & Cariou, M. L. (2002). Diversity and evolution of the alpha-amylase genes in animals. *Biologia*, 57, 181–189.
- D'Amico, S., Gerday, C., & Feller, G. (2000). Structural similarities and evolutionary relationships in chloride-dependent α -amylases. *Gene*, 253, 95–105.
- Darboux, I., Nielsen-LeRoux, C., Charles, J. F., & Pauron, D. (2001). The receptor of *Bacillus sphaericus* binary toxin in *Culex pipiens* (Diptera: Culicidae) midgut: Molecular cloning and expression. *Insect Biochem. Mol. Biol.*, 31, 981–990.
- Davies, G. J., Wilson, K. S., & Henrisat, B. (1997). Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem. J.*, 321, 557–559.
- de Almeida, R. W., Tovar, F. J., Ferreira, I. I., & Leoncini, O. (2003). Chymotrypsin genes in the malaria mosquitoes, *Anopheles aquasalis* and *Anopheles darlingi*. *Insect Biochem. Mol. Biol.*, 33, 307–315.
- Deraison, C., Darboux, I., Duportets, L., Gorjankina, T., Rahbé, Y., & Jouanin, L. (2004). Cloning and characterization of a gut-specific cathepsin L from the aphid *Aphis gossypii*. *Insect Mol. Biol.*, 13, 165–177.
- De Veau, E. J. I., & Schultz, J. C. (1992). Reassessment of interaction between gut detergents and tannins in Lepidoptera and significance for gypsy moth larvae. *J. Chem. Ecol.*, 18, 1437–1453.
- Devenport, M., Alvarenga, P. H., Shao, L., Fujioka, H., Bianconi, M. L., Oliveira, P. L., & Jacobs-Lorena, M. (2006). Identification of the *Aedes aegypti* matrix protein AeIMUCI as a heme-binding protein. *Biochemistry*, 45, 9540–9549.
- Dey, P. M., & Pridham, J. B. (1972). Biochemistry of α -galactosidases. *Adv. Enzymol.*, 36, 91–130.
- Dillon, R. J., & Dillon, V. M. (2004). The insect gut bacteria: An overview. *Annu. Rev. Entomol.*, 49, 71–92.
- Dinglasan, R. R., Devenport, M., Florens, L., Johnson, J. R., McHugh, C. A., et al. (2009). The *Anopheles gambiae* adult midgut peritrophic matrix proteome. *Insect Biochem. Mol. Biol.*, 39, 125–134.
- Dixit, R., Arakane, Y., Specht, C. A., Richard, C., Kramer, K. J., et al. (2008). Domain organization and phylogenetic analysis of proteins from the chitin deacetylase gene family of *Tribolium castaneum* and three other species of insects. *Insect Biochem. Mol. Biol.*, 38, 440–451.
- Dootsdaar, H., McCollum, T. G., & Mayer, R. T. (1997). Purification and characterization of an endo-polygalacturonase from the gut of West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* L.) larvae. *Comp. Biochem. Physiol. B*, 118, 861–867.
- Douglas, A. E. (2006). Phloem-sap feeding by animals: Problems and solutions. *J. Exp. Bot.*, 57, 747–754.
- Dow, J. A. T. (1986). Insect midgut function. *Adv. Insect Physiol.*, 19, 187–328.
- Dow, J. A. T. (1992). pH gradients in lepidopteran midgut. *J. Exp. Biol.*, 172, 355–375.
- Dunn, B. M., Jimenez, M., Parten, B. F., Valler, M. J., Rolph, C. E., et al. (1986). A systematic series of synthetic chromophoric substrates for aspartic proteinases. *Biochem. J.*, 237, 899–906.
- Dunse, K. M., Kaas, Q., Guarini, R. F., Barton, P. A., Craik, D. J., & Anderson, M. A. (2010). Molecular basis for the resistance of an insect chymotrypsin to a potato type II proteinase inhibitor. *Proc. Natl. Acad. Sci. USA*, 107, 15016–15021.
- Edwards, M. J., Lemos, F. J. A., Donnelly-Doman, M., & Jacobs-Lorena, M. (1997). Rapid induction by a blood meal of a carboxypeptidase gene in the gut of the mosquito *Anopheles gambiae*. *Insect Biochem. Mol. Biol.*, 27, 1063–1072.
- Eguchi, M. (1995). Alkaline phosphatase isozymes in insects and comparison with mammalian enzyme. *Comp. Biochem. Physiol. B*, 111, 151–162.
- Eisemann, C., Wijffels, G., & Tellam, R. L. (2001). Secretion of type 2 peritrophic matrix protein, peritrophin-15, from the cardia. *Arch. Insect Biochem. Physiol.*, 472, 76–85.
- Elpidina, E. N., Vinokurov, K. S., Gromenko, V. A., Rudenskaya, Y. A., Dunaevsky, Y. E., et al. (2001a). Compartmentalization of proteinases and amylases in *Nauphoeta cinerea* midgut. *Arch. Insect Biochem. Physiol.*, 48, 206–216.
- Elpidina, E. N., Vinokurov, K. S., Rudenskaya, Y. A., Dunaevsky, Y. E., & Zhuzhikov, D. P. (2001b). Proteinase inhibitors in *Nauphoeta cinerea* midgut. *Arch. Insect Biochem. Physiol.*, 48, 217–222.
- Elpidina, E. N., Tsybina, T. A., Dunaevsky, Y. E., Belozersky, M. A., Zhuzhikov, D. P., & Oppert, B. (2005). A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. *Biochimie*, 87, 771–779.
- Erthal, M., Jr., Silva, C. P., & Samuels, R. I. (2007). Digestive enzymes in larvae of the leaf cutting ant, *Acromyrmex subterraneus* (Hymenoptera: Formicidae: Attinae). *J. Insect Physiol.*, 53, 1101–1111.
- Estébanez-Perpiña, E., Bayés, A., Vendrell, J., Jongsma, M. A., Bown, D. P., et al. (2001). Crystal structure of a novel midgut procarboxypeptidase from the cotton pest *Helicoverpa armigera*. *J. Mol. Biol.*, 313, 629–638.
- Ferreira, A. H. P., Marana, S. R., Terra, W. R., & Ferreira, C. (2001). Purification, molecular cloning, and properties of a β -glycosidase isolated from midgut lumen of *Tenebrio molitor* (Coleoptera) larvae. *Insect Biochem. Mol. Biol.*, 31, 1065–1076.
- Ferreira, A. H. P., Ribeiro, A. F., Terra, W. R., & Ferreira, C. (2002). Secretion of β -glycosidase by middle midgut cells and its recycling in the midgut of *Tenebrio molitor* larvae. *J. Insect Physiol.*, 48, 113–118.
- Ferreira, A. H. P., Terra, W. R., & Ferreira, C. (2003). Characterization of a β -glycosidase highly active on disaccharides and of a β -galactosidase from *Tenebrio molitor* midgut lumen. *Insect Biochem. Mol. Biol.*, 33, 253–265.
- Ferreira, A. H. P., Cristofolletti, P. T., Lorenzini, D. M., Guerra, L. O., Paiva, P. B., et al. (2007). Identification of midgut microvillar proteins from *Tenebrio molitor* and *Spodoptera frugiperda* by cDNA library screenings with antibodies. *J. Insect Physiol.*, 53, 1112–1124.

- Ferreira, A. H. P., Cristofolletti, P. T., Pimenta, D. C., Ribeiro, A. F., Terra, W. R., & Ferreira, C. (2008). Structure, processing and midgut secretion of putative peritrophic membrane ancillary protein (PMAP) from *Tenebrio molitor* larvae. *Insect Biochem. Mol. Biol.*, 38, 233–243.
- Ferreira, C., & Terra, W. R. (1980). Intracellular distribution of hydrolases in midgut caeca cells from an insect with emphasis on plasma membrane-bound enzymes. *Comp. Biochem. Physiol. B*, 66, 467–473.
- Ferreira, C., & Terra, W. R. (1984). Soluble aminopeptidases from cytosol and luminal contents of *Rhynchosciara americana* midgut caeca: Properties and phenanthroline inhibition. *Insect Biochem.*, 14, 145–150.
- Ferreira, C., & Terra, W. R. (1985). Minor aminopeptidases purified from the plasma membrane of midgut caeca cells of an insect (*Rhynchosciara americana*) larva. *Insect Biochem.*, 15, 619–625.
- Ferreira, C., & Terra, W. R. (1986a). The detergent form of the major aminopeptidase from the plasma membrane of midgut caeca cells of *Rhynchosciara americana* (Diptera) larva. *Comp. Biochem. Physiol. B*, 84, 373–376.
- Ferreira, C., & Terra, W. R. (1986b). Substrate specificity and binding loci for inhibitors in an aminopeptidase purified from the plasma membrane of midgut cells of an insect (*Rhynchosciara americana*) larva. *Arch. Biochem. Biophys.*, 244, 478–485.
- Ferreira, C., Bellinello, G. L., Ribeiro, A. F., & Terra, W. R. (1990). Digestive enzymes associated with the glycocalyx, microvillar membranes and secretory vesicles from midgut cells of *Tenebrio molitor* larvae. *Insect Biochem.*, 20, 839–847.
- Ferreira, C., Capella, A. N., Sitnik, R., & Terra, W. R. (1994). Digestive enzymes in midgut cells, endo- and ectoperitrophic contents and peritrophic membranes of *Spodoptera frugiperda* (Lepidoptera) larvae. *Arch. Insect Biochem. Physiol.*, 26, 299–313.
- Ferreira, C., Parra, J. R. P., & Terra, W. R. (1997). The effect of dietary plant glycosides on larval midgut β -glycosidases from *Spodoptera frugiperda* and *Diatraea saccharalis*. *Insect Biochem. Mol. Biol.*, 27, 55–59.
- Ferreira, C., Torres, B. B., & Terra, W. R. (1998). Substrate specificities of midgut β -glycosidases from insects of different orders. *Comp. Biochem. Physiol. B*, 119, 219–225.
- Ferreira, C., Marana, S. R., Silva, C., & Terra, W. R. (1999). Properties of digestive glycosidases and peptidases and the permeability of the peritrophic membranes of *Abracris flavolineata* (Orthoptera: Acrididae). *Comp. Biochem. Physiol. B*, 123, 241–250.
- Fonseca, F. V., Silva, J. R., Samuels, R. I., DaMatta, R. A., Terra, W. R., & Silva, C. P. (2010). Purification and partial characterization of a midgut membrane-bound α -glucosidase from *Quesada gigas* (Hemiptera: Cicadidae). *Comp. Biochem. Physiol.*, 155B, 20–25.
- Fox, L. R., & Macauley, B. J. (1977). Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia*, 29, 145–162.
- Franco, O. L., Rigden, D. J., Melo, F. R., & Grossi-de-Sa, M. F. (2002). Plant α -amylase inhibitors and their interaction with insect α -amylases: Structure, function and potential for crop protection. *Eur. J. Biochem.*, 269, 397–412.
- Fujita, A. (2004). Lysozymes in insects: What role do they play in nitrogen metabolism? *Physiol. Entomol.*, 29, 305–310.
- Fujita, A., Shimizu, I., & Abe, T. (2001). Distribution of lysozyme and protease, and amino acid concentration in guts of a wood-feeding termite, *Reticulitermes speratus* (Kolbe): Possible digestion of symbiont bacteria transferred by trophallaxis. *Physiol. Entomol.*, 26, 116–123.
- Fujita, A., Minamoto, T., Shimizu, I., & Abe, T. (2002). Molecular cloning of lysozyme-encoding cDNAs expressed in the salivary gland of a wood-feeding termite, *Reticulitermes speratus*. *Insect Biochem. Mol. Biol.*, 32, 1615–1624.
- Gaines, P. J., Sampson, C. M., Rushlow, K. E., & Stiegler, G. L. (1999). Cloning of a family of serine protease genes from the cat flea *Ctenocephalides felis*. *Insect Mol. Biol.*, 81, 11–22.
- Gatehouse, L. N., Shannon, A. L., Burgess, E. P. J., & Christeller, J. T. (1997). Characterization of major mid-gut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in response to four proteinase inhibitors in the diet. *Insect Biochem. Mol. Biol.*, 27, 929–944.
- Geer, L. Y., Domrachev, M., Lipman, D. J., & Bryant, S. H. (2002). CDART: Protein homology by domain architecture. *Genome Res.*, 12, 1619–1623.
- Genta, F. A., Terra, W. R., & Ferreira, C. (2003). Action pattern, specificity, lytic activities, and physiological role of five digestive β -glucanases isolated from *Periplaneta americana*. *Insect Biochem. Mol. Biol.*, 33, 1085–1097.
- Genta, F. A., Dillon, R. J., Terra, W. R., & Ferreira, C. (2006a). Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. *J. Insect Physiol.*, 52, 593–601.
- Genta, F. A., Blanes, L., Cristofolletti, P. T., do Lago, C. L., Terra, W. R., & Ferreira, C. (2006b). Purification, characterization and molecular cloning of the major chitinase from *Tenebrio molitor* larval midgut. *Insect Biochem. Mol. Biol.*, 36, 789–800.
- Genta, F. A., Dumont, A. F., Marana, S. R., Terra, W. R., & Ferreira, C. (2007). The interplay of processivity, substrate inhibition and a secondary substrate binding site of an insect exo-beta-1,3-glucanase. *Biochim. Biophys. Acta.*, 1774, 1079–1091.
- Genta, F. A., Bragatto, I., Terra, W. R., & Ferreira, C. (2009). Purification, characterization and sequencing of the major beta-1,3-glucanase from the midgut of *Tenebrio molitor* larvae. *Insect Biochem. Mol. Biol.*, 39, 861–874.
- Gibson, R. P., Gloster, T. M., Roberts, S., Warren, R. A., Storch de Gracia, I., Garcia, A., et al. (2007). Molecular basis for trehalase inhibition revealed by the structure of trehalase in complex with potent inhibitors. *Angew Chem. Int. Ed. Engl.*, 46, 4115–4119.
- Girard, C., & Jouanin, L. (1999a). Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol.*, 29, 1129–1142.
- Girard, G., & Jouanin, L. (1999b). Molecular cloning of a gut-specific chitinase cDNA from the beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol.*, 29, 549–556.
- Giri, A. P., Harsulkar, A. M., Deshpande, V. V., Sainani, M. N., Gupta, V. S., et al. (1998). Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. *Plant Physiol.*, 116, 393–401.

- Gonzales, M. S., Nogueira, N. F. S., Feder, D., de Souza, W., Azambuja, P., et al. (1998). Role of the head in the ultrastructural midgut organization in *Rhodnius prolixus* larvae: Evidence from head transplantation experiments and acdysone therapy. *J. Insect Physiol.*, 44, 553–560.
- Graf, R., Raikhel, A. S., Brown, M. R., Lea, A. O., & Briegel, H. (1986). Mosquito trypsin: Immunocytochemical localization in the midgut of blood-fed *Aedes aegypti* (L.). *Cell Tissue Res.*, 245, 19–27.
- Greenberg, B., & Paretsky, D. (1955). Proteolytic enzymes in the housefly, *Musca domestica* (L.). *Ann. Entomol. Soc. America*, 48, 46–50.
- Grigorten, J. L., Cawford, D. N., & Harvey, W. R. (1993). High pH in the ectoperitrophic space of the larval lepidopteran midgut. *J. Exp. Biol.*, 183, 353–359.
- Grimaldi, D., & Engel, M. S. (2005). *Evolution of Insects*. New York, NY: Cambridge University Press.
- Grossmann, G. A., & Terra, W. R. (2001). Alpha-galactosidases from the larval midgut of *Tenebrio molitor* (Coleoptera) and *Spodoptera frugiperda* (Lepidoptera). *Comp. Biochem. Physiol. B*, 128, 109–122.
- Grossmann, G. L., Campos, Y., Senerson, D. W., & James, A. A. (1997). Evidence for two distinct members of the amylase gene family in the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.*, 27, 769–781.
- Guo, W., Li, G., Pang, Y., & Wang, P. (2005). A novel chitin-binding protein identification from the peritrophic membrane of the cabbage looper, *Trichoplusia ni*. *Insect Biochem. Mol. Biol.*, 35, 1224–1234.
- Halanych, K. M. (2004). The new view of animal phylogeny. *Ann. Rev. Ecol. Evol. Syst.*, 35, 229–256.
- Hanozet, G. M., Giordana, B., & Sacchi, J. F. (1980). K⁺-dependent phenylalanine uptake in membrane vesicles isolated from the midgut of *Philosamia cyntia*. *Biochim. Biophys. Acta.*, 596, 481–486.
- Harper, M. S., & Hopkins, T. L. (1997). Peritrophic membrane structure and secretion in European corn borer larvae (*Ostrinia nubilalis*). *Tissue Cell*, 29, 461–475.
- Harwood, J. L. (1980). Plant acyl lipids: Structure, distribution and analysis. In P. K. Stumpf (Ed.), *The Biochemistry of Plants: A Comprehensive Treatise*, Vol. 4, *Lipids: Structure and Function* (pp. 1–56). New York, NY: Academic Press.
- Harzer, K., Hiraiwa, M., & Paton, B. C. (2001). Saposins (sap) A and C activate the degradation of galactosylsphingosine. *FEBS Lett.*, 508, 107–110.
- Hegedus, D., Erlandson, M., Gillot, C., & Toprak, U. (2008). New insights into peritrophic matrix synthesis, architecture, and function. *Annu. Rev. Entomol.*, 54, 285–302.
- Hirayama, C., Konno, K., Wasano, N., & Nakamura, M. (2007). Differential effects of sugar-mimic alkaloids in mulberry latex on sugar metabolism and disaccharidases of Eri and domesticated silkworms: Enzymatic adaptation of *Bombyx mori* to mulberry defense. *Insect Biochem. Mol. Biol.*, 37, 1348–1358.
- Hooper, N. M. (1994). Families of zinc metalloproteases. *FEBS Lett.*, 354, 1–6.
- Hori, K. (1972). Comparative study of a property of salivary amylase among various heteropterous insects. *Comp. Biochem. Physiol. B*, 42, 501–508.
- Horne, I., Haritos, V. S., & Oakeshott, J. G. (2009). Comparative and functional genomics of lipases in holometabolous insects. *Insect Biochem. Mol. Biol.*, 39, 547–567.
- Huang, S. -W., Zhang, H. -Y., Marshall, S., & Jackson, T. A. (2010). The scarab gut: A potential bioreactor for bio-fuel production. *Insect Science*, 17, 175–183.
- Hughes, J., & Vogler, A. P. (2006). Gene expression in the gut of keratin-feeding clothes moths (*Tineola*) and keratin beetles (*Trox*) revealed by subtracted cDNA libraries. *Insect Biochem. Mol. Biol.*, 36, 584–592.
- Human, H., & Nicolson, S. W. (2003). Digestion of maize and sunflower pollen by the spotted maize *Astylus atromaculatus* (Melyridae): Is there a role for osmotic shock? *J. Insect Physiol.*, 49, 633–643.
- Husebye, H., Artz, S., Burmeister, W. P., Härtel, F. V., Brandt, A., et al. (2005). Crystal structure at 1.1-Å resolution of an insect myrosinase from *Brevicorine brassicae* shows its close relationship to β-glucosidases. *Insect Biochem. Mol. Biol.*, 35, 1311–1320.
- Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Ruppley, J. A. (1972). Vertebrate lysozymes. In P. D. Boyer (Ed.), *The Enzymes* (Vol. 7, pp. 665–868). New York, NY: Academic Press.
- Inward, D., Beccaloni, G., & Eggleton, P. (2007). Death of an order: A comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. *Biol. Lett.*, 3, 331–335.
- Itoh, M., Takeda, S., Yamamoto, H., Izumi, S., Tomino, S., et al. (1991). Cloning and sequence analysis of membrane-bound alkaline phosphatase cDNA of the silkworm, *Bombyx mori*. *Biochim. Biophys. Acta*, 1129, 135–138.
- Itoh, M., Kanamori, Y., Takao, M., & Eguchi, M. (1999). Cloning of soluble alkaline phosphatase cDNA and molecular basis of the polymorphic nature in alkaline phosphatase isozymes of *Bombyx mori* midgut. *Insect Biochem. Mol. Biol.*, 29, 121–129.
- Ivanova, N., Sorokin, A., Anderson, L., Galleron, N., Candellon, B., et al. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. *Nature*, 423, 87–91.
- Janecek, S. (1997). Alpha-amylase family: Molecular biology and evolution. *Progr. Biophys. Mol. Biol.*, 67, 67–97.
- Jasrapuria, S., Arakane, Y., Osman, G., Kraes, K. J., Beerman, R. W., & Muthukrishnan, S. (2010). Genes encoding proteins with peritrophin A-type chitin-binding domains in *Tribolium castaneum* are grouped into three distinct families based on phylogeny, expression and function. *Insect Biochem. Molec. Biol.*, 40, 214–227.
- Jeng, W. -Y., Wang, N. -C., Lin, C. -T., Liaw, Y. -C., Chang, W. -J., et al. (2010). Structural and functional analysis of three β-glucosidases from bacterium *Clostridium cellulovorans*, fungus *Trichoderma reesei* and termite *Neotermes kos-hunensis*. *J. Struct. Biol.*, in press.
- Johnson, K. S., & Felton, G. W. (2000). Digestive proteinase activity in corn earworm (*Helicoverpa zea*) after molting and in response to lowered redox potential. *Arch. Insect Biochem. Physiol.*, 44, 151–161.
- Johnson, K. S., & Rabosky, D. (2000). Phylogenetic distribution of cysteine proteinases in beetles: Evidence for an evolutionary shift to an alkaline digestive strategy in Cerambycidae. *Comp. Biochem. Physiol. B*, 126, 609–619.

- Jones, A. M. E., Winge, P., Bones, A. M., Cole, R., & Rossiter, J. T. (2002). Characterization and evolution of a myrosinase from the cabbage aphid *Brevicorine brassicae*. *Insect Biochem. Mol. Biol.*, 32, 275–284.
- Jordão, B. P., Terra, W. R., & Ferreira, C. (1995). Chemical determinations in microvillar membranes purified from brush borders isolated from the larval midgut from one Coleoptera and two Diptera species. *Insect Biochem. Mol. Biol.*, 25, 417–426.
- Jordão, B. P., Lehan, M. J., Terra, W. R., Ribeiro, A. F., & Ferreira, C. (1996a). An immunocytochemical investigation of trypsin secretion in the midgut of the stablefly *Stomoxys calcitrans*. *Insect Biochem. Mol. Biol.*, 26, 445–453.
- Jordão, B. P., Terra, W. R., Ribeiro, A. F., Lehan, M. J., & Ferreira, C. (1996b). Trypsin secretion in *Musca domestica* larval midguts: A biochemical and immunocytochemical study. *Insect Biochem. Mol. Biol.*, 26, 337–346.
- Jordão, B. P., Capella, A. N., Terra, W. R., Ribeiro, A. F., & Ferreira, C. (1999). Nature of the anchors of membrane-bound aminopeptidase, amylase, and trypsin and secretory mechanisms in *Spodoptera frugiperda* (Lepidoptera) midgut cells. *J. Insect Physiol.*, 45, 29–37.
- Khadeni, S., Guarino, L. A., Watanabe, H., Tokuda, G., & Meyer, E. F. (2002). Structure of an endoglucanase from termite, *Nasutitermes takasagoensis*. *Acta Cryst.*, D 58, 653–658.
- Kim, N., Choo, Y. M., Lee, K. S., Hong, S. J., Seol, K. Y., et al. (2008). Molecular cloning and characterization of a glycosyl family 9 cellulase distributed throughout the digestive tract of the cricket *Teleogryllus emma*. *Comp. Biochem. Physiol.*, 150B, 368–376.
- Kleywegt, G. J., Zou, J. Y., Divne, C., Davies, G. J., Sinning, I., et al. (1997). The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 angstrom resolution, and a comparison with related enzymes. *J. Mol. Biol.*, 272, 393–397.
- Klinkowstrom, A. M., Terra, W. R., & Ferreira, C. (1994). Aminopeptidase A from *Rhynchosciara americana* (Diptera) larval midguts: Properties and midgut distribution. *Arch. Insect Biochem. Physiol.*, 27, 301–305.
- Klinkowstrom, A. M., Terra, W. R., & Ferreira, C. (1995). Midgut dipeptidases from *Rhynchosciara americana* (Diptera) larvae: Properties of soluble and membrane bound forms. *Insect Biochem. Mol. Biol.*, 25, 303–310.
- Knight, P. J. K., Knowles, B. H., & Eller, D. J. (1995). Molecular cloning of an insect aminopeptidase N that serves as a receptor of *Bacillus thuringiensis* Cry 1a (c) toxin. *J. Biol. Chem.*, 270, 17765–17770.
- Koepeke, J., Ermler, V., Warkentin, E., Wenz, G., & Flecker, P. (2000). Crystal structure of cancer chemopreventive Bowman–Birk inhibition in ternary complex with bovine trypsin at 2–3-Å resolution: Structural basis of Janus-faced serine protease inhibitor specificity. *J. Mol. Biol.*, 298, 477–491.
- Koiwa, H., Shade, R. E., Zhu-Salzman, K., D’Urzo, M. P., Murdock, L. L., et al. (2000). A plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases (DuCALs) in the larval midgut of the western corn rootworm (*Diabrotica virgifera virgifera*). *FEBS Lett.*, 471, 67–70.
- Konno, K., Okada, S., & Hirayama, C. (2001). Selective secretion of free glycine, a neutralizer against a plant defense chemical, in the digestive juice of the privet moth larvae. *J. Insect Physiol.*, 47, 1451–1457.
- Krishnamoorthy, M., Jurat-Fuentes, J. L., McNall, R. J., Andacht, T., & Adang, M. J. (2007). Identification of novel CryIAC binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. *Insect Biochem. Mol. Biol.*, 37, 189–201.
- Krishnan, N., & Kodrik, D. (2006). Antioxidant enzymes in *Spodoptera littoralis* (Boisduval): Are they enhanced to protect gut tissues during oxidative stress? *J. Insect Physiol.*, 52, 11–20.
- Lang, T., Hansson, G. C., & Samuelsson, T. (2007). Gel-forming mucins appeared early in metazoan evolution. *Proc. Natl. Acad. Sci. USA*, 104, 16209–16214.
- Le Cahérec, F., Guillemin, M. T., Beuron, F., Cavalier, A., Thomas, D., et al. (1997). Aquaporin-related proteins in the filter chamber of homopteran insects. *Cell Tissue Res.*, 290, 143–151.
- Lee, J. H., Tsuji, M., Nakamura, M., Nishimoto, M., Okuyama, M., et al. (2001). Purification and identification of the essential ionizable groups of honeybee, *Apis mellifera* L., trehalase. *Biosci. Biotechnol. Biochem.*, 65, 2657–2665.
- Lee, M. J., & Anstee, J. H. (1995). Endoproteases from the midgut of *Spodoptera littoralis* include a chymotrypsin-like enzyme with an extended binding site. *Insect Biochem. Mol. Biol.*, 25, 49–61.
- Lehan, M. J. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.*, 42, 525–550.
- Lehan, M. J., Müller, H. M., & Crisanti, A. (1996). Mechanisms controlling the synthesis and secretion of digestive enzymes in insects. In M. J. Lehan, & P. F. Billingsley (Eds.), *Biology of the Insect Midgut* (pp. 195–205). London, UK: Chapman and Hall.
- Lemos, F. J. A., & Terra, W. R. (1991a). Digestion of bacteria and the role of midgut lysozyme in some insect larvae. *Comp. Biochem. Physiol. B*, 100, 265–268.
- Lemos, F. J. A., & Terra, W. R. (1991b). Properties and intracellular distribution of a cathepsin D-like proteinase active at the acid region of *Musca domestica* midgut. *Insect Biochem.*, 21, 457–465.
- Lemos, F. J. A., & Terra, W. R. (1992). A high yield preparation of *Musca domestica* larval midgut microvilli and the subcellular distribution of amylase and trypsin. *Insect Biochem. Mol. Biol.*, 22, 433–438.
- Lemos, F. J. A., Ribeiro, A. F., & Terra, W. R. (1993). A bacteria-digesting midgut lysozyme from *Musca domestica* (Diptera) larvae: Purification, properties and secretory mechanism. *Insect Biochem. Mol. Biol.*, 23, 533–541.
- Leonardi, M. G., Marciani, P., Montorfano, P. G., Cappelloza, S., & Giordana, B. (2001). Effect of fenoxycarb on leucine uptake and lipid composition of midgut brush border membrane in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Pest. Biochem. Physiol.*, 70, 42–51.
- Levitzki, A., & Steer, M. L. (1974). The allosteric activation of mammalian α -amylase by chloride. *Eur. J. Biochem.*, 41, 171–180.

- Li, X., & Brune, A. (2005). Digestion of microbial mass, structural polysaccharides, and protein by the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Soil Biol. Biochem.*, 37, 107–116.
- Lin, G., Bode, W., Huber, R., Chi, C., & Engh, R. A. (1993). The 0.25-nm X-ray structure of Bowman–Birk-type inhibitor from mung bean in ternary complex with porcine trypsin. *Eur. J. Biochem.*, 212, 549–555.
- Linder, M., & Teeri, T. T. (1997). The roles and function of cellulose-binding domains. *J. Biotechnol.*, 57, 15–28.
- Lo, N., Tokuda, G., Watanabe, H., Rose, H., Slaytor, M., et al. (2000). Evidence from multiple gene sequences indicates that termites evolved from wood-feeding cockroaches. *Current Biol.*, 10, 801–804.
- Lopes, A. R., Juliano, M. A., Juliano, L., & Terra, W. R. (2004). Coevolution of insect trypsins and inhibitors. *Arch. Insect Biochem. Physiol.*, 55, 140–152.
- Lopes, A. R., Juliano, M. A., Marana, S. R., Juliano, L., & Terra, W. R. (2006). Substrate specificity of insect trypsins and the role of their subsites in catalysis. *Insect Biochem. Mol. Biol.*, 36, 130–140.
- Lopes, A. R., Sato, P. M., & Terra, W. R. (2009). Insect chymotrypsins: Chloromethyl ketone inactivation and substrate specificity relative to possible coevolutional adaptation of insects and plants. *Arch. Insect Biochem. Physiol.*, 70, 188–203.
- Lu, Y., Zen, K. -C., Muthukrishnan, S., & Kramer, K. J. (2002). Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase. *Insect Biochem. Mol. Biol.*, 32, 1369–1382.
- Luciani, V., Marie-Claire, C., Ruffet, E., Beaumont, A., Roques, B. P., et al. (1998). Characterization of Glu³⁵⁰ as a critical residue involved in the N-terminal amine binding site of aminopeptidase N (EC 3.4.11.2): Insight into its mechanism of action. *Biochemistry*, 37, 686–692.
- Marana, S. R., Ribeiro, A. F., Terra, W. R., & Ferreira, C. (1997). Ultrastructure and secretory activity of *Abracris flavolineata* (Orthoptera: Acrididae) midguts. *J. Insect Physiol.*, 43, 465–473.
- Marana, S. R., Terra, W. R., & Ferreira, C. (2000). Purification and properties of a β -glycosidase purified from midgut cells of *Spodoptera frugiperda* (Lepidoptera) larvae. *Insect Biochem. Mol. Biol.*, 30, 1139–1146.
- Marana, S. R., Jacobs-Lorena, M., Terra, W. R., & Ferreira, C. (2001). Amino acid residues involved in substrate binding and catalysis in an insect digestive β -glycosidase. *Biochim. Biophys. Acta*, 1545, 41–52.
- Marana, S. R., Terra, W. R., & Ferreira, C. (2002a). The role of amino acid residues Q39 and E451 in the determination of substrate specificity of the *Spodoptera frugiperda* β -glycosidase. *Eur. J. Biochem.*, 269, 3705–3714.
- Marana, S. R., Lopes, A. R., Juliano, L., Juliano, M. A., Ferreira, C., et al. (2002b). Subsites of trypsin active site favor catalysis or substrate binding. *Biochem. Biophys. Res. Commun.*, 290, 494–497.
- Marana, S. R., Cançado, F. C., Valerio, A. A., Ferreira, C., Terra, W. R., & Barbosa, J. A. R. G. (2006). Crystallization, data collection and phasing of two digestive lysozymes from *Musca domestica*. *Acta Cryst. F62*, 750–752.
- Markovic, O., & Janecek, S. (2001). Pectin degrading glycoside hydrolases of family 28: Sequence-structural features, specificities and evolution. *Protein Eng.*, 14, 615–631.
- Martin, M. M. (1987). *Invertebrate–Microbial Interactions: Ingested Fungal Enzymes in Anthropod Biology*. Ithaca, NY: Cornell University Press.
- Martin, M. M., & Martin, J. S. (1984). Surfactants: Their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia*, 61, 342–345.
- Matoub, M., & Rouland, C. (1995). Purification and properties of the xylanases from the termite *Macrotermes bellicosus* and its symbiotic fungus *Termitomyces* sp. *Comp. Biochem. Physiol. B*, 112, 629–635.
- Matsumura, I., & Kirsch, J. F. (1996). Is aspartate 52 essential for catalysis by chicken egg white lysozyme? The role of natural substrate-assisted hydrolysis. *Biochemistry*, 35, 1881–1889.
- Mazumdar-Leighton, S., & Broadway, R. M. (2001a). Transcriptional induction of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. *Insect Biochem. Mol. Biol.*, 31, 645–657.
- Mazumdar-Leighton, S., & Broadway, R. M. (2001b). Identification of six chymotrypsin cDNAs from larval midgut of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. *Insect Biochem. Mol. Biol.*, 31, 633–644.
- McNall, R. J., & Adang, M. J. (2003). Identification of novel *Bacillus thuringiensis* Cry1 Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. *Insect Biochem. Mol. Biol.*, 33, 999–1010.
- Miller, D., & Crane, R. K. (1961). The digestive function of the epithelium of the small intestine. II. Localization of disaccharide hydrolysis in the isolated brush border portion of intestinal epithelial cells. *Biochim. Biophys. Acta*, 52, 293–298.
- Mitsumasu, K., Azuma, M., Niimi, T., Yamashita, O., & Yaginuma, T. (2005). Membrane-penetrating trehalase from silkworm *Bombyx mori*. Molecular cloning and localization in larval midgut. *Insect Mol Biol.*, 14, 501–508.
- Murdock, L. L., Brookhart, G., Dunn, P. E., Foard, D. E., Kelley, S., et al. (1987). Cysteine digestive proteinases in Coleoptera. *Comp. Biochem. Physiol. B*, 87, 783–787.
- Nakanishi, K., Yaoi, K., Nagino, Y., Hara, H., Kitami, M., et al. (2002). Aminopeptidase N isoforms from the mid-gut of *Bombyx mori* and *Plutella xylostella*: Their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry 1A toxin. *FEBS Lett.*, 519, 215–220.
- Nakashima, K., Watanabe, H., Saitoh, H., Tokuda, G., & Azuma, J. I. (2002). Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochem. Mol. Biol.*, 32, 777–784.
- Nielsen, J. E., & Borchert, T. U. (2000). Protein engineering of bacterial α -amylases. *Biochim. Biophys. Acta*, 1543, 253–274.
- Nishimoto, M., Kubota, M., Tsuji, M., Mori, H., Kimura, A., et al. (2001). Purification and substrate specificity of honeybee, *Apis mellifera* L., α -glucosidase III. *Biosci. Biotechnol. Biochem.*, 65, 1610–1616.
- Norén, O., Sjostrom, H., Danielsen, E. M., Cowell, G. M., & Skovbjerg, H. (1986). The enzymes of the enterocyte plasma membrane. In P. Desnuelle, H. Sjostrom, & O. Norén (Eds.), *Molecular and Cellular Basis of Digestion* (pp. 355–365). Amsterdam: Elsevier.

- Novillo, C., Castanera, P., & Ortego, F. (1997). Inhibition of digestive trypsin-like proteases from larvae of several lepidopteran species by the diagnostic cysteine protease inhibitor E-64. *Insect Biochem. Mol. Biol.*, 27, 247–254.
- Novotny, V., & Wilson, M. R. (1997). Why are there no small species among xylem-sucking insects? *Evol. Ecol.*, 11, 419–437.
- Okech, B. A., Boudko, D. Y., Linser, P. J., & Harvey, W. R. (2008). Cationic pathway of pH regulation in larvae of *Anopheles gambiae*. *J. Exp. Biol.*, 211, 957–968.
- Oliveira-Neto, O. B., Batista, J. A. N., Rigden, D. J., Fragoso, R. R., Silva, R. O., et al. (2004). A diverse family of serine proteinase genes expressed in cotton boll weevil (*Anthonomus grandis*): Implications for the design of pest-resistant transgenic cotton. *Insect Biochem. Mol. Biol.*, 34, 903–918.
- Ortego, F., Novillo, C., & Catañera, P. (1996). Characterization and distribution of digestive proteases of the stalk corn borer, *Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae). *Arch. Insect Biochem. Physiol.*, 33, 136–180.
- Oviedo, M. N., VanEkeris, L., Corena-Mcleod, M. D. P., & Linser, P. J. (2008). A microarray-based analysis of transcriptional compartmentalization in the alimentary canal of *Anopheles gambiae* (Diptera: Culicidae) larvae. *Insect Mol. Biol.*, 17, 61–72.
- Padilha, M. H. P., Pimentel, A. C., Ribeiro, A. F., & Terra, W. R. (2009). Sequence and function of lysosomal and digestive cathepsin D-Like proteinases of *Musca domestica* midgut. *Insect Biochem. Mol. Biol.*, 39, 782–791.
- Parenti, P., Sacchi, F. V., Hanozet, G. M., & Giordana, B. (1986). Na-dependent uptake of phenylalanine in the midgut of a cockroach (*Blattella gigantea*). *J. Comp. Physiol. B*, 156, 549–556.
- Pauchet, Y., Muck, A., Svatos, A., Heckel, D. G., & Preiss, S. (2008). Mapping the larval midgut lumen proteome of *Helicoverpa armigera*, a generalist herbivorous insect. *J. Proteom. Res.*, 7, 1629–1639.
- Pauchet, Y., Freitak, D., Heidele-Fischer, H. M., Heckel, D. G., & Vogel, H. (2009a). Immunity or digestion: Glucanase activity in a glucan-binding protein family from Lepidoptera. *J. Biol. Chem.*, 284, 2214–2224.
- Pauchet, Y., Muck, A., Svatos, A., & Heckel, D. G. (2009b). Chromatographic and electrophoretic resolution of proteins and protein complexes from the larval midgut microvilli of *Manduca sexta*. *Insect Biochem. Mol. Biol.*, 39, 467–474.
- Pauchet, Y., Wilkinson, P., Vogel, H., Nelson, D. R., Reynolds, S. E., et al. (2010). Pyrosequencing the *Manduca sexta* larval midgut transcriptome: Messages for digestion, detoxification and defense. *Insect Biochem. Mol. Biol.*, 19, 61–75.
- Payan, F. (2004). Structural basis for the inhibition of mammalian and insect α -amylases by plant protein inhibitors. *Biochim. Biophys. Acta*, 1696, 171–180.
- Pechan, T., Cohen, A., Williams, W. P., & Luthe, D. S. (2002). Insect feeding mobilizes a unique defense protease that disrupts the peritrophic matrix of caterpillars. *Proc. Natl. Acad. Sci. USA*, 99, 13319–13323.
- Peng, J., Zhong, J., & Granados, R. R. (1999). A baculovirus enhancer alters the permeability of a mucosal midgut peritrophic matrix from lepidopteran larvae. *J. Insect Physiol.*, 45, 159–166.
- Pereira, P. J.B., Lozanov, V., Patthy, A., Huber, R., Bode, W., et al. (1999). Specific inhibition of insect α -amylases: Yellow mealworm α -amylase in complex with the Amaranth at 2.0 Å resolution. *Structure*, 7, 1079–1088.
- Peters, W. (1992). *Peritrophic Membranes*. Berlin: Springer.
- Peterson, A. M., Barillas-Mury, C. V., & Wells, M. A. (1994). Sequence of three cDNAs encoding an alkaline midgut trypsin from *Manduca sexta*. *Insect Biochem. Mol. Biol.*, 24, 463–471.
- Peterson, A. M., Fernando, G. J. P., & Wells, M. (1995). Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of *Manduca sexta*. *Insect Biochem. Mol. Biol.*, 25, 765–774.
- Pimenta, D. C., Oliveira, A., Juliano, M. A., & Juliano, L. (2001). Substrate specificity of human cathepsin D using internally quenched fluorescent peptides derived from reactive site loop of kallistatin. *Biochim. Biophys. Acta*, 1544, 113–122.
- Ponnuvel, K. M., Nakazawa, H., Furukawa, S., Asaoka, A., Ishibashi, J., Tanaka, H., & Yamakawa, M. (2003). A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. *J. Virol.*, 77, 10725–10729.
- Ponsen, M. B. (1991). *Structure of the digestive system of aphids, in particular Hyalopterus and Coloradoa, and its bearing on the evolution of filter chambers in Aphidoidea*. Wageningen: Wageningen Agricultural University Press, 91–95, pp. 3–61.
- Popova-Butler, A., & Dean, D. H. (2009). Proteomic analysis of the mosquito *Aedes aegypti* midgut brush border membrane vesicles. *J. Insect Physiol.*, 55, 264–272.
- Price, D. R. G., Karley, A. J., Ashford, D. A., Isaacs, H. V., Pownall, M. E., et al. (2007). Molecular characterization of a candidate gut sucrose in the pea aphid, *Acyrtosiphon pisum*. *Insect Biochem. Mol. Biol.*, 37, 307–317.
- Pytelkova, J., Hubert, J., Lepsik, M., Sobotnik, J., Sindelka, R., et al. (2009). Digestive α -amylases from *Ephestia kuehniella* – adaptation to alkaline environment and plant inhibitors. *FEBS J.*, 276, 3531–3546.
- Quicke, D. L. J. (2003). Hymenoptera (ants, bees, wasps). In V. H. Resh, & R. T. Cardé (Eds.). *Encyclopedia of Insects* (pp. 534–546). San Diego, CA: Academic Press.
- Quina, F. H., Politi, M. J., Cuccovia, I. M., Baumgarten, E., Martins-Franchetti, S. M., & Chaimovich, H. (1980). Ion exchange in micellar solutions. 4. “Buffered” systems. *J. Phys. Chem.*, 84, 361–365.
- Rajagopal, R., Agrawal, N., Selvapandian, A., Sivakumar, S., Ahmad, S., et al. (2003). Recombinantly expressed isoenzymic aminopeptidases from *Helicoverpa armigera* (American cotton bollworm) midgut display differential interaction with closely related *Bacillus thuringiensis* insecticidal proteins. *Biochem. J.*, 379, 971–978.
- Ramallo-Ortigão, J. M., Kamhawi, S., Rowton, E. D., Ribeiro, J. M. C., & Valenzuela, J. G. (2003). Cloning and characterization of trypsin- and chymotrypsin-like proteases from the midgut of the sand fly vector *Phlebotomus papatasi*. *Insect Biochem. Mol. Biol.*, 33, 163–171.
- Ramos, A., Mahowald, A., & Jacobs-Lorena, M. (1993). Gut-specific genes from the black-fly *Simulium vittatum* encoding trypsin-like and carboxypeptidase-like proteins. *Insect Mol. Biol.*, 1, 149–163.

- Rana, R. L., & Stanley, D. W. (1999). *In vitro* secretion of digestive phospholipase A2 by midgut isolated from tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.*, 42, 179–187.
- Rawlings, N. D., & Barrett, A. J. (1995). Evolutionary families of metallopeptidases. *Methods Enzymol.*, 248, 183–228.
- Regel, R., Matioli, S. R., & Terra, W. R. (1998). Molecular adaptation of *Drosophila melanogaster* lysozymes to a digestive function. *Insect Biochem. Mol. Biol.*, 28, 309–319.
- Reuveni, M., Hong, Y. S., Dunn, P. E., & Neal, J. J. (1993). Leucine transport into brush border membrane vesicles from guts of *Leptinotarsa decemlineata* and *Manduca sexta*. *Comp. Biochem. Physiol.*, 104A, 267–272.
- Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K. C., & Jones, T. A. (1990). Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. *Science*, 249, 380–386.
- Ryan, C. A. (1990). Proteinase inhibitors in plants: Genes for improving defense against insects and pathogens. *Annu. Rev. Phytopathol.*, 28, 425–449.
- Sakon, J., Irwin, D., Wilson, D. B., & Karplus, P. A. (1997). Structure and mechanism of endo/exocellulase E4 from *Thermomonospora fusca*. *Nature Struct. Biol.*, 4, 810–818.
- Salvucci, M. E. (2003). Distinct sucrose isomerases catalyze trehalose synthesis in whiteflies, *Bemisia argentifolii*, and *Erwinia rhapsodica*. *Comp. Biochem. Physiol. B*, 135, 385–395.
- Salvucci, M. E., Rosell, R. C., & Brown, J. K. (1998). Uptake and metabolism of leaf proteins by the silverleaf whitefly. *Arch. Insect Biochem. Physiol.*, 39, 155–165.
- Santos, C. D., & Terra, W. R. (1986). Midgut alpha-glucosidase and beta-fructosidase from *Erinnyis ello* larvae and imagoes: Physical and kinetic-properties. *Insect Biochem.*, 16, 819–824.
- Santos, C. D., Ribeiro, A. F., & Terra, W. R. (1986). Differential centrifugation, calcium precipitation and ultrasonic disruption of midgut cells of *Erinnyis ello* caterpillars: Purification of cell microvilli and inferences concerning secretory mechanisms. *Can. J. Zool.*, 64, 490–500.
- Sato, P. M., Lopes, A. R., Juliano, L., Juliano, M. A., & Terra, W. R. (2008). Subsite substrate specificity of midgut insect chymotrypsins. *Insect Biochem. Mol. Biol.*, 38, 628–633.
- Scharf, M. E., Kovaleva, E. S., Jadhao, S., Campbell, J. H., Buchman, G. W., & Boucias, D. G. (2010). Functional and translational analyses of a beta-glucosidase gene (glycosyl hydrolase family 1) isolated from the gut of the lower termite *Reticulitermes flavipes*. *Insect Biochem. Mol. Biol.*, 40, 611–620.
- Schechter, I., & Berger, A. (1967). On the size of active site in proteases. *Biochim. Biophys. Res. Commun.*, 27, 157–162.
- Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J., & Crane, R. K. (1973). Purification of the human intestinal brush border membrane. *Biochim. Biophys. Acta*, 323, 98–112.
- Schorderet, S., Pearson, R. D., Vuocolo, T., Eisemann, C., Riding, G. A., et al. (1998). cDNA and deduced amino acid sequences of a peritrophic membrane glycoprotein, “Peritrophin-48,” from the larvae of *Lucilia cuprina*. *Insect Biochem. Mol. Biol.*, 28, 99–111.
- Scrivener, A. M., Slaytor, M., & Rose, H. A. (1989). Symbiont-independent digestion of cellulose and starch in *Panesthia cribrata* Saussure, an Australian wood-eating cockroach. *J. Insect Physiol.*, 35, 935–941.
- Scrivener, A. M., Watanabe, H., & Noda, H. (1997). Diet and carbohydrate digestion in the yellow-spotted longicorn beetle *Psacotha hilaris*. *J. Insect Physiol.*, 43, 1039–1052.
- Shahabuddin, M. (1995). Chitinase as a vaccine. *Parasitol. Today*, 11, 46–47.
- Shao, L., Devenport, M., Fujioka, H., Ghosh, A., & Jacobs-Lorena, M. (2005). Identification and characterization of a novel peritrophic matrix protein, Ae-Aper50, and the microvillar membrane protein, AEG12, from the mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.*, 35, 947–959.
- Shen, Z., & Jacobs-Lorena, M. (1997). Characterization of a novel gut-specific chitinase gene from the human malaria vector *Anopheles gambiae*. *J. Biol. Chem.*, 272, 28895–28900.
- Shen, Z., & Jacobs-Lorena, M. (1998). A type I peritrophic matrix protein from the malaria vector *Anopheles gambiae* binds to chitin: Cloning, expression and characterization. *J. Biol. Chem.*, 273, 17665–17670.
- Shen, Z., Reese, J. C., & Reeck, G. R. (1996). Purification and characterization of polygalacturonase from the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Insect Biochem. Mol. Biol.*, 26, 427–433.
- Shigenobu, S., Watanabe, H., Hattori, M., Sasaki, Y., & Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature*, 407, 81–86.
- Shroya, T. (1963). Metabolism of raffinose in cotton seeds. *Phytochemistry*, 2, 23–46.
- Silva, A., Bacci, M., Jr., Siqueira, C. Q., Bueno, O. L., Pagnoca, F. C., et al. (2003). Survival of *Atta sexdens* workers on different food sources. *J. Insect Physiol.*, 49, 307–313.
- Silva, C. P., & Terra, W. R. (1994). Digestive and absorptive sites along the midgut of the cotton seed sucker bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). *Insect Biochem. Mol. Biol.*, 24, 493–505.
- Silva, C. P., & Terra, W. R. (1995). An α -glucosidase from perimicrovillar membranes of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) midgut cells: Purification and properties. *Insect Biochem. Mol. Biol.*, 25, 487–494.
- Silva, C. P., & Terra, W. R. (1997). Alpha-galactosidase activity in ingested seeds and in the midgut of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). *Arch. Insect Biochem. Physiol.*, 34, 443–460.
- Silva, C. P., & Xavier-Filho, J. (1991). Comparison between the levels of aspartic and cysteine proteinase of the larval midguts of *Callosobruchus maculatus* (F.) and *Zabrotes subfasciatus* (Boh.) (Coleoptera: Bruchidae). *Comp. Biochem. Physiol. B*, 99, 529–533.
- Silva, C. P., Ribeiro, A. F., Gulbenkian, S., & Terra, W. R. (1995). Organization, origin and function of the outer microvillar (perimicrovillar) membranes of *Dysdercus peruvianus* (Hemiptera) midgut cells. *J. Insect Physiol.*, 41, 1093–1103.
- Silva, C. P., Terra, W. R., de Sá, M. F. G., Samuels, R. I., Isejima, E. M., et al. (2001). Induction of digestive α -amylases in larvae of *Zabrotes subfasciatus* (Coleoptera: Bruchidae) in response to ingestion of common bean α -amylase inhibitor 1. *J. Insect Physiol.*, 47, 1283–1290.
- Silva, M. C. P., Terra, W. R., & Ferreira, C. (2004). The role of carboxyl, guanidine and imidazole groups in catalysis by a midgut trehalase purified from an insect larvae. *Insect Biochem. Mol. Biol.*, 34, 1089–1099.

- Silva, M. C. P., Terra, W. R., & Ferreira, C. (2006). Absorption of toxic beta-glucosidases produced by insects and their effect on tissue trehalases from insects. *Comp. Biochem. Physiol.*, 143 B., 367–373.
- Silva, M. C. P., Ribeiro, A. F., Terra, W. R., & Ferreira, C. (2009). Sequencing of *Spodoptera frugiperda* midgut trehalases and demonstration of secretion of soluble trehalase by midgut columnar cells. *Insect Mol. Biol.*, 18, 769–784.
- Silva, M. C. P., Terra, W. R., & Ferreira, C. (2010). The catalytic and other residues essential for the activity of the midgut trehalase from *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.*, 40, 733–741.
- Skibbe, U., Christeller, J. T., Callaghan, P. T., Eccles, C. D., & Laing, W. A. (1996). Visualization of pH gradients in the larval midgut of *Spodoptera litura* using ^{31}P -NMR microscopy. *J. Insect Physiol.*, 42, 777–790.
- Slaytor, M. (1992). Cellulose digestion in termites and cockroaches: What role do symbionts play? *Comp. Biochem. Physiol. B*, 103, 775–784.
- Soares-Costa, A., Dias, A. B., Dellamano, M., de Paula, F. F. P., Carmona, A. K., et al. (2011). Digestive physiology and characterization of a digestive cathepsin L-like proteinase from the sugar cane weevil *Sphenophorus levis*. *J. Insect Physiol.*, 57, 462–468.
- Song, H. K., & Suh, S. W. (1998). Kunitz-type soybean trypsin inhibitor revisited: Refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from *Erythrina caffra* and tissue-type plasminogen activator. *J. Mol. Biol.*, 275, 347–363.
- Spencer, K. C. (1988). Chemical mediation of coevolution in the *Passiflora*–*Heliconius* interaction. In K. C. Spencer (Ed.), *Chemical Mediation of Coevolution* (pp. 167–240). London, UK: Academic Press.
- Strobl, S., Gomis-Ruth, F. X., Maskos, K., Frank, G., Huber, R., et al. (1997). The alpha-amylase from the yellow mealworm: Complete primary structure, crystallization and preliminary X-ray analysis. *FEBS Lett.*, 409, 109–114.
- Strobl, S., Maskos, K., Betz, M., Wiegand, G., Huber, R., et al. (1998a). Crystal structure of yellow meal worm α -amylase at 1.64 Å resolution. *J. Mol. Biol.*, 278, 617–628.
- Strobl, S., Maskos, K., Wiegand, G., Huber, R., GomisRuth, F. X., et al. (1998b). A novel strategy for inhibition of α -amylases: Yellow mealworm α -amylases in complex with the Ragi bifunctional inhibitor at 2.5 Å resolution. *Structure*, 6, 911–921.
- Sugimura, M., Watanabe, H., Lo, N., & Saito, H. (2003). Purification, characterization, cDNA cloning and nucleotide sequencing of a cellulase from the yellow-spotted longicorn beetle, *Psacothea vilaris*. *Eur. J. Biochem.*, 270, 3455–3460.
- Sumida, M., Yuan, X. L., & Matsubara, F. (1994). Purification and some properties of soluble of soluble beta-fructofuranosidase from larval midgut of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol. B. Biochem. Molec. Biol.*, 107, 273–284.
- Tellam, R. L. (1996). The peritrophic matrix. In M. J. Lehane, & P. F. Billingsley (Eds.), *Biology of the Insect Midgut* (pp. 86–114). London, UK: Chapman and Hall.
- Tellam, R. L., Wijffels, G., & Willadsen, P. (1999). Peritrophic matrix proteins. *Insect Biochem. Mol. Biol.*, 29, 87–101.
- Tellam, R. L., Vuocolo, T., Eisemann, C., Briscoe, S., Riding, G., et al. (2003). Identification of an immunoprotective mucin-like protein, peritrophin-55, from the peritrophic matrix of *Lucilia cuprina* larvae. *Insect Biochem. Mol. Biol.*, 33, 239–252.
- Terra, W. R. (1988). Physiology and biochemistry of insect digestion: An evolutionary perspective. *Braz. J. Med. Biol. Res.*, 21, 675–734.
- Terra, W. R. (1990). Evolution of digestive systems of insects. *Annu. Rev. Entomol.*, 35, 181–200.
- Terra, W. R. (2001). The origin and functions of the insect peritrophic membrane and peritrophic gel. *Arch. Insect Biochem. Physiol.*, 47, 47–61.
- Terra, W. R. (2009). Digestion. In V. H. Resh, & R. T. Cardé (Eds.), *Encyclopedia of Insects* (2nd edn, pp. 271–273). San Diego, CA: Academic Press.
- Terra, W. R., & Ferreira, C. (1994). Insect digestive enzymes: Properties, compartmentalization and function. *Comp. Biochem. Physiol. B*, 109, 1–62.
- Terra, W. R., & Ferreira, C. (2005). Biochemistry of digestion. In L. I. Gilbert, K. Iatrou, & S. S. Gill (Eds.), *Comprehensive Molecular Insect Science, Vol. 4, Biochemistry and Molecular Biology* (pp. 171–224). Oxford, UK: Elsevier.
- Terra, W. R., & Ferreira, C. (2009). Digestive system. In V. H. Resh, & R. T. Cardé (Eds.), *Encyclopedia of Insects* (2nd edn, pp. 273–281). San Diego, CA: Academic Press.
- Terra, W. R., & Regel, R. (1995). pH buffering in *Musca domestica* midguts. *Comp. Biochem. Physiol. A*, 112, 559–564.
- Terra, W. R., Ferreira, C., & De Bianchi, A. G. (1978). Physical properties and Tris inhibition of an insect trehalase and a thermodynamic approach to the nature of its active site. *Biochim. Biophys. Acta*, 524, 131–141.
- Terra, W. R., Terra, I. C. M., Ferreira, C., & de Bianchi, A. G. (1979). Carbodiimide-reactive carboxyl groups at the active site of an insect midgut trehalase. *Biochim. Biophys. Acta*, 571, 79–85.
- Terra, W. R., Terra, I. C. M., & Ferreira, C. (1983). Inhibition of an insect midgut trehalase by dioxane and d-gluconolactone: Enzyme pKa values and geometric relationships at the active site. *Intl. J. Biochem.*, 15, 143–146.
- Terra, W. R., Ferreira, C., & Garcia, E. S. (1988). Origin, distribution, properties and functions of the major *Rhodnius prolixus* midgut hydrolases. *Insect Biochem.*, 18, 423–434.
- Terra, W. R., Valentin, A., & Santos, C. D. (1987). Utilization of sugars, hemicellulose, starch, protein, fat and minerals by *Erinnyis ello* larvae and digestive role of their midgut hydrolases. *Insect Biochem.*, 17, 1143–1147.
- Terra, W. R., Ferreira, C., Jordão, B. P., & Dillon, R. J. (1996). Digestive enzymes. In M. J. Lehane, & P. F. Billingsley (Eds.), *Biology of the Insect Midgut* (pp. 153–194). London, UK: Chapman and Hall.
- Terra, W. R., Costa, R. H., & Ferreira, C. (2006). Plasma membranes from insect midgut cells. *An. Acad. Bras. Cien.*, 86, 1–15.
- Theischinger, G. (1991). Megaloptera. In I. D. Naumann, P. B. Carne, J. F. Lawrence, E. S. Nielsen, J. P. Spradbery, et al. (Eds.), *The Insects of Australia* (2nd edn., pp. 516–520). Melbourne, Australia: Melbourne University Press.

- Titarenko, E., & Chrispeels, M. J. (2000). cDNA cloning, biochemical characterization and inhibition by plant inhibitors of the alpha-amylases of the Western corn rootworm, *Diabrotica virgifera virgifera*. *Insect Biochem. Mol. Biol.*, 30, 979–990.
- Tokuda, G., Saito, H., & Watanabe, H. (2002). A digestive β -glucosidase from the salivary glands of the termite, *Neotermes koshunensis* (Shiraki): Distribution, characterization and isolation of its precursor cDNA by 5'- and 3'-RACE amplifications with degenerate primers. *Insect Biochem. Mol. Biol.*, 32, 1681–1689.
- Tokuda, G., Miyagi, M., Makiya, H., Watanabe, H., & Arakawa, G. (2009). Digestive β -glucosidases from the wood-feeding termite, *Nasutitermes takasagoensis*: Intestine distribution, molecular characterization, and alteration in sites of expression. *Insect Biochem. Mol. Biol.*, 39, 931–937.
- Toprak, U., Baldwin, D., Erlandson, M., Gillott, C., Houx, et al. (2008). A chitin diacetylase and putative insect intestinal lipases are components of the *Mamestra configurata* (Lepidoptera: Noctuidae) peritrophic matrix. *Insect Mol. Biol.*, 17, 573–585.
- Treherne, J. E. (1959). Amino acid absorption in the locust (*Schistocerca gregaria* Forsk.). *J. Exp. Biol.*, 36, 533–545.
- Tunaz, H., & Stanley, D. W. (2004). Phospholipase A2 in salivary glands isolated from tobacco hornworms, *Manduca sexta*. *Comp. Biochem. Physiol.*, 139B, 27–33.
- Uscian, J. M., Miller, J. S., Sarath, G., & Stanley-Samuelson, D. W. (1995). A digestive phospholipase A2 in the tiger beetle *Cincindella circumpecta*. *J. Insect Physiol.*, 41, 135–141.
- Vaaje-Kolstad, G., Horn, S. J., van Aalten, D. M., Synstad, B., & Eijsink, V. G. (2005). The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *J. Biol. Chem.*, 280, 28492–28497.
- Valaitis, A. P. (1995). Gypsy moth midgut proteinases: Purification and characterization of luminal trypsin, elastase and the brush-border membrane leucine aminopeptidase. *Insect Biochem. Mol. Biol.*, 25, 139–149.
- Valaitis, A. P., & Bowers, D. F. (1993). Purification and properties of the soluble midgut trehalase from the gypsy moth, *Lymantria dispar*. *Insect Biochem. Mol. Biol.*, 23, 599–606.
- Venâncio, T. M., Cristofolletti, P. T., Ferreira, C., Verjovski-Almeida, S., & Terra, W. R. (2009). The *Aedes aegypti* larval transcriptome: A comparative perspective with emphasis on trypsins and the domain structure of peritrophins. *Insect Mol. Biol.*, 18, 33–44.
- Vetter, J. (2000). Plant cyanogenic glycosides. *Toxicon*, 38, 11–36.
- Vinokurov, K., Taranushenko, Y., Krishnan, N., & Sehna, F. (2007). Proteinase, amylase, and proteinase-inhibitor activities in the gut of six cockroach species. *J. Insect Physiol.*, 53, 794–802.
- Volpicella, M., Ceci, L. R., Cordewener, J., America, T., Gallerani, R., et al. (2003). Properties of purified gut trypsin from *Helicoverpa zea*, adapted to proteinase inhibitors. *Eur. J. Biochem.*, 270, 10–19.
- Volpicella, M., Cordewener, J., Jongsma, M. A., Gallerani, R., Ceci, J. R., & Beekwilder, J. (2006). Identification and characterization of digestive serine proteases from inhibitor-resistant *Helicoverpa zea* larval midgut. *J. Chromatogr. B*, 833, 26–32.
- Vonk, H. J., & Western, J. R. H. (1984). *Comparative Biochemistry and Physiology of Enzymatic Digestion*. New York, NY: Academic Press.
- Wang, P., & Granados, R. R. (1997). Molecular cloning and sequencing of a novel invertebrate intestinal mucin. *J. Biol. Chem.*, 272, 16663–16669.
- Wang, P., & Granados, R. R. (2000). Calcofluor disrupts the midgut defense system in insects. *Insect Biochem. Mol. Biol.*, 30, 135–143.
- Wang, P., & Granados, R. R. (2001). Molecular structure of the peritrophic membrane (PM): Identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol.*, 47, 110–118.
- Wang, P., Zhang, X., & Zhang, J. (2005). Molecular characterization of four midgut aminopeptidase N isoenzymes from the cabbage looper, *Trichoplusia ni*. *Insect Biochem. Mol. Biol.*, 35, 611–620.
- Ward, C. W. (1975a). Aminopeptidases in webbing clothes moth larvae: Properties and specificities of the enzymes of intermediate electrophoretic mobility. *Biochim. Biophys. Acta*, 410, 361–369.
- Ward, C. W. (1975b). Aminopeptidases in webbing clothes moth larvae: Properties and specificities of enzymes of highest electrophoretic mobility. *Austral. J. Biol. Sci.*, 28, 447–455.
- Watanabe, H., & Tokuda, G. (2010). Cellulolytic systems in insects. *Annu. Rev. Entomol.*, 55, 609–632.
- Wei, Y. D., Lee, K. S., Gui, Z. Z., Yoon, H. J., Kim, I., et al. (2006). Molecular cloning, expression, and enzymatic activity of a novel endogenous cellulase from the mulberry longicorn beetle, *Apriona germari*. *Comp. Biochem. Physiol.*, 145 B, 220–229.
- Whitworth, S. T., Blum, M. S., & Travis, J. (1998). Proteolytic enzymes from larvae of the fire ant, *Solenopsis invicta*: Isolation and characterization of four serine endopeptidases. *J. Biol. Chem.*, 273, 14430–14434.
- Whitworth, S. T., Kordula, T., & Travis, J. (1999). Molecular cloning of Soli EC: An elastase-like serine proteinase from the imported red fire ant (*Solenopsis invicta*). *Insect Biochem. Mol. Biol.*, 29, 249–254.
- Wigglesworth, V. B. (1933). The function of the anal gills of the mosquito larva. *J. Exp. Biol.*, 10, 16–26.
- Withers, S. G., & Rupitz, K. (1990). Measurement of active-site homology between potato and rabbit muscle α -glucan phosphorylases through use of a free energy relationship. *Biochemistry*, 29, 6405–6409.
- Woodring, J., & Lorenz, M. W. (2007). Feeding, nutrient flow, and functional gut morphology in the cricket, *Gryllus maculatus*. *J. Morphol.*, 268, 815–825.
- Wolfersberger, M. G. (2000). Amino acid transport in insects. *Annu. Rev. Entomol.*, 45, 111–120.
- Woods, H. A., & Kingsolver, J. G. (1999). Feeding rate and the structure of protein digestion and absorption in Lepidoptera midguts. *Arch. Insect Biochem. Physiol.*, 42, 74–87.
- Zeng, F., Zhu, Y. C., & Cohen, A. C. (2002). Molecular cloning and partial characterization of a trypsin-like protein in salivary glands of *Lygus hesperus* (Hemiptera: Miridae). *Insect Biochem. Mol. Biol.*, 32, 455–464.
- Zhang, S., Shukle, R., Mittapalli, O., Zhu, Y. C., Reese, J. C., et al. (2010). The gut transcriptome of a gall midge, *Mayetiola destructor*. *J. Insect Physiol.*, 56, 1198–1206.

- Zhu, Y. C., & Baker, J. E. (1999). Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Insect Biochem. Mol. Biol.*, 29, 1053–1063.
- Zhu, Y. C., Kramer, K. K., Oppert, B., & Dowdy, A. K. (2000). cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins. *Insect Biochem. Mol. Biol.*, 30, 215–224.
- Ziegler, H., Garon, C. F., Fischer, E. R., & Shahabuddin, M. (2000). A tubular network associated with the brush-border surface of the *Aedes aegypti* midgut: Implications for pathogen transmission by mosquitoes. *J. Exp. Biol.*, 203, 1599–1611.
- Zimoch, L., & Merzendorfer, H. (2002). Immunolocalization of chitin synthase in the tobacco hornworm. *Cell Tissue Res.*, 308, 287–297.