Comparative Biochemistry and Physiology, Part B 158 (2011) 235–241

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Purification and partial characterization of an aminopeptidase from the midgut tissue of *Dysdercus peruvianus*

Inês A. Costa [a], Richard I. Samuels [b], Thaís D. Bifano [c,1], Walter R. Terra [c], Carlos P. Silva [d,*]

[a] Laboratório de Química e Função de Proteínas e Peptídeos, Universidade Estadual do Norte Fluminense Darcy Ribeiro, 28030-600, Campos dos Goytacazes, RJ, Brazil
[b] Laboratório de Entomologia e Fitopatologia, Centro de Ciências e Tecnologia Agropecuária, Universidade Estadual do Norte Fluminense Darcy Ribeiro, 28015-620, Campos dos Goytacazes, RJ, Brazil
[c] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, 05513-970, São Paulo, SP, Brazil
[d] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, CP 476, 88040-900, Florianópolis, SC, Brazil

ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form 8 December 2010
Accepted 8 December 2010
Available online 15 December 2010

Keywords:
Hemiptera
Perimicrovillar membranes
Insect digestion
Enzyme purification

ABSTRACT

The surface of midgut cells in Hemiptera is ensheathed by a lipoprotein membrane (the perimicrovillar membrane), which delimits a closed compartment with the microvillar membrane, the so-called perimicrovillar space. In *Dysdercus peruvianus* midgut perimicrovillar space a soluble aminopeptidase maybe involved in the digestion of oligopeptides and proteins ingested in the diet. This *D. peruvianus* aminopeptidase was purified to homogeneity by ion-exchange chromatography on an Econo-Q column, hydrophobic interaction chromatography on phenyl-agarose column and preparative polyacrylamide gel electrophoresis. The results suggested that there is a single molecular species of aminopeptidase in *D. peruvianus* midgut. Molecular mass values for the aminopeptidase were estimated to be 106 kDa (gel filtration) and 55 kDa (SDS-PAGE), suggesting that the enzyme occurs as a dimer under native conditions. Kinetic data showed that *D. peruvianus* aminopeptidase hydrolyzes the synthetic substrates LpNA, RpNA, AβNA and AsnMCA (K_ms 0.65, 0.14, 0.68 and 0.74 mM, respectively). The aminopeptidase activity upon LpNA was inhibited by EDTA and 1,10-phenanthroline, indicating the importance of metal ions in enzyme catalysis. One partial sequence of BLAST-identified aminopeptidase was found by random sequencing of the *D. peruvianus* midgut cDNA library. Semi-quantitative RT-PCR analysis showed that the aminopeptidase genes were expressed throughout the midgut epithelium, in the epithelia of V1, V2 and V3, Malphigian tubules and fat body, but it was not expressed in the salivary glands. These results are important in furthering our understanding of the digestive process in this pest species.

1. Introduction

The midgut cells of Hemiptera and Thysanoptera are ensheathed by a lipoprotein membrane, the perimicrovillar membrane (Terra, 1988), which extends toward the luminal compartment of the midgut. The perimicrovillar membrane maintains a constant distance from the inner microvillar membrane and delimits a closed compartment, the so-called perimicrovillar space. According to Silva et al. (1995), the perimicrovillar membranes are formed in the Golgi areas of epithelial cells as internal membranes originating from double membrane vesicles. The double membrane vesicles migrate to the cell apex, where the outer vesicle membrane fuses with the microvillar membrane and the inner vesicle membrane fuses with the perimicrovillar membrane. This model is supported by immunolocalization of α-glucosidase (a marker enzyme of perimicrovillar membranes) in midgut cells of the cotton seed sucker bug *Dysdercus peruvianus* (Silva and Terra, 1995; Silva et al., 1995). However, further studies of other biochemical markers are still necessary to explain the origin of this system.

Aminopeptidases (EC 3.4.11) catalyze sequential hydrolysis of amino acid residues from the N-terminal region of peptides or polypeptides, and are classified according to their dependence on metallic ions (generally $Zn^{2}+$ or $Mn^{2}+$) and their substrate specificity. Aminopeptidase N (EC 3.4.11.2) has a wide specificity, although it removes preferentially alanine and leucine residues, while aminopeptidase A (EC 3.4.11.7) prefers aspartic acid and glutamic acid as substrates (Norén et al., 1986; Ferreira and Terra, 1984, 1986).

In insects, these enzymes are found in the midgut and play an important role in the intermediate and final digestion of proteins, since in these animals they are in general more active than carboxypeptidases (Terra and Ferreira, 1994, 2005). Insect aminopeptidases possess pH optima between 7.2 and 9.0, molecular masses between 90 and 130 kDa and K_m values between 0.78 and 0.13 mM when using LpNA as a substrate (Terra and Ferreira, 2005). Aminopeptidases known from insect sources possess wide specificity, being capable of hydrolyzing a variety of β-naphthylamide substrates,

* Corresponding author. Fax: +55 48 3234 4069.
E-mail address: capsilva@ccb.ufsc.br (C.P. Silva).
[1] Present address: Laboratório de Bioquímica e Imunologia de Artrópodes, Instituto de Ciências Biomédicas, Universidade de São Paulo, 05508-900, São Paulo, Brazil.

1096-4959/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2010.12.002

except acyl-amino-β-naphthylamides, which indicates that they are in general N type aminopeptidases (Terra and Ferreira, 1994). An exception is the soluble aminopeptidase associated to the glycocalix of *Rhynchosciara americana* (Diptera) that is similar to aminopeptidases of vertebrates. This enzyme liberates N-terminal residues of aspartic or glutamic acid from peptides that are not efficiently attacked by other aminopeptidases (Klinkowstrom et al., 1994). In less evolved insects (Orthoptera, Hemiptera, Coleoptera Adephaga), aminopeptidases are mainly found in the soluble form, while in more evolved insects (Coleoptera Polyphaga, Diptera and Lepidoptera), the aminopeptidases are generally associated with the microvillar membranes of the midgut cells (Terra and Ferreira, 1994, 2005). In the majority of these insects, microvillar aminopeptidases are the main proteins present in the membranes. They compose 55% of microvillar proteins in larval *Tenebrio molitor* according to Cristofoletti and Terra (1999), being the target of δ-endotoxins produced by the bacterium *Bacillus thuringiensis*. These toxins, after binding to aminopeptidases and to cadherin receptors, form channels that cause cell breakdown and eventually insect death (Knight et al., 1995; Gill et al., 1995; Denolf et al., 1997).

In Hemipterans, aminopeptidases are found in soluble form and are imprisoned in the perimicrovillar space, as demonstrated in *R. prolixus* (Ferreira et al., 1988), *D. peruvianus* (Silva et al., 1996; Damasceno de Sá et al., 2007) and in *Eurygaster integriceps* (Allahyari et al., 2010). In accordance with these results, aminopeptidases could be used as biochemical markers of the perimicrovillar space, whilst α-glucosidase is a perimicrovillar membrane marker and β-glucosidase, a microvillar membrane marker (Ferreira et al., 1988; Silva and Terra, 1995; Silva et al., 1996; Damasceno de Sá et al., 2007; Allahyari et al., 2010).

The present work was performed to extend our knowledge on the aminopeptidase activity present in the midgut epithelium of *D. peruvianus*. We now describe the purification of the major soluble midgut aminopeptidase and detail its enzymatic characteristics (catalytic properties, substrate specificities and its susceptibility to inhibition) and molecular properties (molecular mass and partial sequence).

2. Material and methods

2.1. Animals

Stock cultures of the cotton stainer bug *D. peruvianus* (Hemiptera: Pyrrhocoridae) were maintained in plastic bottles, covered with a piece of cloth, under natural photoperiod conditions at a relative humidity of 60–70% and a temperature of 25 ± 2 °C. The insects in the colony had ample access to water and cotton seeds (*Gossypium hirsutum*).

2.2. Dissection of insects

Adult females were individually immobilized by placing them on crushed ice (8–10 min) and then dissected in cold 215 mM NaCl. *D. peruvianus* midgut is divided into three main sections or first ventriculus (V1), second ventriculus (V2) and third ventriculus (V3) and is linked through V4 (the fourth ventriculus) to the hindgut (Silva and Terra, 1994). Entire guts were removed and rinsed in distilled water. The midgut was separated from the foregut and hindgut, and the midgut contents were removed after cutting open the midgut first ventriculus (V1) lengthwise. Groups of V1 tissues were pooled as a replicate group and stored at −20 °C for use as enzyme sources in the experiments.

2.3. Preparation of samples

Due to the fact that V1 tissue is known to have the highest enzymatic activities (Silva and Terra, 1994), it was used in the preparation of samples. V1 tissues of fed animals were homogenized in double distilled water (unless otherwise stated) by using a pestle. The homogenate was centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was adjusted to 1 mL and the correspondent pellet was dispersed in double distilled water and the final volume adjusted to 1 mL.

2.4. Protein determination and hydrolase assays

Protein concentration was determined according to Bradford (1976), using ovalbumin as a standard. Most of the enzymatic assays for detecting aminopeptidase were carried out using the synthetic substrate leucine *p*-nitroanilide (LpNa) at a concentration of 1 mM according to Erlanger et al. (1961). The reaction volume for the aminopeptidase assay was of 100 μL of enzyme source, 300 μL of substrate in 100 mM Tris/HCl, pH 7.0 and the reaction was interrupted with 200 μL 30% acetic acid and absorbance of free *p*-nitroaniline was read at 410 nm. The assays against RpNA (arginine *p*-nitroanilide) were accomplished using the same conditions as described for LpNA.

Assays against AβNA (alanine β-naphthylamine) were accomplished in a reaction medium containing 100 μL of the enzyme source plus 200 μL of the substrate. After different intervals of time, the reactions were interrupted with 500 μL of 2% HCl in ethanol and revealed with 500 μL of 0.06% *p*-dimethylaminocinamaldehyde in ethanol. Absorbance was read at 540 nm.

The fluorimetric assays with AsnMCA (asparagine methyl-coumarin) was accomplished in a reaction medium containing 50 μL of enzyme source added to 1.95 mL of the substrate/buffer solution (final volume of reaction of 2 mL). Fluorescence was measured for the free release of methyl-coumarin using a Hitachi F2000 fluorimeter at 440 nm after an excitation at 380 nm.

For each determination, the mixture of the reagents was kept at 30 °C for at least four different time periods and initial velocities were calculated. Controls without enzyme or substrate were carried out and treated in a similar way as the experimental groups. One unit of enzymatic activity was defined as the amount of enzyme that hydrolyses one μmol of substrate per minute.

2.5. Ion-exchange chromatography and hydrophobic interaction chromatography

The crude extract of V1 tissue was applied onto a column (5 mL) of Econo-Q (BioRad) equilibrated with 20 mM Tris/HCl buffer, pH 7.0. The column was eluted with 25 mL of a linear gradient of 0–0.5 M of NaCl, followed by 10 mL of a 0.5–1.0 M gradient and 10 mL of 1 M NaCl. The flow was of 1 mL/min and 1 mL fractions were collected. The fractions were assayed for aminopeptidase activity and for protein determination.

The fractions obtained from the Econo-Q column with aminopeptidase activity were pooled and diluted twice with a solution of 2 M ammonium sulfate (final concentration of 1 M). The sample was applied onto a phenyl-agarose column (1.0 × 5.5 cm i.d.) equilibrated with Tris 20 mM HCl buffer, pH 8.0 containing 1 M ammonium sulfate. The column was eluted with 40 mL of a gradient of 1–0 M of ammonium sulfate, followed by 5 mL of an isocratic elution with the same buffer without ammonium sulfate. The flow rate was 1 mL/min and 50 fractions of 1 mL were collected and assayed for the aminopeptidase activity and for determination of proteins.

2.6. Polyacrylamide gel electrophoresis

Electrophoresis was performed under denaturing conditions (SDS-PAGE) as follows. Samples were combined with the sample buffer containing 500 mM Tris–HCl buffer, pH 6.8, 2.0% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue. Samples were heated for 5 min at 95 °C in a water bath before being loaded onto a 12% polyacrylamide gel slab containing