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Carbohydrate digestion in ticks and a digestive α-L-fucosidase

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ABSTRACT

Digestive carbohydrases are present in many species of hematophagous Arthropoda, including ticks. In this work, *Amblyomma cajennense* (Ixodidae) midgut digestive carbohydrases were tracked with different substrates, resulting in the identification of a chitinase and an N-acetyl-β-glucosaminidase and the first description of a digestive α-L-fucosidase in ticks. α-L-fucosidases are involved in various physiological processes, and digestive α-L-fucosidases have been shown to be present in other types of organisms. *Amblyomma cajennense* α-L-fucosidase activity was isolated using acidic and salting-out precipitations and chromatographic steps in hydrophobic and cation-exchange columns. The specificity of the isolated activity as an α-L-fucosidase was confirmed by the hydrolysis of 4-methylumbelliferyl α-L-fucopyranoside and the natural substrate fucoidan and the inhibition by fucose and deoxyfuconojirimycin. The isolated activity of α-L-fucosidase forms oligomers with molecular mass of 140 kDa or 150 kDa as determined by gel filtration and non-reducing SDS–PAGE, respectively. This particular fucosidase has an optimum pH of 5.3, is stable even at high temperatures (stable for at least 2 h at 50 °C), has a K_m of 45 μM to the substrate 4-methylumbelliferyl α-L-fucopyranoside and IC 50% of 327 μM to fucose and 42 pM to deoxyfuconojirimycin. The presence of digestive fucosidases in hematophagous Arthropoda may be related to defence mechanisms against host–parasite interactions.

1. Introduction

Ticks (suborder Ixodida) are the most important group of vectors of pathogens in the phylum Arthropoda, comparable only to mosquitoes (family Culicidae) (Parola and Raoult, 2001). Ticks and tick-borne diseases cause severe economical losses worldwide by directly affecting animal health and by transmitting a variety of pathogens among animals (Jongejan and Uilenberg, 2004). The currently used chemical acaricides have many limitations, including the emergence of drug-resistant tick populations, environmental contamination, and the potential for chemical residues present in milk and meat. An understanding of the physiology of vital processes such as blood digestion and egg and larvae development can help in the development of new and improved control methods (Willadsen, 2006).

Adult ticks of *Amblyomma cajennense* are commonly known as star ticks or horse ticks. They are three-host ticks (Nunes et al., 2010) of epidemiological importance that can transmit several diseases, including spotted fever, also known as "fever of the mountains," "tickfever", "blackfever" or "blue disease" caused by the bacteria *Rickettsia rickettsii*. Many studies have been performed using *Amblyomma cajennense* salivary glands and fat body (Batista et al., 2008; Maruyama et al., 2010), including infection studies.

The digestive system of *Amblyomma cajennense* has not yet been studied. A series of studies have been performed on tick digestive peptidases, which are expected to be present in hematophagous digestive processes (Clara et al., 2011; Franta et al., 2011; Horn et al., 2009; Sojka et al., 2011). Although it is likely that the major digestive enzymes involved in blood digestion in animals are peptidases, carbohydrases are also present in blood-feeding animals (Souza-Neto et al., 2003, 2007; Terra and Ferreira, 2012). However, studies regarding carbohydrases in ticks are very sparse (Del Pino et al., 1999; Kopacek et al., 1999; Grunclova et al., 2003; Mohamed, 2000, 2005).

L-fucose is one of the most common monosaccharides on the non-reducing end of many glycans, and fucosylation is involved in various physiological phenomena (Altmann et al., 2001; Ma et al., 2006; Pedra et al., 2010; Staudacher et al., 1999). Alpha-fucosidases (GH families 29 and 95) catalyse the removal of L-fucose from the non-reducing end when it is bound to α-1,2; α-1,3; α-1,4; or α-1,6 of oligosaccharides or glycoconjugates. Digestive α-fucosidases have been characterised in the molluscans *Chamelea gallina* (Reglero and Cabezas, 1976), *Pomacea canaliculata* (Endo et al., 1993) and *Pecten maximus* (Berteau et al., 2002). The

Abbreviations: MUFUC, 4-methylumbelliferyl α-L-fucopyranoside; E-64, 1-[L-N-(trans-epoxysuccinyl)leucyl]amino-4-guanidinobutane; TLC, thin layer chromatography.

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physiological role of molluscan digestive fucosidases is very clear. These animals feed on brown algae, which contain many sulphated fucans as components of their cell wall. Bowman (1987) demonstrated the activity of digestive α-fucosidase in the mite *Pergamasus longicornis*. Our group has shown the presence of digestive α-fucosidases in the hepatopancreas of the spider *Nephilengys cruentata* and of the scorpion *Tityus serrulatus* (to be published elsewhere).

In this work, we identified the most active digestive carbohydrases in the gut of *Amblyomma cajennense*. N-acetyl-β-glucosaminidase, chitinase and α-fucosidases are the main enzymes involved in carbohydrate digestion. Digestive *Amblyomma cajennense* α-fucosidase activity was isolated and characterised.

2. Materials and methods

2.1. Animals and samples

2.1.1. Amblyomma cajennense

Amblyomma cajennense (Arachnida; Ixodidae) were maintained in the laboratory in total darkness at 27 °C and 90% relative humidity in an incubation oven and were allowed to feed on the shaved skin of *Oryctolagus cuniculus* rabbit backs (Rechav et al., 1997). After 5 days, partially fed females were removed from the rabbits and immobilised on ice, and the tick midguts were appropriately dissected in order to isolate midgut tissue and midgut contents. The extracted samples from midgut tissue and midgut contents were transferred into microtubes and stored at −20 °C until use. The samples were homogenized in ultrafiltered cold water with the aid of a Potter–Elvehjem homogenizer and centrifuged at 16,000×*g* for 20 min at 4 °C.

2.2. Chemicals

Buffer salts, detergents, protein inhibitors, fucose, deoxyfuconojirimycin, and substrates were purchased from Sigma–Aldrich (USA).

2.3. Protein determination and glycoside hydrolase assays

Protein levels were determined according to Smith et al. (1985) using ovalbumin as a standard. All enzymatic assays were performed at 30 °C. For each measurement, incubations were carried out for four or more different time periods, and the initial rates were calculated. One unit of enzyme (U) is defined as the amount of enzyme that hydrolyses the substrate to generate 1 μmol of product/minute. The glycoside hydrolases tested and their substrate and assay conditions are listed in Table 1. Routine assays of α-L-fucosidase used a solution of 25 μM 4-methylumbelliferyl α-L-fucopyranoside (MUFUC) as a substrate. The released 4-methylumbelliferone was measured by the method of Baker and Woo (1992). Fluorescence measurements were carried out in a Gemini XPS spectrofluorimeter (Molecular Devices). Colorimetric measurements were obtained using a SpectraMax 190 spectrophotometer (Molecular Devices).

2.4. Isolation of Amblyomma cajennense α-L-fucosidase activity

Amblyomma cajennense midgut with their luminal contents was initially homogenised in ultrapure water (MilliQ) with a Potter–Elvehjem homogeniser. Homogenate samples were centrifuged at 16,100×g for 20 min at 4 °C. The soluble portion was then mixed (1:1) with a 3.4 M ammonium sulphate solution containing 100 μM E-64 to avoid proteolysis. This mixture was kept at 4 °C for 22 hs and then centrifuged under the same conditions listed above. Subsequently, the soluble portion was diluted in 0.1 M sodium acetate, pH 3.5, kept for one hour at 4 °C and centrifuged again.

The soluble portion after acidic centrifugation was submitted to hydrophobic chromatography on a HiTrap Butyl column (GE Healthcare) in a FPLC system. The column was equilibrated in 50 mM citrate–phosphate buffer at pH 5.0 containing 1.6 M $(NH_4)_2SO_4$. Proteins were eluted with a 25 mL gradient from 1.6 to 0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 1.0 mL/minute. Fractions of 1.0 mL were collected. Active fractions hydrolyzing MUFUC were pooled, desalted using a HiTrap Desalting column (GE Healthcare) and applied to a Resource S column (Amersham Biosciences) equilibrated with 50 mM citrate-phosphate buffer, pH 5.0. Elution was carried out with a 25 mL 0–0.6 M NaCl gradient in the same buffer at a flow rate of 1.0 mL/minute. Fractions of 1.0 mL were collected. Active fractions using MUFUC as substrate were pooled and used to enzyme characterisation.

2.5. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and gel filtration

Samples containing α-L-fucosidase (approximately 1 ug of protein) were combined with sample buffer containing 60 mM Tris–HCl buffer at pH 6.8, 2.5% SDS, 10% v/v glycerol, and 0.005% (w/v) bromophenol blue. The samples were loaded onto a 12% (w/v) polyacrylamide gel slab containing 0.1% SDS (Laemmli,1970). Non-reducing gels (without β-mercaptoethanol) were run at a constant voltage of 100 V at 10 °C and silver-stained for proteins (Blum et al., 1987). Non-reducing gels were also submitted to on gel activity methods. Filter paper was moistened in a MUFUC solution as described at enzyme assay item. The filter paper was then superimposed to the gel and incubated for one hour at 30 °C. Fluorescense of free methylumbelliferone was photographed on gel Documentation (MiniBis Pro-DNR Bio-Imaging Systems). Mr values

Table 1
Assay conditions and methods used in the determination of hydrolases from *Amblyomma cajennense* females*.

Enzyme	Substrate	Concentration	pH (pH range)	Group determined	Reference
Maltase	Maltose	10 mM	7.0	Glucose	Dahlqvist (1968)
Amylase	Starch	1 %	7.0	Reducing groups	Noelting and Bernfeld, 1948
Trehalase	Trehalose	5 mM	7.0	Glucose	Dahlqvist (1968)
α-Glucosidase	4-MU-α-glucopyranoside	0.05 mM	7.0	4-Methylumbelliferone	Baker and Woo (1992)
α-L-fucosidase	4-MU-α-L-fucopyranoside	0.05 mM	5.3 (2.5–10)	4-Methylumbelliferone	Baker and Woo (1992)
chitinase	4-MU β-D-N,N′,N″-triacetylchitotrioside	0.05 mM	7.0	4-Methylumbelliferone	Baker and Woo (1992)
N-acetyl-β-glucosaminidase	4-Methylumbelliferyl N-acetyl-β-D-glucosaminide	0.05 mM	5.6 (2.5–10)	4-Methylumbelliferone	Baker and Woo (1992)
α-Galactosidase	4-MU-α-galactopyranoside	0.05 mM	7.0	4-Methylumbelliferone	Baker and Woo, 1992

* Assays were performed at 30 °C at the indicated pH values. The buffers (0.05 M) were used: citrate-phosphate (pH 2.5–5.5); MES (6.0–6.5); Tris–HCl (7.0–9.0), and glycine-NaOH (pH 9.0–10). Incubations were carried out for at least four different periods of time and the initial rates calculated. One U of enzyme is defined as the amount that catalyses the cleavage of 1 μmol of substrate (or bond)/min.