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Digestive enzymes from workers and soldiers of termite *Nasutitermes corniger*

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ABSTRACT

The digestive apparatus of termites may have several biotechnological applications, as well as being a target for pest control. This report discusses the detection of cellulases (endoglucanase, exoglucanase, and β-glucosidase), hemicellulases (β-xylosidase, α-L-arabinofuranosidase, and β-D-xylanase), α-amylase, and proteases (trypsin-like, chymotrypsin-like, and keratinase-type) in gut extracts from *Nasutitermes corniger* workers and soldiers. Additionally, the effects of pH (3.0–11.0) and temperature (30–100 °C) on enzyme activities were evaluated. All enzymes investigated were detected in the gut extracts of worker and soldier termites. Endoglucanase and β-xylanase were the main cellulase and hemicellulase, respectively. Zymography for proteases of worker extracts revealed polypeptides of 22, 30, and 43 kDa that hydrolyzed casein, and assays using protease inhibitors showed that serine proteases were the main proteases in worker and soldier guts. The determined enzyme activities and their response to different pH and temperature values revealed that workers and soldiers contained a distinct digestive apparatus. The ability of these termites to efficiently digest the main components of lignocellulosic materials stimulates the purification of gut enzymes. Further investigation into their biotechnological potential as well as whether the enzymes detected are produced by the termites or by their symbionts is needed.

1. Introduction

Termites (order Isoptera) are eusocial insects that can be classified into morpho-physiologically distinct castes called queens, kings, alate forms (reproductive members), workers, soldiers, and immature forms. Termites participate in nutrient recycling by grinding, decomposition, humification, and mineralization of cellulosic resources and their variants, because they have appropriate digestive mechanisms capable of metabolizing different biopolymers found in wood, fruits, tubers, crops, and soil components (Tayasu et al., 1997; Hartke and Baer, 2011).

Lignocellulosic materials are degraded by termites since these insects produce cellulases and hemicellulases. The cellulases are usually classified as endoglucanases, exoglucanases and β-glucosidases. Endoglucanases cleave randomly glycoside bonds within the cellulose molecule, hydrolyzing more quickly amorphous regions, where there are fewer and/or weaker hydrogen bonds. The exoglucanases act on the extremities of cellulose chain, not only mainly producing cellobiose but also glucose and cellotriose. The β-glucosidases, also known as cellobiases, catalyze the hydrolysis of cellobiose released by exoglucanases and endoglucanases (Fischer et al., 2013).

The hemicelulases are enzymes that hydrolyze the complex hemicellulose net. Xylanases are responsible for degrading the xylan fraction producing xylobiose and several types of xylo-oligosaccarides. Next, the β-xilosidases cleave the xylobiose and the oligosaccharides until xylose. The α-L-arabinofuranosidases hydrolyze non-reducing terminals containing L-arabinosil residues of polysaccharides such as arabinoxylans, arabinans and arabinogalactans (Saha, 2000; Moreira and Filho, 2008).

The high efficiency of the lignocellulolytic systems found in the termite gut makes their cellulases and hemicellulases important models to be studied for use in the processing of lignocellulosic biomass for biofuel production, for example (Sun and Scharf, 2010; Scharf et al., 2011; Mathew et al., 2013). Cellulases are also employed in the production of pharmaceuticals and detergents, wastewater treatment, and the processing of fruits and vegetables (Mamma et al., 2009).

In addition to cellulose, termites are able to degrade other glucose polymers such as starch and glycogen through the action of amylases (Waller and La Fage, 1986). The amylases are enzymes that hydrolyze α-1,4-glycosidic bonds in amylose chains producing glucose, maltose, and maltotriose units. These enzymes are biotechnologically applied

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in food, paper, detergent, and pharmaceutical industries, among others (Hmidet et al., 2009; Souza and Magalhães, 2010).

Soil is composed of a large proportion of hydrolyzable peptides, and up to 20% of the total organic carbon adsorbed on soil particles may be from peptidic carbon (Kelley and Stevenson, 1996; Knicker et al., 2000). Ji and Brune (2005) showed that the combined action of extreme alkalinity in the hindgut, autooxidative processes, and proteolytic activity make soil-feeding termites able to use peptidic components of humic acids as a source of carbon and energy. Thus, the gut of soil-feeding termites may be an important source of proteases. These enzymes may have several applications, for example, in leather processing, the production of biodegradable plastics, milk-clotting, meat maturation, and enzymatic synthesis of sweeteners (Naveena et al., 2004; Ogino et al., 2008; Haddar et al., 2009; Merheb-Dini et al., 2009).

In higher termites (family Termitidae), digestive enzymes are encoded by the termite genome or produced by gut symbiotic bacteria, such as spirochetes and fibrobacters (Warnecke et al., 2007). Amino acid sequencing, cloning, and expression of enzymes in heterologous systems allow the production of termite enzymes on a large scale through genetic engineering techniques (Olempska-Beer et al., 2006).

Apart from the biotechnological potential, the characterization of digestive enzymes from termites can contribute to the development of new insecticides. Natural insecticides derived from plants, such as enzyme inhibitors, lectins, and secondary metabolites, are known to interfere in digestive enzyme activities. One study showed that *Microgramma vaccinifolia* rhizome lectin possessed termiticidal activity, and was able to promote imbalances in the activities of trypsin-like protease, acid phosphatase, and cellulase from the gut of termites (Albuquerque et al., 2012).

Nasutitermes corniger (Termitidae) is a soil-feeding and wood-damaging termite species. It is considered a threat to urban centers and has been favored by environmental imbalance (Figueiredo, 2004). However, reports on digestive enzymes from this species are scarce. Napoleão et al. (2011) and Albuquerque et al. (2012) have reported the detection of trypsin, amylase, cellulase, and phosphatase activities in the gut of *N. corniger*, but to our knowledge, there is no other information about the characteristics of these and other digestive enzymes. Tokuda et al. (2012) studied the wood digestion of other species from this genus, *Nasutitermes takasagoensis*, and reported that wood fragments are firstly broken by mastication and salivary β-glucosidases, followed by the digestive action of endoglucanases and β-glucosidases in the midgut luminal space.

This paper describes the assessment and partial characterization of cellulolytic (endoglucanase, exoglucanase, and β-glucosidase), hemicellulolytic (β-xylosidase, α-L-arabinofuranosidase, and β-D-xylanase), amylolytic (α-amylase), and proteolytic (trypsin-like, chymotrypsin-like, and keratinase-type proteases) activities in gut extracts of *N. corniger* workers and soldiers.

2. Materials and methods

2.1. Insects

Colonies of *N. corniger* were collected at an Atlantic Forest fragment located at the campus of the *Universidade Federal Rural de Pernambuco*. The authors have authorization from the *Instituto Chico Mendes de Conservação da Biodiversidade* from Brazilian Ministry of the Environment for termite collection (number 36301-2). Termite colony was selected according to overall integrity criteria and the nest was carefully removed from the trunk of a tree using a machete and transferred to laboratory packaged into a black plastic bags. The colony was maintained at 28 ± 2 °C (70 ± 5% relative humidity) in the dark during 6 h. In this period, the workers and soldiers were collected and separated for use in preparation of termite gut extracts.

2.2. Chemicals

Acrylamide, Avicel, azocasein, *N*-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA), bovine serum albumin, carboxymethylcellulose (CMC), casein from bovine milk, citric acid, Coomassie Brilliant Blue R-250, 3,5-dinitrosalicylic acid (DNS), ethylene-diaminetetraacetic acid (EDTA), D(+)-glucose, glutaraldehyde, *N*,*N*′-methylenebis(acrylamide), ρ-nitrophenyl-α-L-arabinofuranoside (ρNPAraf), ρ-nitrophenyl-β-D-glucopyranoside (ρNPG), ρ-nitrophenyl-β-D-xylopyranoside (ρNPX), ρ-nitrophenol, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), sodium bicarbonate, sodium dodecyl sulphate (SDS), N-succinyl–alanyl–alanyl–alanyl–ρ-nitroanilide (Suc–Ala–Ala–Ala–pNA), N-succinyl–L-phenylalanine–ρ-nitroanilide (Suc–Phe–pNA), trans-epoxysuccinyl-L-leucyl-amido (4-guanidino)butane (E-64), trishydroxymethylaminomethane (Tris), xylan and D(+)-xylose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, HCl, dibasic sodium phosphate, monobasic sodium phosphate, NaCl, and trichloroacetic acid were purchased from Vetec (Rio de Janeiro, Brazil). Ammonium persulphate, calcium chloride, sodium acetate, NaOH, soluble starch, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and Triton X-100 were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.3. Termite gut extracts

Groups of workers or soldiers of *N. corniger* were immobilized by placing them in a freezer at −20 °C for 10–15 min. Each termite was decapitated using an 8-mm-long, 0.3-mm needle (BD Ultra-Fine II from Becton, Dickinson and Company, NJ, USA) and had its gut removed intact by pulling of the last abdominal segments. Next, the guts were stored on ice in 0.1 M sodium acetate pH 5.5 or 0.1 M Tris–HCl pH 8.0, both containing 0.15 M NaCl.

N. corniger gut extracts were obtained according to Napoleão et al. (2011). A group of 100 guts from workers or soldiers was placed in a 2-mL glass tissue grinder and manually homogenized with 1 mL of the buffer solution used in dissection. The homogenates were then centrifuged at 9000 *g* at 4 °C for 15 min. The collected supernatants (worker or soldier gut extracts) were used for evaluation of enzyme activities. The extracts in sodium acetate buffer were used in assays for cellulase, hemicellulase and α-amylase activities while extracts in Tris buffer were used in the assays for protease activities. Protein concentration of extracts was determined according to Lowry et al. (1951) using bovine serum albumin (31.25–500 μg/mL) as standard. For each buffer, three independent extractions (using termite groups from distinct nests) were performed and thus three distinct pools (extracts) were evaluated in the experiments. Extracts in the same buffer had their protein concentrations adjusted to the same values in order to standardize the concentrations and volumes in enzyme assays.

2.4. Cellulase activities

Assays for endoglucanase and exoglucanase activities were carried out according to adaptations of the methods described by Li et al. (2009) and Wood and Bhat (1988), respectively. The reactions started by incubating (50 °C, 10 min) gut extract from workers (100 μL, 330 μg of protein) or soldiers (100 μL, 270 μg of protein) with 400 μL of 1% (w/v) CMC (for endoglucanase activity) or 1% (w/v) Avicel (for exoglucanase activity) in 0.1 M sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 μL of DNS was added to stop the reaction and the mixtures were heated (100 °C, 6 min) and immediately cooled in ice (15 min). Then, the absorbance at 540 nm was measured. The amount of reducing sugars was determined using glucose as standard ($Y = 0.1261X - 0.0157$; Y is the absorbance at 540 nm; X is the glucose concentration in mg/mL). One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of glucose per minute. Blanks were performed submitting worker and soldiers