Eur. J. Soil Biol., 1993, **29** (1), 7-11

Activity and origin of digestive enzymes in gut of the tropical earthworm *Pontoscolex corethrurus*

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Received November 11, accepted January 5, 1993.

Abstract

Activities of glucidic digestive enzymes in the gut (content plus walls) of a tropical endogeic earthworm, *Pontoscolex corethrurus,* have been assayed. In order to determine the origin of the enzymes found in the gut, the wall tissues were cultured *in vitro,* and enzymatic activities were measured both in the cultured tissues and in the culture medium.

The earthworm possesses a weak but quite complete enzyme system. In the gut, the enzymes were capable of degrading the following substrates: heteroside (N-acetylglucosamine), oligosaccharides (maltose, laminaribiose) and polysaccharides (starch, laminaran, pullulan, microcrystalline cellulose, carboxymethylcellulose, mannan, glucomannan and caroub galactomannan, lichenin). The strongest enzymatic activities were located in the foregut and midgut.

Among the main enzymes found in the gut, cellulase and mannanase were neither detected, in the cultured tissues nor in the culture medium, which indicates that these two enzymes were produced by micro-organisms ingested with the soil. The oligosaccharidase and heterosidase activities were higher in the cultured tissues than in the medium, which was not the case for the polysaccharidases.

Keywords: Earthworm, glucidic digestive enzymes, *Pontoscolex corethrurus, in vitro* tissue culture, ingested micro-organisms.

Activité et origine des enzymes digestifs dans l'intestin du ver de terre tropical Pontoscolex corethrurus.

Résumé

Les activités d'enzymes digestifs glucidiques de l'intestin (contenu et paroi) d'un ver de terre endogé tropical, *Pontoscolex corethrurus,* ont été testées. Afin de déterminer l'origine des enzymes identifiés dans l'intestin, les tissus constituant les parois ont été mis en culture *in vitro* et les activités enzymatiques ont été mesurées à la fois dans les tissus en culture et dans le milieu de culture.

Le ver de terre possède un équipement enzymatique assez complet mais de faible niveau d'activité. Dans l'intestin, les enzymes ont été capables de dégrader les substrats suivants : hétéroside (N-acétylglucosamine), oligosaccharides (maltose, laminaribiose) et polysaccharides (amidon, laminarine, pullulane, cellulose microcristalline, carboxyméthylcellulose, mannane, glucomannane et caroube galactomannane, lichénine). Les activités enzymatiques les plus intenses ont été localisées dans l'intestin antérieur et moyen.

Parmi les principaux enzymes mis en évidence dans l'intestin, la cellulose et la mannanase n'ont été détectées ni dans les tissus en culture ni dans le milieu de culture, ce qui indique que ces deux enzymes ont été produits par des micro-organismes ingérés avec le sol. Les activités de l'oligosaccharidase et de l'hétérosidase ont été plus élevées dans les tissus en culture que dans le milieu de culture, ce qui n'a pas été le cas pour les polysaccharidases.

Mots-clés : ver de terre, enzymes digestifs glucidiques, *Pontoscolex corethrurus*, culture tissulaire, micro-organismes ingérés.

Eur. J. Soil Biol., 93/01 07 05/$ 2.50/© Gauthier-Villars

INTRODUCTION

Tropical geophagous earthworms seem to exploit soil organic resources by a mutualist earthworm/microflora digestion system (Lavelle *et al.*, 1983). Earthworms enhance microbial activities by providing in their gut a mucus consisting of energetic and easily metabolizable compounds (Martin *et al.*, 1987) and favourable physico-chemical conditions: neutral pH, high moisture and temperature conditions (Barois and Lavelle, 1986).

Earthworms seem to have poor proper enzymatic systems and they appear to rely upon the ingested micro-organisms to degrade soil organic matter. Although lipase, protease, amylase, lichenase, chitinase and cellulase activities have been reported in earthworms by some authors (cf. Laverack, 1963; Tracy, 1951), the studies were carried out only in temperate earthworms of the family Lumbricidae and quantitative studies have been limited to cellulase and chitinase (Devigne and Jeuniaux, 1961; Parle, 1963; Tracy, 1951; Loquet and Vinceslas, 1987; Urbasek, 1990). Furthermore, the origin of the enzymes and the respective roles of earthworm and ingested micro-organisms in their production have not really been addressed. In fact, Parle (1963) found that the time of a gut transit was too short to allow a significant multiplication of micro-organisms. He therefore concluded that the enzymes were produced by the earthworm rather than by micro-organisms. Loquet and Vinceslas (1987) observed cellulase activities of gut walls washed of gut content, but they did not determine the origin of the enzyme since washing had not eliminated all the cellulolytic bacteria.

The aim of this study was to identify enzymatic activities in the gut of the tropical earthworm *Pontoscolex corethrurus* and to determine whether these enzymes were produced by the worm itself or by the micro-organisms contained in the ingested soil. Enzymatic activities have been measured in the gut (contents plus walls) and in the tissue culture of gut walls (the cultured tissues and the culture medium).

MATERIALS AND METHODS

Adult earthworms and soil for laboratory culture were collected at Palma Sola (Veracruz, Mexico). The experiments were done in 2 steps: the first consisted of assaying the enzyme activities in the gut (walls plus content); the second, of determining the origin of the major enzymes by *in vitro* tissue culture. The foregut and midgut were cultured separately.

Preparation of enzyme solutions

1. Gut walls and contents

After dissection and washing in ice-cold distilled water, the earthworm guts were frozen and separated into two parts. The anterior part comprised the pharynx, œsophagus, crop and gizzard; the posterior part was further divided into three equivalent portions refereed to as foregut, midgut and hindgut. For each of the four sections, tissues and gut contents of nine to ten individuals were grouped and crushed in 5 ml of ice-cold distilled water with a microcrusher. The homogenate was then centrifuged for 20 mn at 15 000 rev. min^{-1} at 4°C, the supernatant was dialysed against distilled water for a night at 4°C to eliminate the reducing sugars and the solution obtained was used as the enzyme source.

2. *In vitro* culture of intestinal tissues

Earthworms were dissected and foregut and midgut wall samples were taken. The gut wall tissues were weighed after having been washed free from intestinal content with a Holtfreter solution containing a bactericide (specilline) and a fungicide (fungizone). The culture medium was composed of Holtfreter solution, agar-agar, glucose, chicken embryo extracts, specilline and fungizone (Lattaud, 1983). It was heated for 30 mn at 65°C so as to destroy any enzymes possibly brought along with the chicken embryo. Kinetics of enzyme secretion were observed over a period of 4 to 144 h in a preliminary experiment. The duration of culture was then fixed to 72 h where the maximum secretion had occurred. At the end of the culture, the tissues and the culture medium were frozen. Enzyme solution from tissues was prepared as indicated for the gut (contents plus walls); the culture medium was dialysed directly and used for determination of the enzymatic activities.

Choice of substrates

A large range of synthetic heteroside, oligosaccharide and polysaccharide substrates have been tested. They represent a range of vegetal materials.

1. Synthesis heterosides:

PNP-α-D-glucopyranoside, ONP-β-D-glycopyranoside, PNP-β-D-glucuronide, ONP-β-xylopyranoside, PNP-N-acetyl-β-D-glucosamine, PNP-β-D-mannopyranoside, ONP-α D-galactopyranoside, PNP-β-D-galactopyranoside

2. Oligosaccharides:

Lactose, saccharose, maltose, cellobiose, laminaribiose, salicin, raffinose, gentiobiose

3. Polysaccharides:

– Starch: this polymer of glucoses with α (1→4) link is a reserve material present in seeds, bulbs and tubers.

– Microcrystalline cellulose: polymer of glucose with β (1→4) link; it is a principal constituent of plant cell walls.

– CMC (carboxymethylcellulose) is a synthetic cellulosic substrate.

– Laminaran: this polymer of glucoses with β (1→3) link, is a reserve of brown seaweeds and a constituent of cell walls of fungi.

– Lichenin is a polymer of glucoses with β (1→4) and β (1→3) links, it is a reserve substance present in moss and lichens.

– Mannan: this polymer of mannoses with α (1→2) and α (1→6) links is present in plant cell walls.

– Glucomannan are polymers of mannose and glucose linked with β (1→4) and are frequent in coniferous woods.

– Galactomannan of caroub and lucerne: polymers of mannoses with β (1→4) link, the polymannosidic central chain is substituted by galactoses linked in α (1→6) fashion. The mannose/galactose ratio of caroub galactomannan is 4, that of lucerne galactomannan is 1. Galactomannan is a constituent of cell walls and plant albumen.

– Xylan: polymer of xyloses in β (1→4) mode, with several ramified arabinose units; it is a predominant constituent in the lignified tissues of angiosperms.

– Pullulan: polymers of fungal origin having α (1→6) and α (1→4) links.

– Arabinogalactan: polymers of galactoses and arabinoses, abundant in tree latex.

Enzyme assays

Enzyme activities were assayed according to the methods described by Rouland (1986). The enzyme solution was incubated with substrates at 37°C in MacIlvain (1921) buffer at pH 6.8 which is that of the gut contents; the duration of incubation was 15, 20, 30 and 60 mn for heterosides, oligosaccharides, soluble polysaccharides and insoluble polysaccharides respectively. At the end of the incubation, for heterosidases, 2% Na_2CO_3 was added and the nitrophenolate released was then determined by reading at 400 nm (PNP glucosides) or 420 nm (ONP glucosides) in a spectrophotometer; for oligosaccharidases, glucose liberated from oligosaccharides was determined by the glucose oxidase method (Werner *et al.*, 1976); for polysaccharidases, reducing sugars released were assayed by the Somogyi-Nelson microdosage technique (Nelson, 1944; Somogyi, 1945). The protein content of the enzyme solution was measured by the Sedmak and Grossberg method (1977). Enzymatic activities were expressed as µg glucose released per mg of protein per minute.

RESULTS

1. Heterosidase and oligosidase activities in the gut.

Among the heterosides and oligosaccharides studied, only a few substrates were significantly degraded in the intestine of the earthworm. N-acetylglucosaminidase was highest among the heterosidases detected (*fig.* 1 A); concerning oligosaccharidases, only maltase, laminaribiase and cellulase activities were detectable (*fig.* 1 B). For both heterosidases and oligosaccharidases, the maximum activities were localized in the foregut and in the midgut.

2. Polysaccharidase activities in the gut.

All the polysaccharides analysed were more-or-less broken down, except arabinogalactan and lucerne galactomannan (*fig.* 2). Like the heterosidases and oligosaccharidases, the maximum polysaccharidase activities were found in the foregut and midgut.

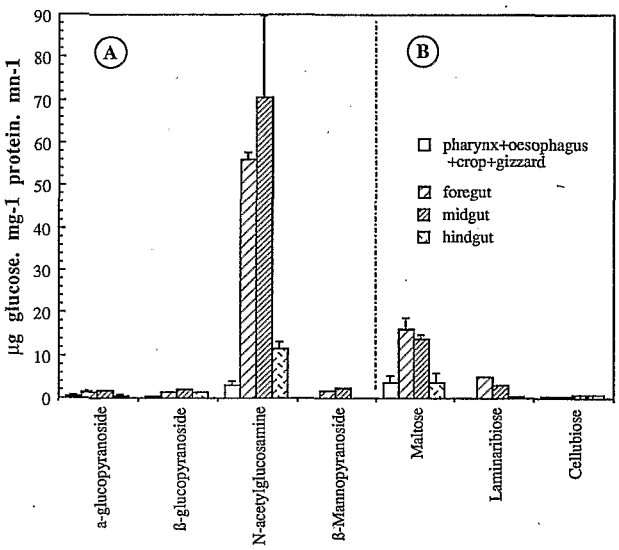

Figure 1. – Glycosidase activities in the intestine of *Pontoscolex corethrurus* measured at pH 6.8 and 37°C. Mean of two independent assays ± standard error. A: heterosidases, B: oligosaccharidases.

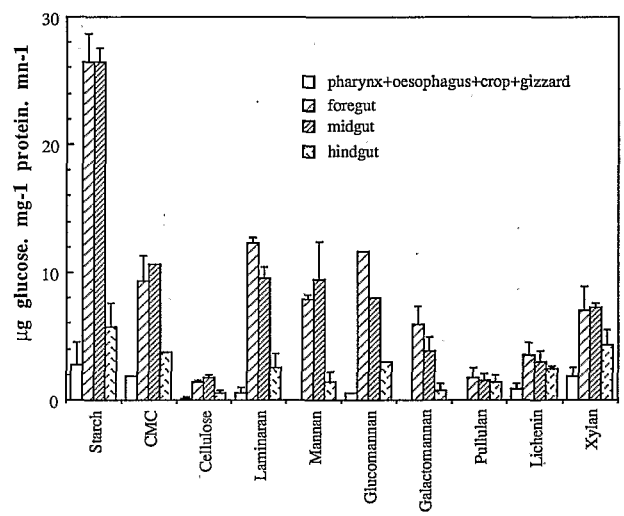

Figure 2. – Polysaccharidases activities in the gut of *Pontoscolex corethrurus* measured at pH 6.8 and 37°C. Mean of two independent assays ± standard error.

3. Enzyme activities in tissues and culture medium.

The quantities of cultured tissues and the culture medium did not allow a quantitative determination of the enzyme activities. Except for cellulase and mannanase, the major enzymes found in the gut were present in the culture tissues and medium (Table 1). For maltase and N-acetylglucosaminidase, activities were more important in the tissues than in the culture medium.

Table 1. – Enzyme activity in the cultured tissues and in the culture medium. 0: Non-detectable activity; +: weak activity; ++: high activity.

	Laminar.	Amylase	Maltase	N-Acetylgluc.	Xylanase
cultured tissues	+	+	++	++	+
culture medium	+	+	+	+	+

	CMCase	Cellulase	Mannanase	Galactoman.
cultured tissues	+	0	0	+
culture medium	+	0	0	+

DISCUSSION

For the first time, a systematic study of the glucidic enzyme activities in a tropical earthworm has been conducted. The results obtained suggest that *Pontoscolex corethrurus* possesses a rather complete enzymatic system. Nonetheless, when compared with the snail *Helix aspera* (cf. Charrier and Rouland, 1992) and xylophagous and fungus-growing termites (cf. Rouland, 1986), this earthworn has a weak enzyme spectrum, especially for cellulase, hemicellulases, cellobiase, and β-D-glucopyranosidase, much like the humivorous termites (cf. Rouland, 1986). The weak enzymatic activities of the earthworm are coherent with its feeding habitats; being an endogeic earthworm, *Pontoscolex corethrurus* feeds on soils poor in organic matter and litter debris. A similar conclusion was formulated by Urbasek (1990) who reported that cellulase activities of endogeic *Lumbricidae* were inferior to those of epigeic ones.

Pontoscolex corethrurus possesses N-acetylglucosaminidase, laminarinase and laminaribiase involving in the degradation of β-1,3 glucan and chitin sub-units which constitute the fungal cell walls. This shows that the earthworm would feed on fungus and it is in concordance with the results obtained by Barois (1986) who reported that the number of fungi decreased during the intestinal transit. It is worth noting that the N-acetylglucosaminidase, which is one of most important enzymes of *Pontoscolex corethrurus*, the did not occur in the temperate earthworm *L. terrestris* Sav. (Li and Shetlar 1965).

Maltase activity was weaker than amylase activity (*fig.* 1 B and *fig.* 2). These may be a possible microbial degradation of maltose through a fermentative process which was not detectable by our methods.

In the cultured tissues and the culture medium, the absence of cellulase and mannanase activities indicated that the earthworm cannot secrete these enzymes and therefore relied on the ingested micro-organisms to degrade cellulose and mannan.

The activities of N-acetylglucosaminase and maltase in the tissues were much higher than those secreted in the culture medium, which indicates that these enzymes are mainly intracellular. In contrast, the polysaccharidase activities were localized equally in the tissues and in the culture medium, which suggests that the polysaccharidases are extracellular enzymes. This is in accordance with the fact that the polysaccharide molecules are too big to pass through the tissue membrane, and their degradation no doubt occurs in the gut content.

CONCLUSIONS AND PERSPECTIVES

The tropical earthworm *Pontoscolex corethrurus* possesses a relatively complete enzyme system although the enzyme activities are rather weak compared with other animals. The respiratory quotient of *Pontoscolex corethrurus* (RQ=0.816, A. Grandval, personal communication) suggests that the alimentation of the worm was a mixture of glucidic metabolites (RQ=1) and more reduced compounds, like lipids and aromatic hydrocarbons (with RQ inferior to 1). Our work was limited to glucidic digestive enzymes; further study will extend to other enzymes such as lipases, proteases and ligninolytic enzymes.

Although the *in vitro* tissue culture lets us conclude that the earthworm secretes numerous enzymes, this species does rely on the ingested microflora to exploit some resources. Under the present conditions of tissue culture, there were only a few enzymes synthesized. Further work is necessary to quantify the enzymes produced by earthworm intestine and by the ingested microflora.

Acknowledgments

The authors wish to thank Dr. Isabelle Barois, Instituto de Ecologia Mexico, for providing the earthworms and soils for laboratory culture. Dr. Spencer Brown, Institut des Sciences Végétales, CNRS, is gratefully acknowledged for the linguistic revision.

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