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The Role of the Earthworm, *Pheretima (Metaphire) hilgendorfi*, in Terrestrial Ecosystem Nutrient Cycling

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Abstract—In the gut tissues and contents of the earthworm *Pheretima (Metaphire) hilgendorfi*, cellulase activity which contributes to the terrestrial ecosystem nutrient cycling has been detected. The origin of the cellulase production, whether it is the earthworm itself or the microorganisms, has not been established at the molecular level. In our study, a cellulase [endo-β-1,4-glucanase (EGase), EC3.2.1.4] gene was identified from the intestine tissue of earthworm *P. (M.) hilgendorfi*. The cDNA encoding EGase of *P. (M.) hilgendorfi* (phhEG) is 1,606 bp with an open reading frame of 449 bp, and belonging to glycoside hydrolase family (GHF) 9. The deduced amino acid sequences of the phhEG showed a 74% identity to another earthworm *Eisenia andrei*, and a 31% identity to the bacterium *Thermobifida fusca*. The recombinant phhEG produced by wheat germ cell-free protein synthesis system showed an enzymatic activity. The result of zymogram analysis of phhEG showed that the band of enzyme activity appears to have a molecular weight of 50 kDa, which is very similar to the value detected from the intestine tissues and contents extracts of *P. (M.) hilgendorfi*. These results suggested that the cellulolytic enzyme which functionally degrading cellulose is secreted by the earthworm themselves and not dependent on the symbiotic and ingested microorganisms.

Keywords: cellulase, Megascolecidae, organic matter decomposition, symbiotic microorganisms

INTRODUCTION

In terrestrial ecosystems, earthworms are very important components since they dominate the invertebrate biomass in the soil. Earthworm activities, e.g. feeding and casting, significantly promote organic matter decomposition and enhance mineralization of soil organic matter in terrestrial environment.

In previous studies, some enzymes (e.g. amylase, protease, phosphatase and cellulase) which decompose soil organic materials were detected in the gut contents of earthworm (Zhang *et al.*, 2000; Tillinghast *et al.*, 2001).

Some studies on the origin of the digestive enzymes in the gut of earthworm have shown that *Hormogaster elisae* (Garvín *et al.*, 2000) and *Pontoscolex corethrurus* (Zhang *et al.*, 1993) cannot produce the cellulase that hydrolyzes cellulose which is the major component of plant cell wall, since these earthworms possess a mutualistic earthworm-microorganisms digestive system (Trigo *et al.*, 1999). On the other hand, *Polypheretima elongata* has been shown to have a potential capacity to synthesize cellulase (Lattaud *et al.*, 1998).

However, it is not yet clarified at molecular level whether the origin of cellulase comes from the earthworms themselves or the symbiotic and/or ingested microorganisms. Our study was done to assess the origin of the cellulase, and to elucidate the role of earthworms alone and their capacity to degrade organic matters in soil ecosystems.

MATERIAL AND METHOD

Animals

P. (M.) hilgendorfi were collected from the surface soil covered with litters at Matsuyama (Ehime, Japan).

After dissection in physiological solution, the gut was removed and divided into six sections: the anterior tract that comprised the pharynx (p), oesophagus (o), crop (c) and gizzard (g); the fore tract (foregut) from the area just posterior to the gizzard to the connection of the intestine caecum; the intestine caecum (i.c.); and the remaining tract which was divided into three tracts (mid-foregut, mid-hindgut and hindgut).

The tissues and contents in the different parts of the gut were homogenated separately in 10 mM sodium phosphate buffer (pH 7.5), centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatants were used for enzymatic analyses.

Screening and cloning of full-length cDNA

Partial cDNA encoding earthworm cellulase was amplified by PCR with degenerated primers designed according to a consensus sequence of other organism cellulase. Full-length cDNA encoding earthworm cellulase was screened from the cDNA library using probes synthesized based from the partial cDNA of the cellulase. cDNA sequencing from positive clone was performed using a Dual CyDye Terminator sequence kit (Amersham Biosciences) and performed on LONG-READ TOWER DNA Sequencer (Amersham Biosciences).

Northern blot analysis

Total RNA isolated from the gut tissues of each of the six sections, were fractionated by 1.0% agarose gel electrophoresis in the presence of formaldehyde, and transferred to a nylon membrane (Amersham Hybond-N^+; GE Healthcare). After overnight hybridization at 50°C with the DIG-labeled probe synthesized using Dig Easy Hyb, the membrane was washed, immunostained with an antibody against DIG, and analyzed using a LAS-1000 mini (Fujifilm).

Table 1. Cellulase activity and expression of mhEG mRNA (signal in northern blot analysis) in the gut tissues of *M. hilgendorfi*.

	p + o + c + g	foregut	i.c.	mid-foregut	mid-hindgut	hindgut
Specific activity	518.9	1211	2314	4253	2212	358.2
(μg glucose/mg protein)	(233.7)	(175.1)	(175.1)	(358.5)	(384.5)	(194.5)
Signal in northern blot analysis	—	—	—	++	+	—

p, pharynx; o, oesophagus; c, crop; g, gizzard; i.c., intestine caecum; —, non-detectable signal; +, weak signal; ++, high signal.

Recombinant protein

The cDNA fragment encoding the open reading frame (ORF) was subcloned into pEU3b expression vector (Sawasaki *et al*., 2002). mRNA was prepared by *in vitro* transcription with SP6 RNA polymerase (Promega), and cell-free protein synthesis was performed with wheat germ extract as previously reported (Madin *et al*., 2000).

Activity measurements

EGase activity of the crude enzyme extracted from gut tissues was measured at 37°C for 24 h in 10 mM sodium phosphate buffer (pH 7.5) with carboxymethyl cellulose (CMC) as substrate. The release of glucose was monitored continuously at 490 nm.

Zymogram analysis

The crude enzyme solutions from gut tissues and contents, and the recombinant phhEG in SDS-PAGE sample buffer were applied to 10% polyacrylamide gel containing 0.1% (w/v) CMC. After electrophoresis the gel was shaken in 10 mM sodium phosphate buffer (pH 7.5) containing 2.5% Triton X-100 for 1h to remove SDS, incubated at 37°C for 15 h with 10 mM sodium phosphate buffer (pH 7.5), and stained with Congo red.

RESULT AND DISCUSSION

Zymogram analysis revealed that the cellulase activity of the extracts from the intestine tissues and contents of *P. (M.) hilgendorfi* was detected as a single band with molecular weight of 51 kDa, which indicates that one major cellulase functions in degrading cellulose (data not shown). This result suggested that *P. (M.) hilgendorfi* has either a species specific cellulase produced by the earthworm itself or a cellulase produced by species specific symbiotic microorganisms.

Therefore, we attempted to investigate the existence of a cellulase gene in earthworm. To clone the full-length cDNA of the earthworm (*P. (M.) hilgendorfi*) cellulase, a cDNA library constructed from the intestine tissue of *P. (M.) hilgendorfi* was screened, and a positive clone (phhEG) was obtained. The

deduced amino-acid sequence of phhEG comprised 1,606 nucleotides including poly(A+)tail and contained a single ORF encoding a protein of 449 amino acids. The N terminal of phhEG was found to contain an 18 amino acids signal peptide based on likelihood predictions using SignalP3.0, World Wide Web Prediction Server, Center for Biological Sequence Analysis (Nielsen *et al*., 1997), resulting in the formation of a mature enzyme composed of 431 amino acids with molecular weight of 47.1 kDa.

Comparison of the deduced amino acid sequence with the available cellulase sequences showed that the phhEG sequence was closely related to the cellulase belonging to GHE 9 of the other earthworm, *Eisenia andorei* and the Japanese sea urchin, *Strongylocentrotus nudus* (Nishida *et al*., 2007). Moreover, the amino acid residues of phhEG have conserved catalytic sites (Tomme *et al*., 1991; Tomme *et al*., 1992; Khademi *et al*., 2002) which are almost invariant among the glycoside hydrolase family (GHF) 9 of the Eukaryota (Davison and Blaxter, 2005). Based on these results, we concluded that the phhEG belongs to GHF9, and phhEG gene is found to be that of the earthworm, and not of the microorganisms.

To confirm the expression of phhEG gene at transcriptional level, Northern blot analysis was performed using total RNA prepared from the different parts of gut tissue. Hybridization signal was detected as a single band specifically in midforegut tissue. Consistently, cellulase specific activity was highest in the midforegut, followed by midhindgut (Table 1). It is possible that the midforegut in *P. (M.) hilgendorfi* is the site where endogenous EGase are mainly synthesized for degrading cellulose.

In addition, the recombinant phhEG produced using wheat germ cell-free protein synthesis system showed an enzymatic activity, which was identified as a single band in the zymogram analysis (data not shown). The molecular size of the recombinant phhEG was approximately the same as the value detected in the crude enzyme from the intestine tissues and contents of *P. (M.) hilgendorfi*. These results suggested that the cellulolytic enzymes which play functionally in degradation of cellulose in the gut of earthworm are secreted by the earthworm, and not by the symbiotic and ingested microorganisms.

Thus, in addition to a mutualistic earthworm-microorganisms digestive system, *P. (M.) hilgendorfi* alone has the capacity to degrade cellulose, similarly with *P. elongate* (Lattaud *et al*., 1997). Hence, earthworms play an important role in the nutrient cycling which is essential for environmental preservation and agricultural sustainability.

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