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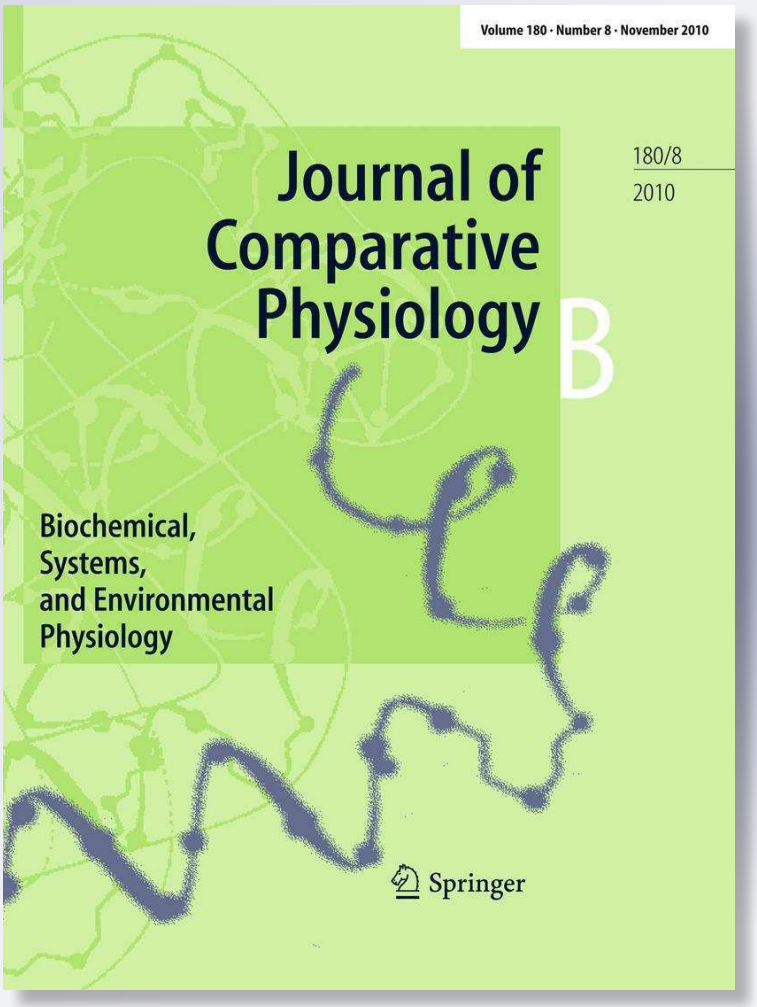

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ORIGINAL PAPER

Endogenous origin of endo-β-1,4-glucanase in common woodlouse *Porcellio scaber* (Crustacea, Isopoda)

Rok Kostanjšek · Maša Milatovič · Jasna Štrus

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Abstract Because endogenous cellulases have been observed in arthropods, the potential ability to produce cellulose degrading enzymes was examined in the terrestrial isopod *Porcellio scaber*, an important decomposer of decayed plant material. cDNA fragments encoding portions of two novel endo-β-1,4-glucanase amino acid sequences were amplified by RT-PCR, and the amino acid sequences predicted were affiliated to endo-β-1,4-glucanases from other arthropods, where they cluster with endo-β-1,4-glucanases of decapod crustaceans. Hybridization in situ reveals the hepatopancreas to be the primary site of gene expression and provides direct evidence of the endogenous origin of endo-β-1,4-glucanase in *P. scaber*. Conservation of catalytically important amino acid residues suggests that both sequences translate into functional cellulases. Cellulolytic activity was detected in hepatopancreatic extract after separation by SDS-PAGE, which included CMC as substrate. This is the first evidence of endogenous cellulases in peracarid crustaceans and gives strong support for the involvement of isopod endo-β-1,4-glucanases in the degradation of cellulose in their diet.

Keywords Cellulase · Endo-β-1,4-glucanase · Hepatopancreas · Digestive system · Isopod · *Porcellio scaber*

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Introduction

Cellulose, produced mainly by terrestrial plants and some marine algae, is the most abundant renewable organic compound on earth. It is therefore not surprising that this complex carbohydrate is utilized as a food source by many organisms, including arthropods. Such organisms must be able to break cellulose down to an utilizable energy source, usually glucose. Due to its complex structure, the degradation of cellulose requires synergistic action of several enzymes known as cellulases, which include endoglucanases (endo-β-1,4-glucanase, EC 3.2.1.4.), exoglucanases (cellulose 1,4-cellobiosidase, EC 3.2.1.91) and cellobiases (β -glucosidases, EC 3.2.1.21) (Béguin and Aubert 1994).

In spite of circumstantial evidence suggesting the endogenous origin of cellulases in higher animals (Watanabe and Tokuda 2001), the cellulolytic capability of arthropods has been traditionally assigned to symbiotic protozoa and bacteria in the arthropods' digestive systems. Direct evidence of the endogenous origin of cellulases in arthropods appeared only in the past decade, when the endogenous cellulase gene was identified in the termite *Reticulitermes speratus* (Watanabe et al. 1998). After this initial report, cellulase genes were characterized in other arthropods, mainly termites and cockroaches (Tokuda et al. 1999; Lo et al. 2000; Nakashima et al. 2002; Zhou et al. 2007; Shimada and Maekawa 2008), beetles (Girard and Jouanin 1999; Sugimura et al. 2003; Lee et al. 2004; Kim et al. 2008) and herbivorous decapod crustaceans (Byrne et al. 1999; Linton et al. 2006; reviewed in Davison and Blaxter 2005). Currently numerous diverse animal cellulases have been recognized and belong to five glycosyl hydrolase families (GHF), namely GHF5, GHF6, GHF10, GHF45 and GHF9 (Watanabe and Tokuda 2001, 2010; Davison and Blaxter 2005). While the first four GHF families include

relatively few or no arthropod-derived members, numerous arthropod cellulases have been reported in the GHF9 family, especially in the subgroup E2, which besides arthropods also include endoglucanase genes from cnidarians, mollusks, annelids, echinoderms and chordates (Watanabe and Tokuda 2001, 2010; Davison and Blaxter 2005).

Terrestrial isopods, including the common woodlouse *Porcellio scaber*, Latreille 1804, are ubiquitous and effective herbivorous scavengers. They feed mainly on decayed plant material and contribute significantly to the decomposition of cellulose and other complex organic compounds in their diet (Zimmer 2002). Due to easy handling and ecological importance of terrestrial isopods, a substantial amount of data on their role in cycling of organic matter has been gathered in previous decades. They contribute indirectly to decomposition by promoting microbial cellulolytic activity, which includes fragmentation of the substrate, increasing the number of ingested microorganisms in their gut and distributing microbes with cellulolytic properties throughout the terrestrial ecosystems (reviewed by Zimmer 2002; Kostanjšek et al. 2006). Data on degradation of ingested cellulose by isopods are, however, fragmentary and inconclusive, due mainly to the shortcomings of the methods used. Despite early suggestions of the endogenous origin of cellulases in the tissues of the isopod *Oniscus asellus* (Hartenstein 1964), the cellulolytic properties were generally assigned to microbial activity in the digestive system of terrestrial isopod or in food (Hassall and Jennings 1975; Kozlovskaja and Striganova 1977; Kukor and Martin 1986; reviewed by Zimmer 2002). Indirect measurements of cellulolytic activity and bacterial counts in the isopod digestive glands (Zimmer and Topp 1998) led to the hypothesis of the presence of resident bacterial microbiota with cellulolytic properties in the lumen of digestive glands (Zimmer and Topp 1998; Zimmer 2002). Although neither permanent presence in the digestive glands nor cellulolytic properties of these bacteria have been proven, it is suggested that they provide the isopod with cellulases and are assigned a crucial role in the transition of isopods from sea to land (Zimmer et al. 2001; Zimmer 2002).

Apart from presumed bacterial symbionts, the successful transition from aquatic to land environment required several adaptations of the digestive system of terrestrial isopod crustaceans. These include morphological and physiological adaptations, which enabled them to utilize terrestrial food sources and reduce water loss during digestion (Warburg 1987). The digestive system of terrestrial isopods consists of a short foregut, comprising an esophagus and a stomach, and a tube-like hindgut, both lined with cuticle. The midgut of terrestrial isopods is reduced to two or three pairs of blind-ended tubular digestive glands, also known as hepatopancreas. The wall of each tube includes single-cell epithelium, which is the key site for the secretion of digestive fluids and of nutrient absorption. Digestive enzymes produced in the hepatopancreas include different carbohydrases (e.g., amylase, glycogenase, glucosidase, galactosidase, fructosidase, maltase, chitinase), dehydrogenases, esterases, lipases, arylamidases, oxidases, phosphatases and catalase (Hartenstein 1964, reviewed in Zimmer 2002). The hepatopancreas is connected to the anterior part of the hindgut, where digestive fluids are mixed with food and digestion takes place. Digested nutrients are passed through a system of filters back into hepatopancreas, where absorption takes place, while food particles and a majority of bacteria are retained in the gut (Hames and Hopkin 1989). Although the diameter of individual bacterial cells exceeds the gaps in the filter apparatus, various bacteria can avoid the filter system and colonize the hepatopancreas (Hames and Hopkin 1989, 1991; Wang et al. 2004a, b). The digestive tract of isopods lack the compartmentalization found in most of the other herbivorous arthropods (Breznak 1982), where food persists for prolonged periods, and where massive resident microbiota collaborating in food degradation could become established. Resident gut microbiota in isopods are restricted to bacteria colonizing the hepatopancreas (Kostanjšek et al. 2004, 2006; Wang et al. 2004a, b) and attached to the gut wall (Kostanjšek et al. 2007).

The aim of this study was to determine whether isopod crustaceans produce endogenous cellulases in their digestive system and directly degrade the cellulose in their diet. Endogenous production of cellulases was confirmed by cloning part of the endo-β-1,4-glucanase gene from cDNA prepared from RNA isolated from the hepatopancreas of *P. scaber*. Sequences of putative endo-β-1,4-glucanase genes from *P. scaber* were analyzed and affiliated with endogenous cellulase genes of other arthropods in phylogenetic analysis. The site of expression of endo-β-1,4-glucanase gene in digestive tissues of *P. scaber* was determined by in situ hybridization and a plausible role of endogenous cellulases in the digestion of terrestrial isopods is discussed.

Materials and methods

Maintenance of the animals

The animals were collected in their natural environment from a location near Ljubljana in central Slovenia. They were kept at 20°C in glass tanks filled with soil under conditions of high humidity and a 16-h/8-h day/night cycle. Leaf litter from the same collection site was provided as food ad libitum. Healthy adult animals of both sexes were used in the experiment.

Isolation of RNA

Total RNA was isolated from legs, hepatopancreas and hindgut of five adult animals in an Rnase-free environment. The surface of the narcotized animals was sterilized by submersion in absolute alcohol and a small burn. Animals were decapitated by removing the cephalothorax with the attached stomach and all four hepatopancreatic lobes from the rest of the body. All seven pairs of pereopods were carefully dissected from the body. The cephalothorax and the stomach were removed from the glands. The hindguts were removed from decapitated animals with fine-tipped forceps treated with RNaseZap (Ambion, Austin, Texas) by gentle pulling through the posterior end of the body. Hindguts were opened longitudinally and washed with DEPC-treated water to remove the gut contents. Isolated legs, hepatopancreas and washed hindguts, weighing approximately 60, 50 and 30 mg, respectively, were separately transferred in 1 ml of TRI Reagent (Sigma) and homogenized with RNaseZap-treated homogenizer. Total RNA was isolated according to the protocol for the isolation of tissue RNA with TRI Reagent (Sigma). The concentration and purity of total RNA suspended in DEPC-treated water was measured at 260 and 280 nm with ND-1000 spectrophotometer (Nano-Drop). To obtain eukaryotic mRNA from the samples, Poly(A^+) RNA was isolated from approximately 0.25 µg of each total RNA using PolyATract mRNA Isolation System III (Promega, Madison, WI) following the manufacturer's protocol for small-scale mRNA isolation.

Reverse transcription, cloning and sequencing

In a PCR tube, 8 µl of isolated mRNA was mixed with 1 µl of 50 µM oligo$(dT)_{20}$ primer and 1 µl of 10 mM dNTP mix to synthesize the first-strand cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, USA). The presence of bacterial genes in first-strand cDNA obtained from the hepatopancreas, hindgut and legs was established by amplification of bacterial 16S rRNA genes using a combination of evolutionarily conserved bacterial primer fD1 (Weisburg et al. 1991) and universal primer Uni-522 (Giovannoni et al. 1988) as described previously (Kostanjšek et al. 2007). The first-strand cDNA from hepatopancreas, devoid of bacterial genes, was used as a template for PCR amplification of a part of the endoglucanase gene using a combination of previously described degenerate primers termed F2 and R4 (Linton et al. 2006). This primer combination enables amplification of a section approximately 900 nt in length within the endo-β-1,4-glucanase gene in various arthropods including crustaceans (Linton et al. 2006; Byrne et al. 1999). To reduce the degeneracy of the primers on their 5' ends and improve the amplification rate, highly degenerate primers F2 and R4 were modified by consensus-degenerate hybrid oligonucleotide strategy (Rose et al. 2003). Modified primers were synthesized at Jena Bioscience (Germany) at our request and designated F2-hop (5′-GG GCCGGCCCGARGAYAT-GAC-3′) and R4-hop (5′-GGC GTTGTAGTCGGTGGCN ACYTCRTT-3′).

Primers were used with a touchdown PCR protocol recommended for degenerate primers designed with the Codehop algorithm (Rose et al. 2003). An initial 3 min denaturation at 94°C was followed by 20 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 66°C with 0.5°C drop per cycle and 1 min elongation at 72°C. The initial 20 cycles were followed by 20 additional cycles consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final 5 min extension at 72°C.

All PCR reactions were performed in a Mastercycler epgradient thermal cycler (Eppendorf, Germany) in 20 µl reaction volume. From each sample. 1 µl of obtained cDNA was amplified in 20 µl reaction mixture consisting of 1 U reaction buffer (Biotools, Spain), 1 µM of each primer, 10 mM of dNTP, 2.5 mM of $MgCl_2$ and 1 U of *Taq* polymerase (Biotools, Spain). PCR products were separated by gel electrophoresis in 1% agarose gel. Amplicons of expected size (approximately 900 bp) were excised from the gel and purified with a QIAquick Gel Extraction Kit (Qiagen).

Purified PCR products were cloned into pJET1.2/Blunt vector using CloneJET™ PCR Cloning Kit (Fermentas) and transformed into competent JM109 *E. coli* cells (Promega). Cloned plasmids were isolated from the cells using Wizard® *Plus* SV Minipreps DNA Purification System (Promega) and plasmids containing a PCR product were sequenced at Microsynth (Balgach, Switzerland).

The sequence data have been deposited in the GenBank sequence database under accession numbers GU120094 and GU120095 for nucleotide, and ACY70393 and ACY70394 for amino acid sequences.

Sequence analysis

The nucleotide sequences obtained were edited by ChromasPro 1.33 (Technelysium Pty Ltd.) and searches were conducted through the GenBank/EMBL/DDBJ database with the BLASTn algorithm. Protein sequence translations were conducted by MEGA 4 software (Tamura et al. 2007) and compared to the sequences deposited in GenBank, Swiss-Prot and EMBL databases using the BLASTp search algorithm.

Sequence data were aligned using Muscle multiple alignment software, version 3.6 (Edgar 2004) and checked manually. A data set of 65 unambiguously aligned amino acid sequences was used for the construction of a phylogenetic

tree. The most appropriate model of amino acid substitution was selected by testing alternative models of protein evolution using the Modelgenerator program (Keane et al. 2006). The Bayesian inference of phylogeny was generated with MrBayes software package 3.1.2 (Ronquist and Huelsenbeck 2003), using the Whelan and Goldman empirical model of protein evolution (Whelan and Goldman 2001), with gamma distributed rate heterogeneity and a significant proportion of invariable sites. The analysis was run in duplicate with four chains for 2×10^6 generations with a sampling frequency of 100 generations. The initial 500,000 generations were discarded as a burn-in.

In situ hybridization

Freshly dissected hepatopancreases of *P. scaber* were fixed in R-F fixative (Hasson et al. 1997) for 24 h and embedded in paraffin (J. T. Baker, UltraPar, Holland). Tissue sections of 8–10 µm were transferred to poly L-lysine coated slides (Menzel GmbH & Co KG, Braunschweig, Germany). Rehydrated sections were treated with 1 µg/ml proteinase K (Sigma) for 6 min at 37°C. Proteinase K activity was terminated by immersing the sections in 0.2% glycine solution. Sections were postfixed in 4% paraformaldehyde and acetylated with 0.1 M triethanolamine and 0.5% acetic anhydride for 10 min at room temperature.

Sections were prehybridized in prehybridization buffer (IsHyb In Situ Hybridization (ISH) Kit, BioChain) for 3 h at 55°C and then hybridized overnight at 58°C in hybridization buffer (IsHyb In Situ Hybridization (ISH) Kit, BioChain) containing 10 nM of LNA probe (Vester and Wengel 2004). Antisense LNA probe, homologous to cloned cDNA sequences (5′-GTACAGCCATAATGCTC CCCAAAC-3′), and scrambled LNA probe (5′-GTGTAA CACGTCTATACGCCCA-3′), used as a negative control, were designed and synthesized by Exiqon (Vedbaek, Denmark) at our request.

Post-hybridization washes were performed in 0.1× SSC with 0.1% Triton X-100, twice for 60 min at 58°C and twice for 10 min at room temperature, and in 1× ELF wash buffer (ELF® 97 mRNA In Situ Hybridization Kit, Invitrogen) for 5 min at room temperature. After washing, slides were blocked for 30 min in ELF blocking reagent (ELF® 97 mRNA In Situ Hybridization Kit, Invitrogen) followed by 1 h incubation in ELF blocking reagent with added 1 mM levamisole (Sigma-Aldrich) to quench endogenous alkaline phosphatase activity. Slides were then incubated with anti-DIG-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) diluted in ELF blocking reagent (1:500) for 30 min at room temperature. After washing in 1× ELF wash buffer, slides were incubated in the dark with ELF substrate working solution (ELF® 97 mRNA In Situ Hybridization Kit, Invitrogen) for 90 min at room temperature, quickly washed in ELF wash buffer, post-fixed in post-fixation solution (2% paraformaldehyde in PBS with 20 µg/ml BSA) and mounted with ELF mounting medium (ELF® 97 mRNA In Situ Hybridization Kit, Invitrogen). Fluorescent precipitates were observed under UV light with microscope Axioimager.Z1 (Zeiss, Germany).

Detection of the cellulolytic activity

Cellulolytic activity was detected on zymograms after SDS-PAGE separation of crude protein extract from hepatopancreas of *P. scaber*. Crude extract was prepared in three replicates, each containing isolated hepatopancreatic lobes from ten animals homogenized in 500 µl of ice cold physiological solution for *P. scaber* (Hagedorn and Ziegler 2002). The hepatopancreases were dissected during the first 16 h of the day period. After removal of larger tissue particles by centrifugation at 10,000×*g* for 10 min, crude protein extracts in supernatant were denatured for 10 min at 80°C and subjected to 10% SDS-PAGE according to Laemmli (1970), with 0.1% carboxymethylcellulose (CMC) incorporated in the electrophoresis gel. Following electrophoresis, enzymes were renatured in 50 mM Tris–HCl buffer (pH = 6.8) and 5 mM mercaptoethanol with 1 mM EDTA and incubated for 4 h at 37°C, allowing separated cellulases to digest the CMC in the gel. Cellulolytic activity was detected as clearing zones after staining of gel with Congo red (Sigma-Aldrich) (Béguin 1983). Molecular mass was determined according to the calibration curve with PageRuler SM0661 protein marker (Fermentas).

Results

A combination of modified degenerate primers, originally named F2 and R4 (Linton et al. 2006), was used to amplify an approximately 900 bp long region of endo-β-1,4-glucanase gene from RNA isolated from the legs, the hepatopancreas and the hindgut of *P. scaber*. The cDNA product of the expected length was amplified only from RNA template isolated from the hepatopancreas; amplification of cDNA from the legs and the hindgut using the same set of primers was not successful (Fig. 1). Amplification of bacterial 16S rRNA genes from cDNA using bacteria-specific set of primers, fD1 and Uni-522, confirmed the presence of amplicons of expected size (approximately 510 bp) in cDNA isolates from legs and hindgut, while bacterial RNA genes were not detected in the cDNA from hepatopancreas (Fig. 2). Sequence analysis of cloned cDNA products amplified from mRNA from the hepatopancreas yielded two similar sequences, extending for 891 (*Ps-EG1*) and 699 nt (*Ps-EG2*), respectively (Fig. 3). Comparison revealed that the sequences obtained were 98% identical, sharing

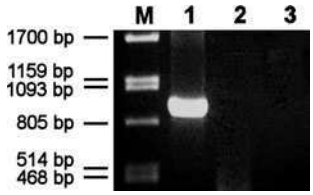

Fig. 1 PCR amplification of endo-β-1,4-glucanase fragment from c-DNA isolated from the hepatopancreas (*lane 1*), hindgut (*lane 2*) and leg of *P. scaber* (*lane 3*), using the modified pair of primers, F2 and R4. DNA markers (M) were Lambda DNA/PstI Marker (Fermentas)

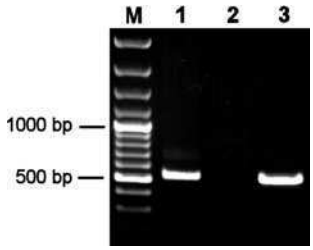

Fig. 2 PCR amplification of bacterial 16S rRNA gene fragment from cDNA isolated from the leg (*lane 1*), hepatopancreas (*lane 2*) and hindgut of *P. scaber* (*lane 3*), using the primer pair fD1 and Uni-522. DNA markers (M) were GeneRuler™ 100 bp Plus DNA Ladder (Fermentas)

685 of 699 nucleotides. Both sequences revealed the highest similarity to arthropod endo-β-1,4-glucanase gene sequences, with maximum sequence coverage of 36%, bit scores up to 109 and expected p values below $3e^{-20}$ for Ps-EG1, while for the Ps-EG2 the highest query coverage was 46%, highest bit scores were 120 and lowest expected p values $1e^{-23}$ to the nearest known sequence deposited in public databases. The comparison also revealed the presence of three catalytically important residues and two conserved regions characteristic of GHF9 in the sequence *Ps-EG1* (Fig. 3).

Translation of the cDNA sequences obtained into putative amino acid sequences confirmed the high similarity between both sequences. They share 228 of 233 compared amino acids, a 97.8% identity, with a bit score of 461 and expected p value of $3e^{-128}$. Comparison of translated sequences with amino acid sequences in public databases confirmed the highest similarity of both sequences to arthropod EG-ases from the conserved domain of glycosyl hydrolase family (GHF) 9, with the highest bit scores up to 335 and the lowest expected p value of $4e^{-90}$ for Ps-EG1, and the highest bit score of 253 and the lowest expected p value of $2e^{-65}$ for Ps-EG2.

To determine the phylogenetic position of putative endogenous endoglucanase from *P. scaber*, a phylogenetic analysis with other available arthropod endoglucanase sequences from GHF9 was conducted using a Bayesian tree method. The analyzed data set consisted of two putative amino acid sequences retrieved from *P. scaber*, endoglucanase genes from termites (33 sequences from 12 species), cockroaches (19 sequences from 8 species), other hexapods (5 sequences from 5 species) and crustaceans (5 sequences from 4 species). A single endoglucanase sequence from mollusk *Ampullaria crossean* was used as an outgroup. Sequences of insufficient length and expressed sequence tags available in public databases were not included in the analysis.

A phylogenetic analysis based on endo-β-1,4-glucanase gene sequences of arthropods in general reflects their phylogenetic affiliation. The arthropod endoglucanase genes form a monophyletic group, consisting of six deeply branched and statistically strongly supported clusters. However, the support of deep branching points within arthropod cluster is low and therefore depicted as polytomy in Fig. 4. The largest of arthropod clusters consists of termite endoglucanase genes including three sequences from the cockroach *Cryptocercus clevelandi*. The second group consists of endoglucanases from cockroach *Periplaneta ameriacana* and cricket *Teleogryllus emma*, while other cockroach endoglucanases are grouped in two separated and deeply branched clusters. The fourth cluster includes endoglucanase sequences from other hexapods (lice *Pediculus humanis*, bee *Apis melifera*, wasp *Nasonia vitripennis,* beetle *Tribolium castaneum* and pea aphid *Acyrthosiphon pisum*), whereas the last cluster includes crustacean endoglucanases. In the latter, both putative amino acid sequences from *P. scaber* cluster closely together, forming a sister group to endoglucanase sequences from decapod crustaceans of the genera *Austrothelphusa* and *Cherax* (Fig. 4). Despite the relatively long genetic distances between decapod and *P. scaber* endoglucanases, the high values of the Bayesian posterior probabilities strongly support the affiliation of the latter to endo-β-1,4-glucanase genes of other crustaceans.

To localize the expression site of putative endoglucanase genes in tissues of *P. scaber*, endoglucanase transcripts were detected by in situ hybridization. Tissue sections were hybridized by antisense LNA probe, homologous to 24 nucleotide long segments of *Ps-EG1* and *Ps-EG2* cDNA sequences (Fig. 3). The expression of endoglucanase gene was localized in the cytoplasm of hepatopancreatic epithelial cells. Observed transcripts were distributed unevenly among the hepatopancreatic cells, regardless of cell shape, and with the strongest expression localized to the apical parts of larger, dome-shaped cells. No transcripts were detected in other tissues (Fig. 5). No signal was observed after hybridization with scrambled probe, which was used as the negative control.

Cellulolytic activity was detected in crude protein extract from the hepatopancreas of *P. scaber* after separation by SDS-PAGE, which included CMC as substrate. The developed zymogram revealed almost identical patterns of clearings in all three replicates, consisting of a distinctive

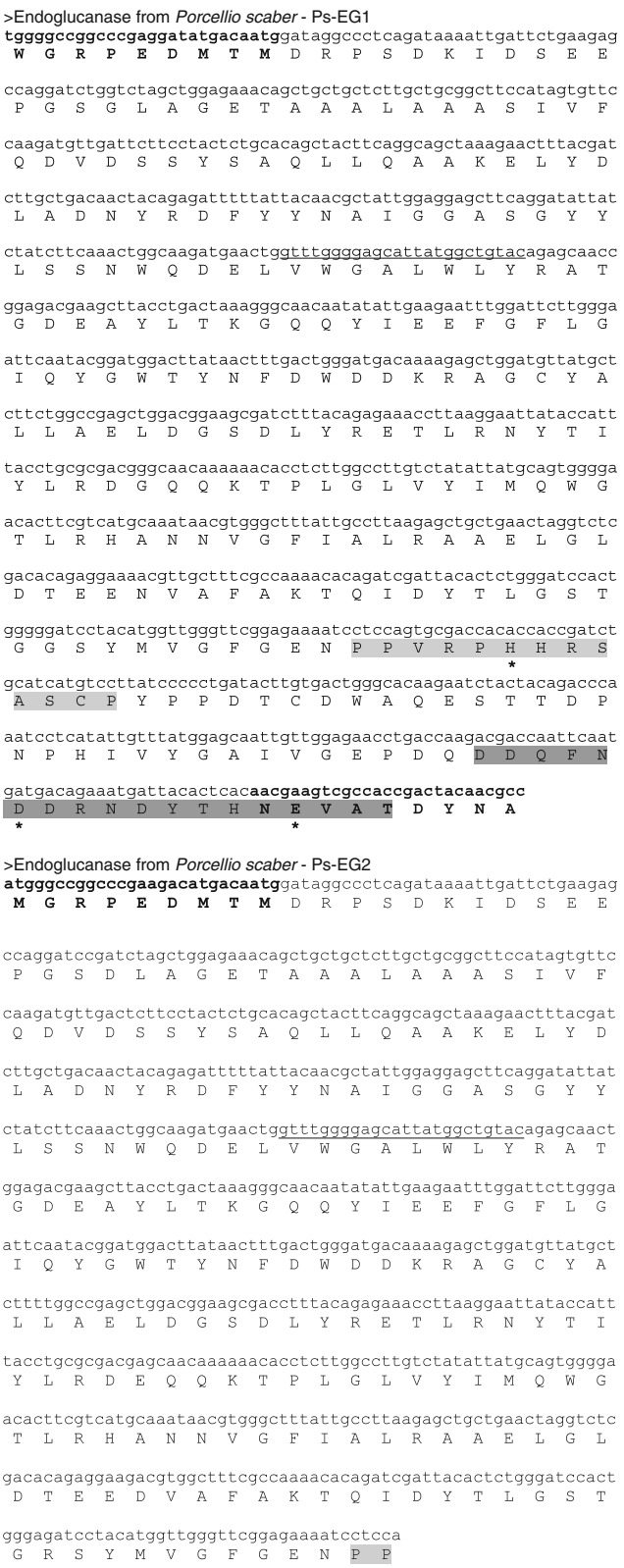

Fig. 3 Nucleotide and predicted amino acid sequences of 891 nt and 699 nt long endo-β-1,4-glucanase PCR product amplified from cDNA prepared from mRNA isolated from the hepatopancreas of *P. scaber*. Sequences homologous to primers used (F2hop and R4hop) are highlighted in *boldface*. Sites of specific oligonuclotide probe used in in situ hybridization are *underlined*. Catalytically important residues are indicated by *asterisks*. Conserved regions II and III of endo-β-1,4-glucanase gene (Davison and Blaxter 2005) are highlighted in *lighter* and *darker gray colors*

band at 26.7 kDa and several smaller bands with cellulolytic activity with molecular mass estimated between 21.4 and 17.5 kDa (Fig. 6).

Discussion

A substantial amount of direct evidence on endogenous cellulases in animals has been reported in recent years, by characterization of cellulase genes in genomes of a number of invertebrate groups including cnidarians, platyhelminths, mollusks, annelids, nematodes, echinoderms, chordates and arthropods (reviewed by Watanabe and Tokuda 2001, 2010; Davison and Blaxter 2005). In view of the ecological role of terrestrial isopods and the suggested absence of endogenous cellulases in these crustaceans (Zimmer 2002), their ability to produce endogenous cellulases was examined with a molecular approach.

Since the occurrence of endogenous cellulases has not yet been confirmed in peracarid crustaceans, a combination of modified primers, designed for amplification of a region of endo-β-1,4-glucanase gene in arthropods (Linton et al. 2006), was applied to *P. scaber* tissues to amplify a region of endo-β-1,4-glucanase gene by RT-PCR from mRNA isolated from the tissues. Obtained cDNA amplicons of expected length indicated the expression of the gene in isopod hepatopancreas and suitability of the described set of primers for detection of endo-β-1,4-glucanase gene in peracarid crustaceans as well.

The expression of the cellulase genes in the hepatopancreas itself does not unequivocally indicate their endogenous origin. Since the majority of cellulolytic activity is assigned to microorganisms, such as bacteria, fungi and protozoa, the detected cellulases might just as well originate from diverse microbiota in the isopod environment or its digestive system (Kostanjšek et al. 2002, 2004, 2006; Wang et al. 2004a, b), rather than from the animal itself. The possibility of amplification of microbial cellulases was primarily reduced by sterilization of the animal surface, dissection under sterile conditions and removal of gut contents by washing. The presence of bacterial RNA was further reduced by isolation of eukaryotic mRNA from total RNA based on polyA tail presence, and the use of a primer set designed for specific amplification of endo-β-1,4-glucanase genes of arthropods (Linton et al. 2006). Finally, the absence of bacterial transcripts in cDNA from hepatopancreas was established by PCR, using a combination of universal primers designed for the amplification of a region of

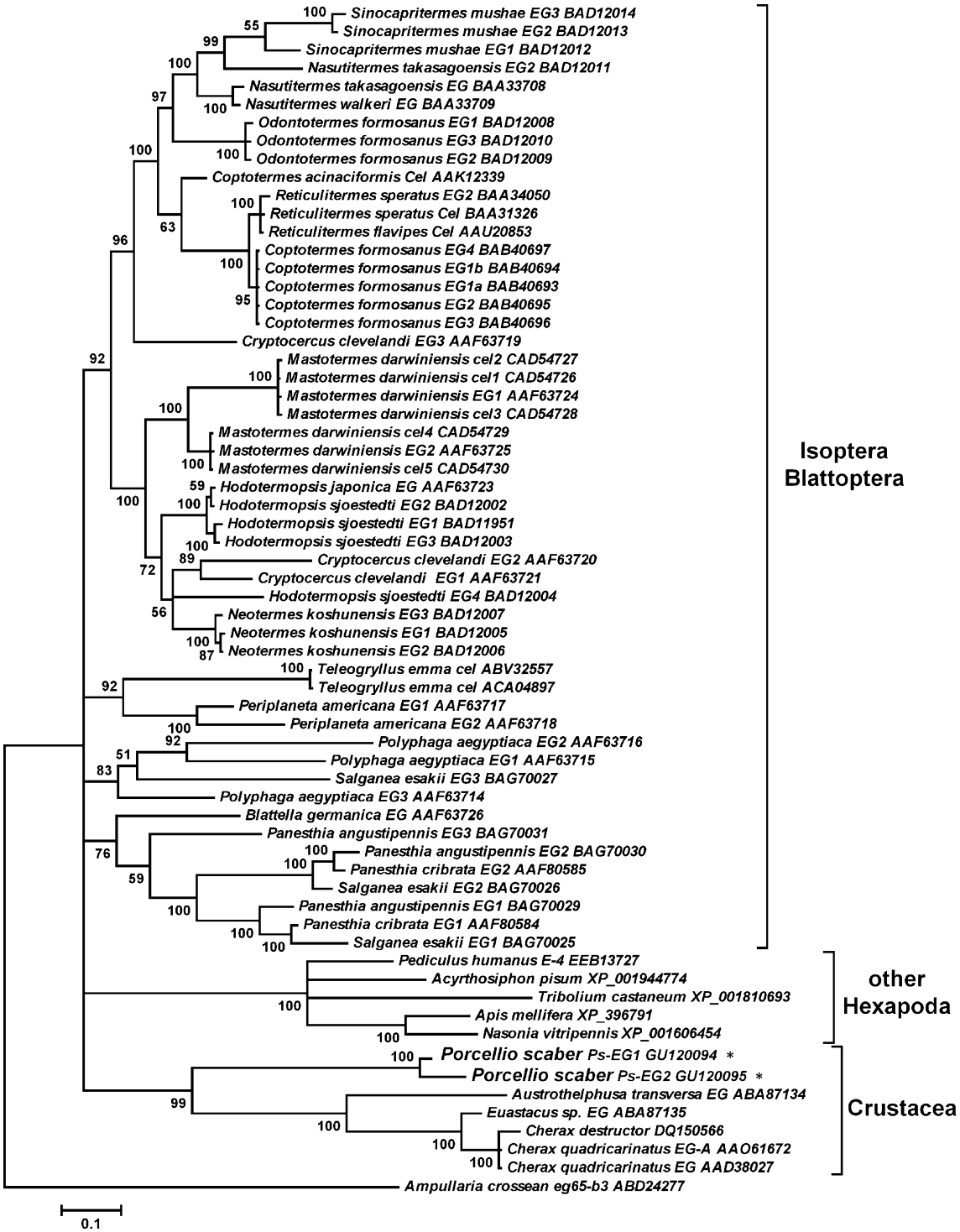

Fig. 4 Bayesian phylogenetic tree based on 297 amino acid-long segment of arthropod endo-β-1,4-glucanase genes. Each name of the species is followed by the name and accession number of the gene. The support for branching points is given as posterior Bayesian probabilities in percentages. Branches with less than 50% support have been collapsed. Sequences retrieved in the present study are marked with an *asterisk*

bacterial 16S rRNA gene. At the same time, the presence of bacterial 16S rRNA genes in cDNA transcripts from the legs and the hindgut can most probably be assigned to diverse microbiota associated with the substrate and gut wall (Kostanjšek et al. 2004).The initial indication of the endogenous origin of endo-β-1,4-glucanase in *P. scaber* was further confirmed by cloning and analysis of the cDNA sequences. Putative amino acid sequences obtained by

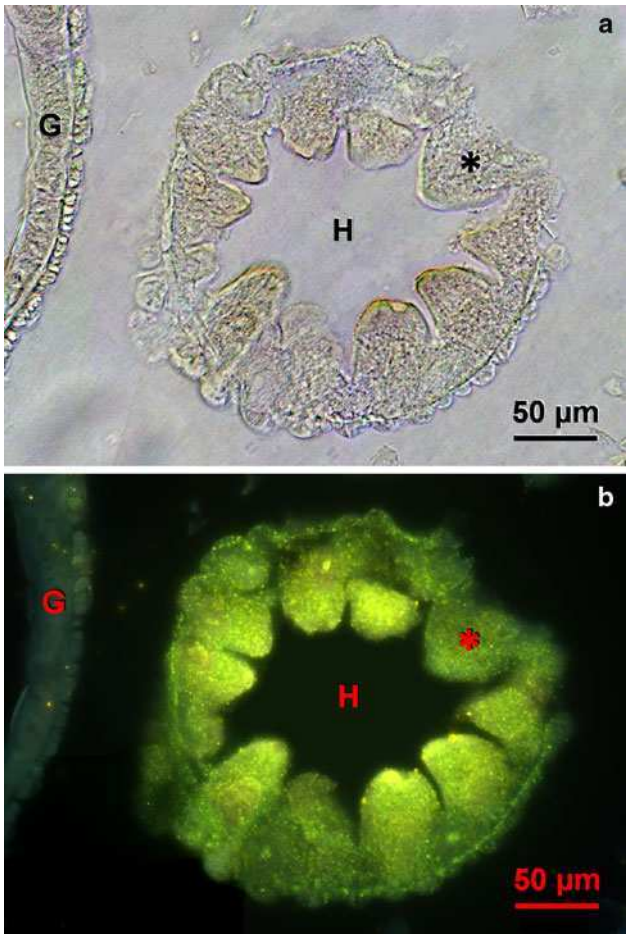

Fig. 5 Detection of endo-β-1,4-glucanase gene expression in tissues of *P. scaber* by in situ hybridization. Cross section of hepatopancreas observed by **a** differential interference contrast and **b** fluorescence, after hybridization with antisense probe homologous to Ps-EG mRNA. (*G* gut, *H* hepatopancreas, *asterisk* hepatopancreatic cell; *scale bar* 50 µm)

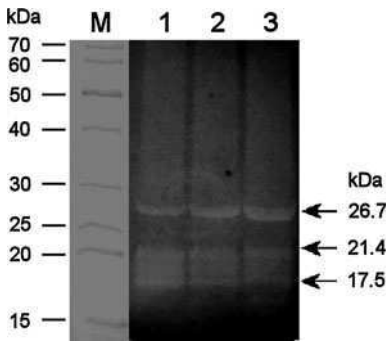

Fig. 6 Zymogram obtained with Congo red staining after incubation of renatured SDS-PAGE gels containing 0.1 MCM. Cellulolytic activity of the crude extract prepared from the hepatopancreas of *P. scaber* in three replicates (*lanes 1–3*) is indicated as clearing zones (*arrows*). Molecular mass markers (M) were PageRuler SM0661 (Fermentas)

translation of cDNA showed the highest similarity toward the GHF9 endo-β-1,4-glucanases from other arthropods. The association of the sequences obtained from GHF9 endoglucanase was further confirmed by the presence of two GHF9 specific conservative domains and most of catalytically important residues (Davison and Blaxter 2005). As in other arthropods where multiple copies of the GHF9 genes are common (Watanabe and Tokuda 2001; Davison and Blaxter 2005; Shimada and Maekawa 2008), two almost identical copies of the gene, named *Ps-EG1* and *Ps-EG2*, were detected in the genome of *P. scaber*.

Due to a substantial amount of arthropod endoglucanase sequences available in public databases and the recent evidence (Davison and Blaxter 2005) that GHF9 sequences isolated from the same animal phyla tend to group together, phylogenetic analysis has been used to reveal the affiliation of endoglucanase genes from *P. scaber* with GHF9 cellulases of other arthropods. Strong statistical support of the affiliation of *P. scaber* endoglucanase genes to endo-β-1,4-glucanase genes of other crustaceans confirms the endogenous origin of endoglucanases in *P. scaber*. Apart from minor inconsistencies, as for example the position of GHF9 sequences from cricket *Teleogryllus emma* indicated also in previous studies (Shimada and Maekawa 2008), the general topology of phylogenetic tree based on GFH9 endo-β-1,4-glucanase corresponds to currently accepted evolutionary relationships among arthropod groups, supporting the hypothesis of the presence of cellulases in a common ancestor (Lo et al. 2003; Davison and Blaxter 2005) and also indicating the potential of endo-β-1,4-glucanase gene as a genetic marker in phylogenetic studies.

Localization of the endo-β-1,4-glucanase mRNA by in situ hybridization strongly implicates the hepatopancreas as a primary site of cellulase expression in *P. scaber*. Although hepatopancreatic cells occur in different physiological states and stages of differentiation, two morphologically distinct types of epithelial cells are generally recognized in the hepatopancreas of terrestrial isopods (Hames and Hopkin 1991; reviewed in Zimmer 2002). Expression of endogenous GHF9 endoglucanases was detected in all hepatopancreatic cells, regardless of their shape. However, certain variation in the level of the endoglucanase gene expression was observed in hepatopancreatic cells. According to their physiological role, the strongest expression was localized in apical parts of large, dome-shaped B cells, involved in secretion and absorption (Bettica et al. 1984; Hames and Hopkin 1991). This result is in accordance with the localization of endogenous endoglucanases in *Cherax quadricarinatus*, where endoglucanases were detected in a subset of hepatopancreatic cells known as F cells, which are the key sites of synthesis and secretion of digestive enzymes in decapod crustaceans (Byrne et al. 1999). Otherwise, considerable variations in

the site of expression and levels of activity of GHF9 endoglucanases were demonstrated in arthropod digestive systems. Thus, cellulases can be expressed in hepatopancreas (Byrne et al. 1999), salivary glands (Watanabe et al. 1998), midgut glands (Tokuda et al. 1999), both, salivary and midgut glands (Nakashima and Azuma 2000; Nakashima et al. 2002; Tokuda et al. 2004), the salivary and foregut glands (Zhou et al. 2007), or even through the entire digestive tract (Kim et al. 2008). However, the expression of GHF9 endoglucanases in arthropods is in general associated with tissues involved in the synthesis of digestive enzymes. From this perspective, the expression in hepatopancreatic cells observed in *P. scaber* is consistent with cellulase expression in other arthropods.

Although caution should be exercised when drawing conclusions on the catabolic properties of enzymes based on molecular evidence, the preserved conservative domains and most of catalytically active residues (Davison and Blaxter 2005) of endo-β-1,4-glucanase transcripts, Ps-EG1 and Ps-EG2, suggest their cellulolytic activity. Due to the plausible occurrence throughout the digestive tract of microbial cellulases ingested with food (Hassall and Jennings 1975) and localization of endo-β-1,4-glucanase mRNA by in situ *hybridization*, the detection of endogenous cellulolytic activity has been focused on the hepatopancreatic tissue. The secretion activity of cells in isopod hepatopancreas is influenced by feeding behavior and changes dramatically during the 24-h digestive cycle (Hames and Hopkin 1991). To reduce the possibility of low secretion activity of gland cells at the moment of their removal, the samples for enzymatic analysis were collected as three replicates in the first 16 h of the daily cycle, when secretion of gland cells is the most intense (Hames and Hopkin 1991). According to isopod diet, the detection of cellulolytic activity in the hepatopancreas established by developed zymograms was expected and in accordance with previous reports on enzymatic activity in the digestive system of terrestrial isopods (Hartenstein 1964; Kukor and Martin1986; Zimmer and Topp 1998). Identical pattern of clearings in all replicates indicated the consistent presence of multiple cellulases in the hepatopancreas of *P. scaber*, consisting of cellulase with molecular mass estimated at 26.7 kDa and a group of smaller cellulases with molecular masses between 21.4 and 17.5 kDa. Although the molecular mass of carboxymethyl cellulose degrading enzymes generally exceeds the size of cellulases detected by zymogram (Tomme et al. 1995), shorter endoglucanases of approximately the same size as those observed in *P. scaber* are not uncommon among invertebrates (Watanabe and Tokuda 2001; Xu et al. 2000). However, since the molecular mass of known arthropod endoglucanases range between 36 and 54 kDa (Allardyce and Linton 2009) and the estimated molecular mass of endo-β-1,4-glucanase transcripts, Ps-EG1 and Ps-EG2, exceeds the size of clearings in zymogram as well, the observed clearings may also correspond to fragments with preserved catalytic domains produced by cleavage of the native cellulases during analysis.

Endo-β-1,4-glucanases are the most frequently characterized group of endogenous cellulases in arthropods (Watanabe and Tokuda 2010). Successful cellulose degradation, however, also includes the action of several other cellulases (Béguin and Aubert 1994). Although some of the latter are produced endogenously by arthropods (Watanabe and Tokuda 2001, 2010) and their presence in isopod tissues is not excluded, the role of microbial cellulolytic activity in isopod digestion cannot be ruled out. Since the digestive tract of isopods lack compartments in which massive microbiota could remain for prolonged periods and collaborate in cellulose digestion, the role in cellulose degradation has been assigned to presumably obligate endosymbiotic bacteria in the hepatopancreas (Zimmer and Topp 1998). Several inconsistencies challenge the suggested cellulolytic properties and consequential role of these endosymbionts in *P. scaber* digestion. Firstly, the direct observations revealed insufficient number or even occasional absence of bacteria in the lumen of hepatopancreas (Hames and Hopkin 1991; Millaku et al. 2010). Further, phylogenetic affiliation of hepatopancreatic endosymbionts has related them to bacterial groups Rickettsiales (Wang et al. 2004a) and Mycoplasmatales (Wang et al. 2004b). Members of both groups are known parasites and pathogens of plants and animals, with no known cellulolytic activities or mutualistic relationships with their hosts. Finally, the suggested role of hepatopancreatic symbionts in cellulose degradation is challenged by the absence of bacterial 16S rRNA genes in cDNA and expression of endogenous endo-β-1,4-glucanase in hepatopancreas. According to multiple copies of the endo-β-1,4-glucanase gene, the adaptation to degradation of various substrates in the isopod diet may be suggested, as shown in other arthropods where endogenous GHF9 endoglucanases commonly appear in multiple copies (Lo et al. 2000; Shimada and Maekawa 2008; Tokuda et al. 1999). Our results suggest that cellulolytic activity in the digestive system of *P. scaber* is not necessarily reliant upon an endosymbiotic community (Zimmer and Topp 1998). Therefore, the suggested influence of hepatopancreatic endosymbionts on isopod's digestion and evolution (Zimmer et al. 2001; Zimmer 2002) should be reconsidered.

Isopods exhibit strong preference toward leaf litter heavily colonized by microorganisms (Gunnarsson 1987). As suggested earlier, these probably serve as a source of nutrients and vitamins (Gunnarsson 1987; Ullrich et al. 1991), as well as of enzymes including cellulases that are required for litter degradation. Cellulases might be obtained by isopods via ingested microorganisms (Hassall and

Jennings 1975) or their extracellular cellulases (Hassall and Rushton 1985; Kukor and Martin 1986). Either way, the cellulose degradation in the digestive tract of terrestrial isopods is influenced by ingested microbial cellulases, probably of fungal and bacterial origin. In this regard, microbial cellulases might assist in the degradation of insoluble or crystalline cellulose in plant material, while crustacean endoglucanases would take part in digestion of amorphous cellulose, as suggested in decapod crustaceans (Xue et al. 1999; Crawford et al. 2004). Although the mechanism of maintaining slightly acid conditions in the anterior hindgut (Zimmer and Brune 2005) required for optimal cellulolytic activity (Hartenstein 1964, Zimmer and Brune 2005) is not fully understood, it is likely that microbial activity in ingested food takes part in the maintenance of acidic milieu. At the same time, degradation of the cellulose in the anterior hindgut by endogenous cellulases may not only be important for direct glucose absorption by isopods, but also provide substrates for numerous and diverse bacterial microbiota in the posterior parts of the hindgut (Kostanjšek et al. 2002, 2006). Although further studies will be necessary to characterize the enzymatic properties of endogenous cellulases and to clarify their detailed role in cellulose degradation, it seems likely that cellulose degradation in terrestrial isopods includes the synergistic effects of endogenous glucanases, from the hepatopancreas observed in the present study, and microbial enzymes ingested with food.

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