Available online at www.sciencedirect.com
SCIENCE DIRECT
Developmental and Comparative Immunology 30 (2006) 381–392
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

# Relationship between hemolytic molecules in *Eisenia fetida* earthworms

Petra Procházková[a,*], Marcela Šilerová[a], Jürgen Felsberg[b], Radka Josková[a], Alain Beschin[c], Patrick De Baetselier[c], Martin Bilej[a]

[a]*Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic*
[b]*Department of Biogenesis and Biotechnology of Natural Compounds, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic*
[c]*Department of Cellular and Molecular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

Received 11 May 2005; revised 8 June 2005; accepted 13 June 2005
Available online 12 July 2005

**Abstract**

The coelomic fluid of the earthworm *Eisenia fetida* has been reported to contain a variety of proteins causing the lysis of red blood cells—EFAF (*Eisenia fetida andrei* factor), fetidin, lysenin, eiseniapore, and hemolysins isolated either from coelomic fluid (H1, H2, H3) or from cell lysate ($CL_{39}$ and $CL_{41}$). We document the presence of two distinct genes with a high level of homology. These genes encode fetidin and lysenin but their level of expression differs in individual *E. fetida andrei* animals.


*Keywords:* Fetidin; Lysenin; Eisenia; Eiseniapore; Hemolysins; Earthworms; Invertebrates

## 1. Introduction

The coelomic fluid of *Eisenia fetida* earthworms exhibits numerous biological activities including hemolytic activity. The majority of the proteins with hemolytic activity identified so far show bactericidal and/or bacteriostatic activities against pathogenic soil bacteria [1–4] and they represent a heterogeneous set of proteins described by different groups in the last 35 years.

Hemolytic *E. fetida* proteins were described for the first time in 1968 by Du Pasquier and Duprat [5]. Later on, they were named EFAF (*Eisenia fetida andrei* factors), and characterized as two glycoproteins of 40 and 45 kDa, secreted by chloragocytes and eleocytes, which differ in the extent of glycosylation [1,6,7] and constitute a polymorphic system. Accordingly, the 45-kDa protein was characterized as having a pI of 6.0 and, possibly, being encoded by one

*Abbreviations:* CF, coelomic fluid; PCR, polymerase chain reaction; LBSS, *Lumbricus* balanced salt solution; PBS, phosphate buffered solution; EFAF, *Eisenia fetida andrei* factor; CL, coelomocyte lysate; SRBC, sheep red blood cells.

* Corresponding author. Tel.: +420 2 4106 2367; fax: +420 2 4172 1143.
*E-mail address:* kohler@biomed.cas.cz (P. Procházková).

0145-305X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2005.06.014

nonpolymorphic gene, while the 40-kDa protein was defined as being encoded by a gene having four alleles encoding four isoforms with pI of 6.3, 6.2, 5.95 and 5.9. All animals possess either 2 or 3 isoforms of the 40-kDa molecule, with one isoform, with pI of 6.0, invariably present. As a result of the polymorphism, six different hemolytic patterns were found in *E. fetida* individuals [1,8].

In 1997, the 40 and 45-kDa proteins were named fetidins and the gene coding for the 40-kDa was cloned [9,10]. Fetidin amino acid sequence comprises an N-glycosylation site (position 250–252) and a peroxidase motif (position 52–62) and both fetidins display peroxidase activity. Fetidins bind to sphingomyelin, which is a major lipid constituent of plasma membranes of most mammalian cells. Then they polymerize and form channels of 10 nm through the lipid bilayer [7,11]. Moreover, they display antibacterial activity against both Gram-negative and Gram-positive bacteria [12,13].

Independently, a 41-kDa hemolytic protein produced by coelomocytes and causing the contraction on rat vascular smooth muscle, was cloned and described as lysenin by Sekizawa et al. [14,15]. Simultaneously, two 42-kDa lysenin-related proteins with weak contraction activity were identified. Lysenin displays a high amino acid sequence homology (89% identity, 95% positive) with fetidin and with lysenin-related protein-1 (76% identity, 89% positive). Moreover, lysenin-related protein-2 was found to correspond at the amino acid level to fetidin. Lysenin specifically recognizes sphingomyelin and formes oligomers, which leads to the formation of pores in target membranes with a diameter of $\sim$3 nm [16,17]. Lysenin protein is expressed in large coelomocytes and in free large chloragocytes present in the lumen of the typhlosole [18].

At the same time, another 38 kDa cytolytic protein was identified in the coelomic fluid of *E. fetida* earthworms and named eiseniapore [19]. This protein requires sphingomyelin or galactosylceramide to bind to red blood cell membranes, inducing lysis. Lange et al. showed that eiseniapore forms stable oligomers and induces pores in sphingomyelin-containing membranes with a central channel with outer and inner diameters of 10 and 3 nm, respectively [19,20]. The molecular identity of eiseniapore has not been elucidated.

Eue et al. [21] described 3 hemolytic proteins from the coelomic fluid of *E. fetida*, H1, H2, and H3, with molecular masses of 46, 43, and 40 kDa. They consist of several isoforms (with pI of 6.2, 6.0, 5.8 and 5.4 for H1, 6.0 and 5.2 for H2, and 6.1 and 5.1 for H3). While H3 exerts lytic and hemagglutinating activity, H1 and H2 show only lytic activity. Moreover, H3 splits into two fragments of 18 and 21 kDa.

In 2003, Koenig et al. [22] have isolated native hemolytic proteins both from coelomocyte lysate ($CL_{39}$ and $CL_{41}$) and from the coelomic fluid (H1, H2, H3) of *E. fetida* earthworms. Using mass spectrometric analyses, they have demonstrated the identity of $CL_{39}$ with fetidin and $CL_{41}$ with lysenin, and the probable identity of H1-3 hemolysins with fetidin. Hence, different proteins from *E. fetida* cause lysis of red blood cells, but there is no consensus concerning their nomenclature, identity, and relationships. The major task in this respect is therefore to convincingly document that fetidin and lysenin are either protein isoforms encoded by alleles of one gene or, alternatively, they are products of two related genes. Here, we show that fetidin/$CL_{39}$ and lysenin/$CL_{41}$ are encoded by two individual genes with high homology, whose level of expression differs from individual to individual.

## 2. Material and methods

### *2.1. Earthworms and harvesting of the coelomic fluid (CF) and coelomocytes*

Adult *E. fetida andrei* earthworms (*Oligochaeta; Annelida*) kept in compost were transferred 3 days before experiments on filter paper soaked with isotonic *Lumbricus* balanced salt solution (LBSS; [23]). Coelomic fluid containing coelomocytes of 25 individual worms was obtained by puncturing post-clitellum segments of the coelomic cavity with a Pasteur micropipette and kept on ice. Sample from each individual was centrifuged ($500\times g$, 10 min, 4 °C), and settled coelomocytes were promptly used for isolation of RNA. Supernatant containing the cell-free coelomic fluid was centrifuged again ($7000\times g$, 10 min, 4 °C), and stored at −20 °C in the presence of the protease inhibitor Complete™ (Roche). The same procedure was used for *Lumbricus terrestris*,

*Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta* and *Lumbricus rubellus*.

### 2.2. Hemolysis assay

To evaluate the hemolytic activity, 100 μl of suitably diluted coelomic fluid (CF) of 25 individual worms with Complete™ (Roche) was further serially diluted in 145 mM NaCl (pH 7.4) and incubated in 96-well microtiter plates with 100 μl of sheep erythrocyte suspension (3% in 145 mM NaCl, pH 7.4) for 2 h at room temperature. The plates were centrifuged ($100\times g$, 10 min, 4 °C) and OD of collected supernatants was measured at 405 nm. Optimum dilution of CF (1:8000) of individual worms was that showing the highest resolution among samples. To exclude that a difference in hemolytic activity was due to a difference in the protein level of the coelomic fluids, protein concentration of all samples was assayed according to Bradford [24]. The differences did not exceed the level of statistical significance ($P<0.05$, data not shown).

### 2.3. Lysis of SRBC in agarose after native PAGE

To visualize differences in the pattern of hemolytic proteins, 8 μl samples of coelomic fluid were run for 5 h at 300 mA constant current at 4 °C on native 10% PAGEs without SDS (16×20 cm gel). After separation, the gels were washed in 145 mM NaCl, pH 7.4 and applied on plates containing 1% low melting agarose (Invitrogen) and 0.9% NaCl with 1% sheep erythrocyte suspension. The lysis was observed after a 3-h incubation at room temperature as transparent bands in front of the reddish background.

### 2.4. Genomic DNA isolation

Genomic DNA was isolated from coelomocytes of six individuals (*E. fetida andrei*). Coelomocytes were lysed (10 mM Tris–HCl, pH 8; 100 mM EDTA, pH 8; 0.5% SDS, 20 μg/ml RNase A), and genomic DNA was extracted twice with buffered phenol (Merck), precipitated with isopropanol, washed in ethanol and resuspended in TE buffer (pH 7.4).

### 2.5. RNA isolation, cDNA synthesis, PCR and cloning

Total RNA was prepared from coelomocytes using 1 ml of Trizol reagent as recommended by the supplier (Invitrogen). DNAse I-treated total RNA (2 μg) was reverse-transcribed with Oligo(dT)$_{12-18}$ (Invitrogen) and SUPERSCRIPT™ II Rnase H$^-$ Reverse Transcriptase (Invitrogen). Resulting cDNAs were used in PCR with primers corresponding to the nucleotide sequence for fetidin (GenBank accession no. U02710) and for lysenin (GenBank accession no. D85846, Table 1). The specifity of primers was tested on plasmids containing either fetidin or lysenin sequence. PCR reactions were performed with cDNA templates (0.5 μl of the RT reaction product) or with genomic DNA (0.25 μg) to which 2 units of Taq polymerase (Invitrogen),

Table 1
Oligonucleotides specific for fetidin and/or lysenin cDNA (sequences listed 5–3′) used in PCR and sequencing

| Primer | Sequence | Direction | Occurrence in fetidin (nucleotide pos.) | Occurrence in lysenin (nucleotide pos.) |
|---|---|---|---|---|
| Lys1up | ACG GCT TCG CAT AGT ATT GGT TCT | sense | 256–279 | 300–323 |
| Lys1low | CCT TCC TGT CCG CAT GCT TTA TTC | antisense | 622–599 | 666–643 |
| Lys2up | AAG CAT GCG GAC AGG AAG GAG TAT | sense | 604–627 | 648–671 |
| Lys2low | GCG GAA GCA AAT GTG TAA TGG TG | antisense | 1042–1021 | 1090–1069 |
| Lys3up | CAG CGC TGG GCA ATC AAT AAG TCA | sense | 820–843 | 864–887 |
| Lys3low | CGG CCG CGC AGA ACA ATA GG | antisense | 1473–1454 | 1510–1491 |
| Lysen4up | CTC AGA TGC AGT TAT GTA TCT A | sense | – | 1–24 |
| VAV1 | TGT GAG CGA TGT CGT CTA GAG CAG GAA TCG | sense | 80–109 | – |
| VAV2 | CCC CAT TTC AGG GGT TAA CCA ACC A | antisense | – | 1045–1021 |
| VAV3 | TCG CTA TTA CAA TCT ACA CCG C | antisense | – | 1154–1133 |
| VAV4 | TGG TTG TAC ACT TGG TAA AG | antisense | 1310–1291 | – |

0.2 mM dNTP, 1 × company-supplied buffer, 1.5 mM $MgCl_2$ and 0.4 μM primer pairs were added. PCR conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55–63 °C for 40 s, 72 °C for 120 s, and 72 °C for 7 min.

Alternatively, 100 ng of DNAse I-treated total RNA was used in One-Step RT-PCR (Invitrogen) with primers Lysen4up and Lys2low amplifying both fetidin and lysenin. Conditions were 45 °C for 45 s, and 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 40 s, 72 °C for 120 s, and 72 °C for 7 min as recommended by the supplier (Invitrogen).

PCR products were purified with PCR purification kit (Qiagen) and sequenced in both directions with different primers (Table 1), preferably with Lysen4up and Lys2low primers. All possible combinations of available primers were used in PCR with cDNA of other worm species (Table 1).

### 2.6. Relative quantification of fetidin and lysenin gene expression

To measure the levels of fetidin and lysenin mRNA in coelomocytes from *E. fetida andrei* earthworms, a quantitative real-time RT-PCR assay was performed using the iCycler™ iQ real-time PCR detection system (Bio-Rad). The cDNA samples of four individuals (no. 1–4) synthesized as described above (0.2 μl) were used for iQ™ SYBR® green (Biorad) real-time PCR, in the presence of 400 nM fetidin gene-specific forward primers Lys2up or Lys3up and reverse primer VAV4, or 400 nM lysenin gene-specific forward primer Lys2up and reverse primers VAV2 or VAV3 (Table 1). 'No template controls' were included for every primer set and non-specific amplification was excluded by a follow-up dissociation assay (melting point curves). The PCR conditions were 95 °C for 5 min followed by 40 cycles of 95, 58, and 72 °C for 60, 45 and 70 s, respectively, and 72 °C for 7 min. Quantitative measurements were normalized using *E. fetida* myosin mRNA levels as a housekeeping gene (GenBank accession no. AF537290). The myosin primers used were as follows: forward, 5′-GCG AAA TCG AGG ATG TTC GT-3′, and reverse 5′-CTA CTT CTC GGG AGG TCC AGC-3′ giving an amplicon of 0.453 kb. The differences in the $C_T$ values of fetidin or lysenin (threshold PCR cycle) and the internal control myosin were calculated. Relative expression of fetidin and lysenin was determined as fold expression relative to individual no. 1. The SYBR® green assay was done in duplicate and repeated in three independent experiments.

### 2.7. Sequencing

Sequence analysis was performed by the dideoxy chain termination method [25] in the cycle sequencing technique [26] with the ABI PRISM BigDye terminator v3.1 cycle sequencing kit (Applied Biosystem) according to the manufacturer's protocol, using the automated DNA sequencer (ABI PRISM 3100 DNA sequencer, Applied Biosystems). Primers listed in Table 1 were used for sequencing. Sequence analysis was edited using LaserGene (DNASTAR Inc., Madison, WI, USA) and analyzed using NCBI Blast [27].

### 2.8. Statistical analysis

Paired Student's *t*-test using GraphPad Prism software was performed to evaluate the significance of the data. Differences were considered significant when $P < 0.05$.

## 3. Results

### 3.1. Comparison of fetidin and lysenin sequences

Both cDNA and amino acid sequences of fetidin and lysenin display high homology, with differences being found in 5′UTR (Figs. 1–3). To elucidate the relationships between the two molecules we designed lysenin-specific primers based on the published nucleotide sequences of fetidin and lysenin cDNA (Fig. 1). RNA from coelomocytes of 25 adult *E. fetida andrei* earthworms was isolated, used in One-Step RT-PCR with Lys2low and Lysen4up primers and all amplified fragments were sequenced. Unexpectedly, we found that primer Lysen4up is not specific for lysenin, but amplifies also fetidin cDNA due to errors in the published 5′UTR sequence of fetidin [9]. The sequence in 5′UTR fetidin cDNA we have amplified corresponded rather to the published sequence of lysenin cDNA. Comparison of 5′UTR nucleotide sequences published for fetidin and lysenin cDNA

with fetidin cDNA found in 20 samples tested here is shown in Fig. 3.

Comparison of amino acid sequences of fetidin and/or lysenin proteins of 25 individual worms with published amino acid sequences of fetidin and lysenin protein is shown in Table 2. Twenty out of 25 tested samples correspond to the amino acid sequence of fetidin, the others corresponding to the amino acid sequence of lysenin.

Based on our finding we designed a new set of specific primers based on the presence of deletions in sequences of lysenin and fetidin cDNA. Gaps starting at nucleotide positions 148 and 1381 in the sequence of lysenin were used to design primers specific for fetidin cDNA, VAV1 and VAV4. Next, gaps in cDNA sequence of fetidin starting at nucleotide positions 977 and 1070 were used to design primers specific for lysenin, VAV2 and VAV3. The use of these specific

```
                          Lysen4up
lys     1        ctc agatgcagtt attgtatcta ataggcatct cggtcttccg ctggtttctc

lys    54 gcagatacat caaaagacta gaagaggcca tcaagatcct gtt/gaattg agtgcactcc
fet     1 gaattcccgg ccgcgggcta gaagaggcca tcaagatcct ggca------ -c--------
                                          VAV1
lys   113 tgtttgc//t tcagaaactt gtgagcgatg tcggctaaag cag//////c agagggatat
fet    61 -------ca- g------t-t gtgagcgatg tcgtctagag caggaatcg- ----------

lys   165 gaacagatag aagtagatgt ggtggcagta tggaaagaag gctatgtata cgaaaatcgc
fet   121 ---------- ---------- ---------- ---------- ----c--t-- ---------g

lys   225 ggaagcacca gtgtggatca gaagatcaca ataacaaaag gcatgaaaaa tgtgaattca
fet   181 ---------- -------g-- --------a- ---------- ------g--- -t--------
                                    Lys1up
lys   285 gaaacaagga cagtgactgc tacgcatagt attggttcta ctattagcac tggagatgca
fet   241 -------a-- --t--acggc ttcgcatagt attggttcta ---------- -------ct-

lys   345 ttcgaaattg gaagcgtgga ggttagctac agccattcac atgaagaatc ccaagttagc
fet   301 --t-----a- c--c------ t--------- ---t-c---- ---------- ---------t

lys   405 atgacggaaa ctgaagttta tgaatcaaag gtgatcgaac acactataac gattccacct
fet   361 ---------- ---------- ---------- -aa------- ---------- ----------

lys   465 acttcaaaat tcacaagatg gcaactgaat gctgacgttg gtggagcgga tattgaatac
fet   421 ---------- ---------- ---------- ---------- ---------- ----------

lys   525 atgtatttga ttgatgaagt cacacccata ggagggactc agagtattcc acaggtcatc
fet   481 ---------- ---------- ---------- ---------- t--------- ----------

lys   585 acaagtcggg ctaaaattat agttggccga cagataatcc ttggaaaaac agaaattcga
fet   541 -a-------- --------c- ---------- g-a---ta-- -----g---- --------ga
          Lys1low          Lys2up
lys   645 attaagcatg cagaaaggaa ggagtacatg acagtcgttt caagaaaaag ttggccagct
fet   601 ataaagcatg cggacaggaa ggagtat--- ---------- ---------- c---------

lys   705 gcaactcttg gacatagcaa acttttcaag tttgtgctct atgaagattg ggggggattt
fet   661 ---------- ---------- -----a---- ---------- ---------t -tat------

lys   765 cgaattaaaa cgctgaacac catgtattcg ggctatgagt atgcctattc ctctgatcaa
fet   721 ---------- ---------- ---------- ---------- ---------- ----------
                                                              Lys3up
lys   825 ggaggaatct actttgatca gggtactgat aatccgaaac agcgctgggc aatcaataag
fet   781 ---------- ---------- -----g---- ---------c agcgctgggc aatcaataag

lys   885 tcattgcctc ttcgtcatgg tgacgtagtc accttcatga ataagtactt cactcgcagt
fet   841 tca------- ---------- ---------- ---------- ---------- ----------
```

Fig. 1. Comparison of nucleotide sequences of fetidin (GenBank accession no. U02710) and lysenin cDNA (GenBank accession no. D85846). The start (atg) codons are boxed and the stop (tga, taa) codons are indicated with asterisks. Oligonucleotides used as primers specific for the fetidin and lysenin mRNA are underlined and their names are in bold. Nucleotides of fetidin cDNA that match with the lysenin cDNA are indicated by a dash, gaps are indicated by a slash.

```
lys   945 gggctgtgct acgatgatgg accggcaaca aacgtgtact gtctggacaa acgtgaagac
fet   901 --t------- --t------- ---------- g--------- --t------- ----------
                                      * VAV2
lys  1005 aagtggattt tggaagtggt tggttaaccc ctgaaatggg gtgtcatgat tggtcagcaa
fet   961 ---------- -a----//// tggttaaacc ctgaaatggg g--------- ----------
                    Lys2low                 *
lys  1065 gcatcaccat ttcgcatttg cttctgtcat atatttcacc tttagcacaa tgatgaggtg
fet  1017 ----caccat tacacatttg cttccg/--- g--------- -g-------c ---a//////
                          VAV3
lys  1125 caactaaagc ggtgtagatt gtaatagcga ctcatttatg tattattcag cattatttac
fet  1070 ////////// ////////// ////////ga ---------- c--------- at--------

lys  1185 ataagacatt aagaacattc ggtttattta tttatatgct tttgtaggcc ttatattgat
fet  1102 ---------- ---------- a-c-a-g-g- -----c---- -------a-- ----------

lys  1245 tattaacagg cgacctaatt cagcaaacat gcagggtctg caaacatttg taggccgatg
fet  1162 --c--g--a- ---------- g------t-- --t-a----- ---gg----- -//////---

lys  1305 tcaatgttta tattcacgag aactttaaac cttatgatag cttcgctttg cttaactagg
fet  1216 c--------- -------a-- --tg------ ---------- ---------- ----------
                                VAV4
lys  1365 cctaaactaa tatag///// //////gt/c aaccagctat gtatctaaac taaccgagga
fet  1276 ---------- -----cttta ccaagtgtac aacca----- --ca------ ---t------

lys  1413 ctatatttaa ccattagcat tagcattctt gttaacccaa gtcctaaaat gtcttgtacg
fet  1336 --g--c---- ---a-----a -g-----t-- -----t---- ca--ct--c- ----a----t
                                Lys3low
lys  1473 gagatgcagt ttgttgttcc aattgttctg caaaaaaaca aggataggcc atgacatgtt
fet  1436 aa-----ga- ----ca-ccc tattgttctg cgcggccg-g -attc

lys  1533 aaactagtat atctcaaagc tattgaaaaa taaatggaga tcaataaatc
```

Fig. 1 (*continued*)

primers together with stringent annealing temperature in PCR provides a simple tool for amplifying only the desired sequence (either fetidin or lysenin). Using specific primers for fetidin (VAV1, VAV4) or lysenin (VAV2, VAV3) we could amplify cDNA of both fetidin and lysenin in the 25 earthworms tested, suggesting that the coelomocytes of each individual *E. fetida andrei* contain mRNA for both fetidin and lysenin.

### 3.2. The presence of both genes coding for fetidins and lysenin in individual E. fetida andrei earthworms

Genomic DNA of six individual *E. fetida andrei* earthworms was isolated. Using the common primer Lys3up and the specific primers VAV3 or VAV4, we amplified partial DNA sequences of genes coding for lysenin or fetidin, respectively, and compared them with nucleotide sequences published in NCBI Blast database. The presence of both sequences in every individual (Fig. 4) suggests that each protein is encoded by a different gene. Amplification of longer fragments was not successful because of the possible presence of intron-exon structure in the genes and thus an incorrect match of the primers with the template.

### 3.3. Different expression of fetidin and lysenin

To see the quantitative differences in the expression of genes encoding lysenin and fetidin, the cDNA of four individual worms was used in real-time PCR with fetidin-specific forward primers Lys2up or Lys3up and reverse primer VAV4 and with lysenin-specific forward primer Lys2up and reverse primers VAV2 or VAV3 (Table 1). Relative expression of fetidin and lysenin was determined as fold expression as compared to individual no. 1 that was chosen as a reference value. As summarised in Fig. 5, there was a significantly higher expression of lysenin in cDNA from individual no. 3 and 4 than no. 1 and 2. Expression of lysenin gene in individuals no. 3 and 4 was 23–26 times higher than in individuals no. 1 and 2 while the expression of fetidin gene was similar in all individuals. The fact that individuals no. 3 and 4 contain a higher level of lysenin mRNA corresponds to the results of sequencing, with these

```
Lysenin 1     MSAKA//AEG  YEQIEVDVVA  VWKEGYVYEN  RGSTSVDQKI  TITKGMKNVN  SETRTVTATH  SIGSTISTGD  AFEIGSVEVS  YSHSHEESQV  SMTETEVYES
Fetidin 1     --SR-GI---  ----------  ----------  ------E---  K-----R-L-  ---K-L--S-  ----------  L---AT-D--  --Y-------  ----------
                ++                                      +         + +       + +  +                      + +        +

Lysenin 99    KVIEHTITIP  PTSKFTRWQL  NADVGGADIE  YMYLIDEVTP  IGGTQSIPQV  ITSRAKIIVG  RQIILGKTEI  RIKHAERKEY  MTVVSRKSWP  AATLGHSKLF
Fetidin 101   -E--------  ----------  ----------  ----------  ----L-----  -K-----L--  -E-Y--E---  -----D----  ----------  ---------Y
                                                                          +          +    +          +

Lysenin 199   KFVLYEDWGG  FRIKTLNTMY  SGYEYAYSSD  QGGIYFDQGT  DNPKQRWAIN  KSLPLRHGDV  VTFMNKYFTR  SGLCYDDGPA  TNVYCLDKRE  DKWILEVVG/
Fetidin 201   -------MY-  ----------  ----------  ---------S  ----------  ----------  ----------  -----Y----  -D--------  --------KP
                                                           +                                                    +
```

Fig. 2. Amino acid sequence alignment of fetidin and lysenin proteins. Identical amino acids are indicated by dash (−), missing amino acids are indicated by slash (/). Similar residues identified by the ClustalW program are indicated by plus (+).

```
Lysenin       1    ctcagatgca gttattgtat ctaataggca tctcggtctt ccgctggttt ctcgcagata catcaaaaga
Fetidin       1                                                          gaattcc cggccgcggg
5´UTR         1    ctcagatgca gttattgtat ctaataggca tctcggtctt ccgctggttt ctcgcagata catcagaggg
                   ++++++++++ ++++++++++ ++++++++++ ++++++++++ ++++++++++ +++:+:++++ :++:+/+/+/

Lysenin      81    ctagaagagg ccatcaagat cctg-ttgaa ttgagtgcac tcctgtttgc --ttcagaaa cttgtgagcg atg
Fetidin      28    ctagaagagg ccatcaagat cctggcagaa ttgactgcac tcctgtttgc catgcagaaa tttgtgagcg atg
5´UTR        81    ctagaagagg ccatcaagat cctg-cagaa ttgactgcac tcctgtttgc cat-cagaaa tttgtgagcg atg
                   :::::::::: :::::::::: ::::+//::: ::::/::::: :::::::::: //: :::::: +:::::::::: :::
```

Fig. 3. Comparison of nucleotide sequence of 5′UTRs of published sequences of lysenin and fetidin cDNA with the sequence of fetidin cDNA found in 20 individuals tested (5′UTR). The start (atg) codons are in bold. Nucleotides identical in both sequences are indicated with colon (:), nucleotides corresponding to nucleotide sequence of lysenin cDNA are indicated with plus (+), nucleotides corresponding to nucleotide sequence of fetidin cDNA are indicated with slash (/).

Table 2
Comparison of amino acid sequences of fetidin and/or lysenin proteins of 25 individual *E. fetida* earthworms with published amino acid sequences of fetidin (GenBank accession no. U02710) and lysenin (GenBank accession no. D85846) protein

| | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa | aa |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lysenin** | **3-4** | **--** | **35** | **39** | **45** | **47** | **52** | **54** | **57** | **69** | **73-5** | **76** | **81** | **101** | **100** | **150** | **155** | **160** | **162** | **165** | **174** | **198** | **205-7** | **238** | **274** | **280** | **297-9** |
| **Fetidin** | **3-4** | **6-7** | **37** | **41** | **47** | **49** | **54** | **56** | **59** | **71** | **75-6** | **78** | **83** | **102** | **145** | **152** | **157** | **162** | **164** | **167** | **176** | **200** | **207-8** | **240** | **276** | **282** | **299-0** |
| **Fetidin** | **SRA** | **GI** | **E** | **K** | **R** | **L** | **K** | **L** | **S** | **L** | **AT** | **D** | **Y** | **E** | **L** | **K** | **L** | **E** | **Y** | **E** | **D** | **Y** | **MY** | **S** | **Y** | **D** | **KP** |
| **No. 1** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | **Q** | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **2** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **4** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **5** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **6** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **7** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **8** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | **Q** | Y | E | D | Y | MY | S | Y | D | KP |
| **10** | SRA | GI | E | K | R | L | K | **V** | S | **A** | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **11** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **12** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **13** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **14** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **15** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **17** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **18** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **21** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **22** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **23** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | **Q** | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **24** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **25** | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | E | D | Y | MY | S | Y | D | KP |
| **Lysenin** | **AKA** | **--** | **D** | **T** | **K** | **V** | **R** | **V** | **T** | **A** | **GS** | **E** | **H** | **V** | **Q** | **T** | **I** | **Q** | **I** | **K** | **E** | **F** | **WG** | **T** | **D** | **N** | **G-** |
| **No. 3** | AKA | -- | D | T | K | V | R | V | T | A | GS | E | H | V | Q | T | I | Q | I | K | E | F | WG | T | D | N | G- |
| **9** | AKA | -- | D | T | K | V | R | V | T | A | GS | E | H | V | Q | T | I | Q | I | K | E | F | WG | T | D | N | G- |
| **16** | AKA | -- | D | T | K | V | R | V | T | A | GS | E | H | V | Q | T | I | Q | I | K | E | F | WG | T | D | N | G- |
| **19** | AKA | -- | D | T | K | V | R | V | T | A | GS | E | H | V | Q | T | I | Q | I | K | E | F | WG | T | D | N | G- |
| **20** | AKA | -- | D | T | K | V | R | V | T | A | GS | **D** | H | V | Q | T | I | Q | I | K | E | F | WG | T | D | N | G- |
| **Positive** | --A | -- | + | - | -- | + | -- | -- | + | - | - + | -- | + | - | - | - | + | + | - | + | + | + | -- | + | - | + | - |

Differences in amino acid sequences between individual worms (no. 1–25) are shown. Differences between obtained and presumed sequences are indicated in shadow. Similar amino acid residues identified by the ClustalW program are indicated by plus (+), non-similar by minus (-).

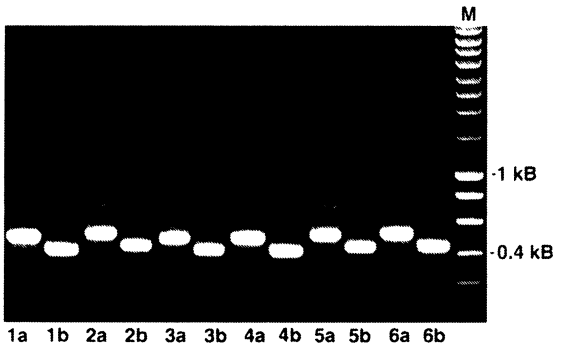



Fig. 4. PCR analysis reveals the presence of the fetidin and lysenin genes in genomic DNA of individual *E. fetida* earthworms. Genomic DNA of 6 specimens (1–6) was amplified using primers Lys3up, VAV4 and VAV3 specific for fetidin (lanes a) or lysenin (lanes b). DNA ladder marker is on the right margin (M).

samples being sequenced as lysenin, while the others were sequenced as fetidin.

### 3.4. Hemolysis assay and hemolytic pattern

To address the relationships between hemolytic activity and expression of fetidin and lysenin, coelomic fluids of 25 individual earthworms were separated in native PAGEs and gels were applied on sheep erythrocyte suspensions embedded in agarose. Patterns of hemolytic proteins of coelomic fluids were

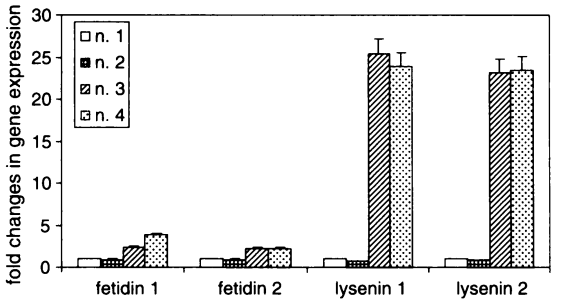



Fig. 5. Gene expression levels of fetidin and lysenin differ in individual *E. fetida* earthworms. Gene expression levels of fetidin and lysenin in 4 individual *E. fetida* earthworms (no. 1–4) were determined by quantitative RT-PCR and normalized for the housekeeping gene myosin. Fold changes in gene expression are relative to expression of cDNA no. 1. For both genes, different combinations of primers specific for fetidin and/or lysenin genes were used. Fetidin 1 was amplified with primers Lys2up and VAV4, fetidin 2 with Lys3up and VAV4, lysenin 1 with primers Lys2up and VAV2, lysenin 2 with primers Lys2up and VAV3. The values are means of 3 experiments (±SD) performed in duplicate.

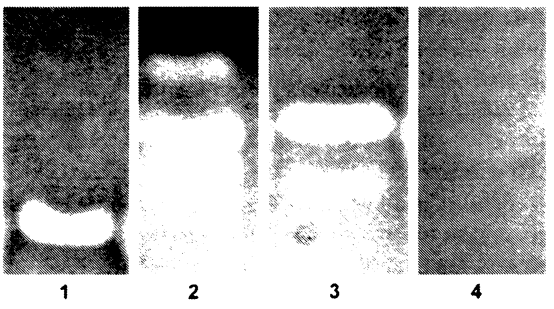



Fig. 6. Four different hemolytic patterns of coelomic fluid proteins were found in 25 individuals of *E. fetida* earthworms. The proteins were separated on native PAGE. After separation the gel was applied on agarose plates containing sheep erythrocyte suspension. The lysis was observed after a 3-h incubation at room temperature as transparent bands in front of the reddish background.

observed as transparent bands in front of the reddish background (Fig. 6). In this way, we determined four different hemolytic patterns. Pattern no. 1 consists of one clear hemolytic band, pattern no. 2 is formed by at least five faint bands including the one present in pattern no. 1, pattern no. 3 consisted of 2 bands that are most likely present also in pattern no. 2, and pattern no. 4 did not develop any visible hemolysis. Out of the 25 tested worm coelomic fluids, three generated pattern no. 1, twelve pattern no. 2, three pattern no. 3 and seven did not develop any visible hemolytic pattern (no. 4).

In parallel, the hemolytic activity of the 25 samples was quantified by incubating coelomic fluids with sheep erythrocyte suspension (Fig. 7). The distinct hemolytic patterns correlated with differences in the level of hemolytic activity of the coelomic fluids. The coelomic fluids of animals developing hemolytic pattern no. 2 have higher hemolytic activity (0.42–3.0 O.D.) than coelomic fluids from animals displaying pattern no. 1, 3 or 4. Also, the hemolytic pattern no. 2 showed a higher number of bands on agarose-embedded erythrocytes, reflecting probably the presence of more hemolytic proteins in the coelomic fluids of these earthworms.

### 3.5. Unique appearance of fetidin and/or lysenin in E. fetida andrei earthworms

All available fetidin and/or lysenin primers were used to address the presence of the corresponding

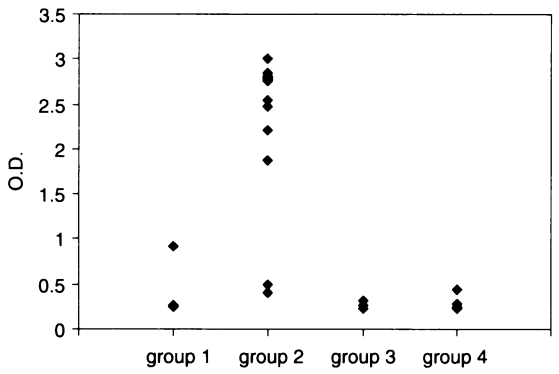



Fig. 7. The intensity of the hemolytic activity of the coelomic fluids of *E. fetida* earthworms distributed into 4 groups based on the hemolytic patterns (see Fig. 6) varies. Coelomic fluids from 25 individuals were incubated with sheep erythrocyte suspension and OD of collected supernatants was measured at 405 nm. Values are the means of three experiments.

mRNA in other earthworm species (*L. terrestris*, *A. caliginosa*, *A. icterica*, *A. longa*, *A. rosea*, *D. veneta*, *L. rubellus*). No PCR amplification occurred in these latter species, suggesting that fetidin and lysenin are proteins unique for *E. fetida andrei* earthworm (data not shown). These findings are in compliance with the fact that coelomic fluids from these earthworm species were not hemolytic either on agarose-embedded RBC or on RBC suspension.

## 4. Discussion

Many *E. fetida* proteins of that cause lysis of red blood cells were described and even though their terminology differs (for review see [28,29]), their further characterization indicates that they are the - same or similar molecules. Indeed, all hemolytic molecules described so far share biochemical analogies, having a similar molecular mass around 40 kDa, similar pI and ability to bind sphingomyelin and create pores in lipid membranes. The controversy concerning the nomenclature and identity of hemolytic proteins of *E. fetida* was partially elucidated by Koenig et al. [22], who characterized hemolysins from coelomocyte lysate ($CL_{39}$ and $CL_{41}$) and coelomic fluid (H1-3). Using mass spectrometry, they demonstrated the identity of $CL_{39}$ and $CL_{41}$ with fetidin and lysenin, respectively. Moreover, they reported the identity of H1-3 with fetidin. On the other hand, another hemolytic protein named eiseniapore, which has not yet been sequenced, shows common characteristics with lysenin [19,20]. Thus, when all the data are viewed together, it is most likely that *E. fetida* earthworms have two main hemolytic proteins fetidin and lysenin, although it has not yet been convincingly shown whether these proteins are isoforms or encoded by two independent genes.

Lassegues et al. [9] assumed that every *E. fetida andrei* worm possesses 4 alleles of a gene encoding 4 isoforms of the 40-kDa fetidin protein. Moreover, they presumed that the second described 45-kDa fetidin is encoded by 1 gene which, however, was not sequenced. It can be expected that this 45-kDa fetidin is identical with the other hemolytic protein lysenin identified by Sekizawa et al. [15]. We showed that all 25 experimental animals used in the present study express mRNA for both fetidin and lysenin. Furthermore, the presence of DNA coding for both proteins at the genomic level was observed in six selected animals, suggesting that fetidin and lysenin do not result from posttranscriptional splicing or other modification of the transcript. The possibility that both proteins are encoded by different alleles of one gene is also improbable, since all experimental specimens used would have to be heterozygotes. Therefore, our data confirm that fetidin and lysenin are two different proteins encoded by two distinct genes, which have very high sequence and functional homology.

By testing the hemolytic activity in the coelomic fluid of 25 individual *E. fetida andrei*, we found three different hemolytic patterns plus one group of seven animals with hemolytic pattern below the detection limit. The absence of the hemolytic pattern in the fourth group is in a good agreement with the very low hemolytic activity assessed in suspension (Fig. 7). However, Roch [1] found six different hemolytic patterns based on the presence of four isoforms of the 40-kDa hemolysin. The difference in the number of hemolytic patterns can arise from the use of different techniques used by the two groups. Whereas we separated coelomic fluid proteins in native PAGE before evaluating the hemolytic activity, Roch separated these proteins according to their isoelectric points. Most coelomic fluids tested in our study (12 out of 25) generated a

hemolytic pattern (no. 2) formed by at least five bands. However, the identity of the proteins in hemolytic spots could not be determined by proteome techniques due to the low amount of coelomic fluid, which can be obtained from one individual earthworm and loaded on the native PAGE. Seven samples did not reveal any significant hemolytic pattern on agarose with red cells; this was in accordance with the measurement of hemolytic activity, which was very low in these samples.

It was proposed that fetidin is an intracellular and secreted protein while lysenin is only intracellular [9,10,14]. Fetidin and lysenin not only differ in their location within the earthworm but, as found in this study, the expression of fetidin and lysenin in coelomocytes may differ from individual to individual. By determining the relative expression of the two genes in real-time PCR, we found that fetidin was expressed to similar levels in all tested animals, while the expression of lysenin varied from animal to animal. Accordingly, the hemolytic activity of coelomic fluid from individuals with a higher expression of lysenin was higher and the samples generated hemolytic pattern no. 2 (data not shown).

Save for *E. fetida andrei*, we did not find any expression of fetidin or lysenin mRNA in any other earthworm species tested (*L. terrestris*, *A. caliginosa*, *A. icterica*, *A. longa*, *A. rosea*, *D. veneta*, *L. rubellus*). Accordingly, only the coelomic fluid from *E. fetida andrei* exerts detectable hemolytic activity. This suggests unique appearance of fetidin and/or lysenin in *E. fetida* earthworms. Alternatively, gene sequences coding for these proteins may differ so that any primer designed for *E. fetida* fetidin and lysenin mRNA of would be inefficient in the other species. The unique occurrence of fetidin and lysenin in *E. fetida* species could reflect their living conditions. *E. fetida* earthworms living in the compost could have developed distinct defence mechanisms to better tolerate a wide range of environmental conditions and their fluctuations.

## Acknowledgements

This work, supported by the Czech Science Foundation (310/04/0806), an Institutional Research Concept (AV0Z50200510) and a bilateral international scientific and technological cooperation grant from the Ministry of the Flemish Community (BOF-BWS 03/06), was performed within the frames of an Interuniversity Attraction Pole Program. Authors are grateful to Ph. Roch for helpful discussion.

## References

[1] Roch P. Protein analysis of earthworm coelomic fluid: I) polymorphic system of the natural hemolysin of *Eisenia fetida andrei*. Dev Comp Immunol 1979;3(4):599–608.

[2] Roch P, Lassegues M, Valembois P. Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: III-Relationship within the polymorphic hemolysins. Dev Comp Immunol 1991; 15(1–2):27–32.

[3] Valembois P, Roch P, Lassegues M. Antibacterial molecules in annelids. In: Brehelin M, editor. Immunity in invertebrates. Berlin: Springer; 1986. p. 74–93.

[4] Valembois P, Roch P, Lassegues M, Cassand P. Antibacterial activity of the haemolytic system from the earthworm *Eisenia fetida andrei*. J Invert Pathol 1982;40:21–7.

[5] Du Pasquier L, Duprat P. Humoral and cellular aspects of a non-specific natural immunity in the oligochetee *Eisenia foetida Sav (Lumbricinae)*. C R Acad Sci Hebd Seances Acad Sci D 1968;266(5):538–41.

[6] Roch P. Isolation of agglutinins and lysins from earthworm coelomic fluid by gel filtration followed by chromatofocussing. J Chromat 1984;290:231–5.

[7] Roch P, Valembois P, Davant N, Lassegues M. Protein analysis of earthworm coelomic fluid II. Isolation and biochemical characterization of the *Eisenia foetida andrei* factor (EFAF). Comp Biochem Physiol 1981;69B:829–36.

[8] Roch P, Valembois P, Lassegues M. Genetic and biochemical polymorphism of earthworm humoral defenses. Prog Clin Biol Res 1987;233:91–102.

[9] Lassegues M, Milochau A, Doignon F, Du Pasquier L, Valembois P. Sequence and expression of an *Eisenia-fetida*-derived cDNA clone that encodes the 40-kDa fetidin antibacterial protein. Eur J Biochem 1997;246(3): 756–62.

[10] Milochau A, Lassegues M, Valembois P. Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei*. Biochim Biophys Acta 1997;1337(1): 123–32.

[11] Roch P, Canicatti C, Valembois P. Interactions between earthworm hemolysins and sheep red blood cell membranes. Biochim Biophys Acta 1989;983(2):193–8.

[12] Hirigoyenberry F, Lassalle F, Lassegues M. Antibacterial activity of *Eisenia fetida* andrei coelomic fluid: transcription

and translation regulation of lysozyme and proteins evidenced after bacterial infestation. Comp Biochem Physiol B 1990; 95(1):71–5.

[13] Lassegues M, Roch P, Valembois P. Antibacterial activity of *Eisenia fetida andrei* Coelomic fluid: evidence, induction, and animal protection. J Invert Pathol 1989;53:1–6.

[14] Sekizawa Y, Kubo T, Kobayashi H, Nakajima T, Natori S. Molecular cloning of cDNA for lysenin, a novel protein in the earthworm *Eisenia foetida* that causes contraction of rat vascular smooth muscle. Gene 1997;191(1):97–102.

[15] Sekizawa Y, Hagiwara K, Nakajima T, Kobayashi H. A novel protein, lysenin, that causes contraction of the isolated rat aorta: its purification from the coelomic fluid of the earthworm, *Eisenia foetida*. Biomed Res 1996;17: 197–203.

[16] Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, Umeda M. Lysenin, a novel sphingomyelin-specific binding protein. J Biol Chem 1998;273(9): 5300–6.

[17] Yamaji-Hasegawa A, Makino A, Baba T, Senoh Y, Kimura-Suda H, Sato SB, Terada N, Ohno S, Kiyokawa E, Umeda M, Kobayashi T. Oligomerization and pore formation of a sphingomyelin-specific toxin, lysenin. J Biol Chem 2003; 278(25):22762–70.

[18] Ohta N, Shioda S, Sekizawa Y, Nakai Y, Kobayashi H. Sites of expression of mRNA for lysenin, a protein isolated from the coelomic fluid of the earthworm *Eisenia foetida*. Cell Tissue Res 2000;302(2):263–70.

[19] Lange S, Nussler F, Kauschke E, Lutsch G, Cooper EL, Herrmann A. Interaction of earthworm hemolysin with lipid membranes requires sphingolipids. J Biol Chem 1997; 272(33):20884–92.

[20] Lange S, Kauschke E, Mohrig W, Cooper EL. Biochemical characteristics of Eiseniapore, a pore-forming protein in the coelomic fluid of earthworms. Eur J Biochem 1999;262(2): 547–56.

[21] Eue I, Kauschke E, Mohrig W, Cooper EL. Isolation and characterization of earthworm hemolysins and agglutinins. Dev Comp Immunol 1998;22(1):13–25.

[22] Koenig S, Wagner F, Kauschke E, Peter-Katalinic J, Cooper E L, Eue I. Mass spectrometric analyses of CL(39), CL(41) and H(1), H(2), H(3) confirm identity with fetidin and lysenin produced by earthworm leukocytes. Dev Comp Immunol 2003;27(6–7):513–20.

[23] Stein E, Cooper EL. The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. Dev Comp Immunol 1981;5(3):415–25.

[24] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[25] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74(12):5463–7.

[26] Murray V. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Res 1989; 17(21):8889.

[27] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215(3): 403–10.

[28] Bilej M, De Baetselier P, Beschin A. Antimicrobial defense of the earthworm. Folia Microbiol (Praha) 2000;45(4): 283–300.

[29] Cooper EL, Kauschke E, Cossarizza A. Digging for innate immunity since Darwin and Metchnikoff. Bioessays 2002; 24(4):319–33.

## 4.2. Characterization of CCF-like pattern recognition proteins in different Lumbricid species

Coelomic fluid of *E. fetida* earthworms contains a pattern recognition molecule named coelomic cytolytic factor (Bilej *et al.* 1995; Beschin *et al.* 1998). Upon binding of microbial cell wall components CCF triggers the activation of the prophenoloxidase cascade (proPO), an important invertebrate defense pathway. The broad recognition specificity of CCF is based on the presence of two lectin-like domains, each recognizing different motifs (Bilej *et al.* 2001). Moreover, CCF was found to contribute to the cytolytic as well as trypanolytic activity of the coelomic fluid (Bilej *et al.* 1995; Olivares Fontt *et al.* 2002).

The aim of our study was to characterize CCF-based biological properties of the coelomic fluids of other Lumbricid species: *Aporrectodea caliginosa*, *Apporectodea icterica*, *Apporectodea longa*, *Apporectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus*, *Lumbricus terrestris.*

Western blot analysis revealed that coelomic fluids of all tested species contain a protein cross-reacting with a monoclonal antibody elicited against *E. fetida* CCF. Knowing that *E. fetida* CCF is involved in cytolytic and trypanolytic activities of the coelomic fluid, we tested whether coelomic fluids of other earthworm species are able to lyse TNF-sensitive tumor cell line L929 and *Trypanosoma brucei brucei* parasites. Interestingly, we did not detect either cytolytic or trypanolytic activity in coelomic fluid of any species except that of *E. fetida*.

Since it is known that coelomic fluid of *E. fetida* triggers the proPO cascade, we tested coelomic fluids of other earthworm species to do so upon binding either laminarin or *N, N'*-diacetylchitobiose as these sugar compounds are recognized by spatially distinct CCF domains. All coelomic fluids are able to activate the proPO cascade in the presence of laminarin, whereas only the coelomic fluid of *E. fetida* triggers the cascade in the presence of *N, N'*-diacetylchitobiose suggesting a broader pattern recognition specificity.

The cDNA encoding CCF-like proteins of all other earthworm species was sequenced and 80-90 % identity between the sequences was revealed. A high level of homology was found in the central part of CCF, which is considered as a lectin-like domain capable of binding β-1,3-glucans, which is in coincidence with the ability of CCF and all CCF-like molecules to bind laminarin and consequently to initiate the activation of proPO cascade. The C-terminal part of CCF-like molecules was found to be the most variable supporting the fact that *N, N'*-diacetylchitobiose is recognized by *E. fetida* CCF only.

The phylogenetic analysis of CCF and CCF-like molecules revealed that these molecules of earthworms belonging to the genus *Lumbricus* fall into a well-supported group having the same progenitor, while CCF-like molecules of earthworms belonging to the genus *Apporectodea* are heterogeneous, forming a paraphyletic group. *E. fetida* CCF was found to be closely related to that of *D. veneta*.

Each earthworm species lives in a different part of the soil and thus is subjected to different microbial conditions. The highest microbial activity is definitely found in the places were the organic matter is the most abundant. It is therefore obvious that *E. fetida* living in decaying organic matter, in compost and in mold appears to be best equipped to resist microbial activity, as reflected in the broader CCF pattern recognition specificity.

To sum up, we have identified the presence of CCF-like molecules in other earthworm species both on mRNA and protein level. Furthermore, we found that the CCF of *E. fetida* is particular – it is the only one that recognizes *N, N'*-diacetylchitobiose and has cytolytic and trypanolytic activities. We suggest that the broad recognition repertoire of *E. fetida* CCF reflects a particular microbial environment this species lives in.

**Šilerová M., Procházková P., Josková R., Josens G., Beschin A., De Baetselier P., Bilej M.: Comparative study of the CCF-like pattern recognition protein in different Lumbricid species. *Dev. Comp. Immunol.* 30: 765-771 (2006).**

Šilerová M., Procházková P., Josková R., Josens G., Beschin A., De Baetselier P., Bilej M.: Comparative study of the CCF-like pattern recognition protein in different Lumbricid species. *Dev. Comp. Immunol.* 30: 765-771 (2006).

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Developmental and Comparative Immunology 30 (2006) 765–771
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

# Comparative study of the CCF-like pattern recognition protein in different Lumbricid species

Marcela Šilerová[a], Petra Procházková[a], Radka Josková[a], Guy Josens[b], Alain Beschin[c], Patrick De Baetselier[c], Martin Bilej[a,*]

[a]*Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic*
[b]*Department of Organism Biology, Université Libre de Bruxelles, Av. Roosevelt 50, B-1050 Brussels, Belgium*
[c]*Department of Cellular and Molecular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium*

Received 23 August 2005; received in revised form 1 November 2005; accepted 17 November 2005
Available online 12 December 2005

**Abstract**

Coelomic fluid of the Lumbricid *Eisenia fetida* contains a 42-kDa pattern recognition protein named coelomic cytolytic factor (CCF) that binds microbial cell wall components and triggers the activation of the prophenoloxidase cascade, an important invertebrate defense pathway. Here we report on the sequence characterization of CCF-like molecules of other Lumbricids: *Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus* and *Lumbricus terrestris*, and show that CCF from *E. fetida* has a broader saccharide-binding specificity, being the only one recognizing *N,N'*-diacetylchitobiose. We suggest that the broad recognition repertoire of *E. fetida* CCF reflects a particular microbial environment this species lives in.


*Keywords:* Innate immunity; CCF; Lumbricids; Earthworms; Annelids; Invertebrates

## 1. Introduction

Defense strategies of invertebrates, which naturally lack the adaptive immune system comprising antibodies and lymphocytes, are based on innate mechanisms including wound healing, clotting and coagulation responses, phagocytosis, and encapsulation reactions. Lectin-like molecules and pattern recognition receptors contribute to non-self recognition processes involved in host innate defense not by discriminating between individual antigens but by recognizing surface determinants common to potential pathogens [1]. Coelomic fluid (CF) of *Eisenia fetida* earthworms (*Oligochaeta*, *Annelida*) contains a 42-kDa pattern recognition protein named coelomic cytolytic factor (CCF; [2]). CCF binds, via lectin-like interactions, cell wall components of Gram-negative bacteria (O-antigen of lipopolysaccharide), Gram-positive bacteria (the

*Abbreviations:* CCF, coelomic cytolytic factor; TNF, tumor necrosis factor; CF, coelomic fluid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered solution

*Corresponding author. Tel.: +420 2 4106 2367; fax: +420 2 4172 1143.
*E-mail address:* mbilej@biomed.cas.cz (M. Bilej).

0145-305X/$ - see front matter 
doi:10.1016/j.dci.2005.11.002

peptidoglycan constituents muramic acid and muramyl dipeptide) and yeast ($\beta$-1,3-glucans and *N,N'*-diacetylchitobiose). It also triggers the activation of the prophenoloxidase cascade [3,4], a major invertebrate defense pathway resulting in the production of melanin that exerts antimicrobial and cytotoxic activities [5].

The broad recognition specificity of *E. fetida* CCF is based on the presence of two distinct lectin-like domains [4]. The first domain, localized in the central part of the molecule, has a homology with the polysaccharide-binding motif and glucanase motif of $\beta$-1,3-glucanases and other invertebrate defense molecules; it is implicated in interactions with lipopolysaccharide and $\beta$-1,3-glucans. The C-terminal tryptophan-rich domain interacts with *N,N'*-diacetylchitobiose and peptidoglycan constituents such as muramic acid and muramyl dipeptide.

CCF was originally described as a cytolytic protein that lyses tumor necrosis factor (TNF)-sensitive tumor L929 cell line in a protease-independent way and shows other functional analogies with the mammalian cytokine TNF. CCF is secreted by macrophage-like coelomocytes upon lipopolysaccharide stimulation [6] while TNF is produced by macrophages [7]. Moreover, CCF and TNF have opsonizing properties [2] and both proteins bind $\beta$-1,3-glucans and *N,N'*-diacetylchitobiose via lectin-like interactions [3,8]. The lectin-like domain of TNF is involved in the killing of African and American trypanosomes [9–11] and so is the purified CCF as well as the CF of *E. fetida* earthworms [12]. Importantly, despite their functional analogies, CCF and TNF have neither gene nor amino acid sequence homology, which indicates that they have a different evolutionary origin [13].

The aim of our study was to characterize CCF-based biological properties of CFs of other Lumbricid species: *Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus* and *Lumbricus terrestis* and to link them with their taxonomical classification, taking into account the microbial environment they are subjected to. We show that all species express CCF-like molecules having a high level of homology, particularly in polysaccharide and glucanase-binding motifs. The CF of *E. fetida* is particular—it is the only one that shares TNF-like activities such as cytolytic and trypanolytic activity owing to its lectin-like specificity for *N,N'*-diacetylchitobiose. *E. fetida* is the only species living in mold, an environment with strong antigenic pressure that can result in the broader saccharide recognition capacity of CCF.

## 2. Materials and methods

### 2.1. Animals, isolation of CF and coelomocytes

Adult earthworms (*A. caliginosa* (Savigny), *A. icterica* (Savigny), *A. longa* (Ude), *A. rosea* (Savigny), *D. veneta* (Rosa), *E. fetida* (Savigny), *L. rubellus* (Hoffmeister) and *L. terrestris* (Linne); Oligochaeta, Annelida) were collected from their natural habitats with an electrical expeller [14] and maintained in soil at 4 °C. They were transferred on moist paper towels 2 days before experiment. CF containing coelomocytes from 10 earthworms of each species was harvested by puncturing post-clitellum segments of the coelomic cavity with a Pasteur micropipette. Coelomocytes were separated by centrifugation (500*g*, 10 min, 4 °C) and the coelomocyte pellet was washed twice with Lumbricus balanced salt solution (71.5 mM NaCl, 4.8 mM KCl, 3.8 mM $CaCl_2$, 1.1 mM $MgSO_4$, 0.4 mM $KH_2PO_4$, 0.4 mM $NaH_2PO_4$, 4.2 mM $NaHCO_3$; pH 7.3; [15]) and then used for RNA isolation. The cell-free CF was centrifuged again (7000*g*, 10 min, 4 °C) and the supernatant was stored at −20 °C after having determined protein concentration according to Bradford [16] using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories).

### 2.2. Polyacrylamide gel electrophoresis and Western blot analysis

Gel electrophoresis of CF (10 µl, i.e. 100 µg of protein) was performed in 12% SDS-polyacrylamide gel according to Laemmli [17]. Proteins were transferred on a nitrocellulose membrane and free sites were blocked with 2% bovine serum albumin. Anti-CCF monoclonal antibody 12C9 [2] was added (10 µg/ml) and, after repeated washings, peroxidase-labeled anti-mouse IgG antibody (Sigma-Aldrich) was used for visualization (4-chloro-1-naphtol as substrate).

### 2.3. Cytotoxic L929 assay

L929 fibrosarcoma TNF-sensitive cell line was cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, $1 \times 10^6$ U/l of penicillin, 100 mg/l of streptomycin and 250 µg/l of amphotericin B. One hundred microliters of $4 \times 10^5$ cells/ml cell suspension was

adhered in 96-well flat-bottomed culture plates for 1 h at 37 °C and 5% $CO_2$. Then 100 µl of serially diluted CF was added and after 18 h of incubation at 37 °C cell viability was assessed by crystal violet uptake: cells were stained for 10 min with 100 µl of a 0.5% solution of crystal violet dissolved in 22% ethanol and 8% formaldehyde. The plates were rinsed in water, 100 µl of 30% acetic acid was added and dye uptake was measured at 620 nm.

### 2.4. Trypanosomes and trypanolytic assay

*Trypanosoma brucei brucei* (AnTat 1.1E) was maintained by intraperitoneal inoculations in BALB/c mice. Trypomastigotes were obtained from the blood (supplemented with 10 U of heparin/ml) of infected mice by ion-exchange chromatography on DEAE-cellulose equilibrated with phosphate-buffered saline (PBS) supplemented with 1.5% D-glucose, pH 8.0. Parasites were centrifuged (1800*g*, 10 min) and resuspended at $2 \times 10^6$/ml in glucose-supplemented PBS. Parasite suspension (100 µl) was mixed with 100 µl of serially diluted CF and incubated at 30 °C and 5% $CO_2$ for 3 h. Trypanolysis was quantified by counting the remaining intact parasites under a light microscope.

### 2.5. Prophenoloxidase-activating assay

The level of prophenoloxidase activation was assessed as described previously [3,4]. Briefly, 10 µl of CF (with or without 1 mM serine protease inhibitor Pefabloc (4-(2-aminoethyl)benzene sulfonyl hydrochloride; Boehringer Mannheim), 65 µl of 0.1 M Tris, pH 8.0, containing 50 mM $CaCl_2$ and 10 µl of 15 mM L-DOPA (3-(3,4-dihydroxylphenyl)-L-alanine; Fluka) were incubated at room temperature for 6 h in the absense or presence of laminarin or *N,N'*-diacetylchitobiose (1 µg/ml). Proteolytic enzymes are necessary for activating the cascade; therefore the level of the prophenoloxidase activation was estimated as the difference of L-DOPA oxidation in the absence and presence of the serine protease inhibitor. The oxidation of L-DOPA was measured at 492 nm and expressed as the difference between the values with or without Pefabloc.

### 2.6. RNA isolation, cDNA synthesis, PCR and rapid amplification of cDNA ends (RACE)

Total RNA was isolated from $5 \times 10^6$ coelomocytes using 1 ml of TRIZOL reagent (Gibco BRL) according to the manufacturer's protocol. Three micrograms of DNAse I-treated total RNA was reverse-transcribed using Oligo$(dT)_{12-18}$ primer and Superscript II RNase $H^-$ Reverse Transcriptase (Gibco BRL). Primers for *E. fetida* CCF used in the polymerase chain reaction (PCR) were as follows: sense 5′-TTCACTGACTGGGATCAGCACC-3′ and antisense 5′-ATTGCGTTTGTAGACTCG-GAT-3′. An approximately 1100 bp fragment was amplified using the following cycling parameters: 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 58 °C and 90 s at 72 °C and a final extension for 7 min at 72 °C. The PCR product was ligated in pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The remaining 3′ end of CCF-like cDNA was obtained using 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). Total RNA (3 µg) was reverse-transcribed using an adapter primer (5′-GGCCACGCGTCGACTAG-TACTTTTTTTTTTTTTTTT-3′). Universal amplification primer (5′-CUACUACUACUAGGCC-ACGCGTCGACTAGTAC-3′) and a CCF-specific internal sense primer (5′-CTGACCAGTCTTCC-GAAACA-3′) were used in the subsequent PCR reaction. Similarly, the 5′ end of CCF-like cDNA was obtained using 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). Reverse transcription was carried out with 3 µg of total RNA using an antisense CCF internal primer (5′-TCAGTTGCGCTTGTAGACTCG-3′). Terminal deoxynucleotidyl transferase was used to add homopolymeric oligo-dC tails to the 3′ end of purified cDNA. Primers used in subsequent PCR were: sense abridged anchor primer (5′-GGCCACGCG-TCGACTAGTACGGGIIGGGIIGGGIIG-3′) and antisense CCF specific primer (5′-CTCGATCGG-GCGAGATCGAC-3′). Both 3′ and 5′ RACE products were cloned in pCR2.1-TOPO and sequenced.

### 2.7. Sequencing

Isolated and purified plasmid DNA was sequenced with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The chain termination reaction [18] was performed by the cycle sequencing technique [19] according to the manufacturer's protocol. Finally, sequences were determined with an ABI PRISM 3100 DNA sequencer (Applied Biosystems).

### 2.8. Phylogenetic analysis

The amino acid sequences of CCF and CCF-like molecules were aligned using the CLUSTALW program [20]. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.0 software [21]. The reliability of branching pattern was assessed by bootstrap analysis (100 or 10,000 replications).

## 3. Results and discussion

We have shown the presence of CCF-like proteins, originally reported in *E. fetida* as CCF [2,4], in other earthworm species: *A. caliginosa*, *A. icterica*, *A. longa*, *A. rosea*, *D. veneta*, *L. rubellus* and *L. terrestris*. Using Western blot analysis we detected a 42-kDa protein in CFs of all tested species cross-reacting with 12C9, a monoclonal antibody elicited against *E. fetida* CCF (Fig. 1a).

Knowing that *E. fetida* CCF is involved in cytolytic and trypanolytic activities of the CF [2,12], we wanted to address whether CFs of other earthworm species have the same properties. CFs of all eight species were tested, in the concentration range of 10 ng/ml–10 mg/ml, for the ability to lyse fibrosarcoma TNF-sensitive cell line L929 and *T. brucei brucei* parasites. Whereas the CF of *E. fetida* causes the lysis of 50% L929 cells in the concentration 100 μg/ml (LD50) and 50% parasites in the concentration 1.25 μg/ml, we were not able to detect either cytolytic or trypanolytic activity in the CF of any other earthworms (data not shown).

It was documented that the CF of *E. fetida* triggers the prophenoloxidase cascade which can be activated by a broad range of microbial compounds such as lipopolysaccharide, saccharides bearing β-1,3-glucosidic link (laminarin, curdlan, zymosan), *N,N′*-diacetylchitobiose or peptidoglycan constituents muramyl dipeptide and muramic acid [3]. In a related Lumbricid, originally classified as *L. terrestris*, CF has a narrower pattern recognition specificity—neither *N,N′*-diacetylchitobiose nor peptidoglycan compounds can activate the cascade [4].

We therefore examined the ability of CFs of *A. caliginosa*, *A. icterica*, *A. longa*, *A. rosea*, *D. veneta*, *E. fetida*, *L. rubellus* and *L. terrestris* to trigger the prophenoloxidase pathway upon binding laminarin and *N,N′*-diacetylchitobiose as these sugar compounds are recognized by spatially distinct CCF domains in *E. fetida* earthworms [4]. We found that the CFs of all tested earthworm species were able to induce the oxidation of L-DOPA, i.e. to initiate the prophenoloxidase cascade in the presence of laminarin, whereas the CF of *E. fetida* is the only one triggering the cascade in the presence of *N,N′*-diacetylchitobiose (Table 1). This indicates that the pattern recognition specificity of CCF in *E. fetida* earthworms is broader than in other earthworm species. We already reported [4] that recognition of lipopolysaccharide and β-1,3-glucans—mediated by the central part of CCF encompassing the polysaccharide-binding and glucanase motif—leads to the activation of the prophenoloxidase cascade both in *E. fetida* and *L. terrestris* earthworms. Yet, *N,N′*-diacetylchitobiose and peptidoglycan trigger prophenoloxidase cascade only in *E. fetida* earthworms. Sequence differences in the C-terminal carbohydrate-binding domain were suggested to account for the inability of *L. terrestris* CCF to bind *N,N′*-diacetylchitobiose and peptidoglycan [4]. Therefore, we sequenced the CCF-like gene in all other species.

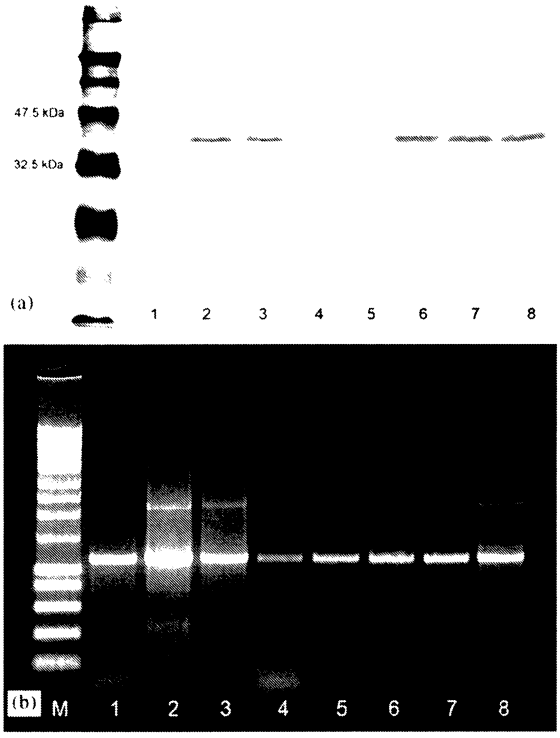



Fig. 1. (a) Detection of CCF and CCF-like molecules in the coelomic fluids by Western blot using anti-*E. fetida* CCF monoclonal antibody. (b) PCR analysis using primers specific for *E. fetida* CCF reveals the presence of CCF-like mRNA. Lane 1: *A. caliginosa*, lane 2: *A. icterica*, lane 3: *A. longa*, lane 4: *A. rosea*, lane 5: *D. veneta*, lane 6: *E. fetida*, lane 7: *L. rubellus* and lane 8: *L. terrestris*. Protein markers or DNA ladder marker are on the left margin (M).

Table 1
Activation of the prophenoloxidase cascade in the coelomic fluid of different earthworm species by laminarin and *N,N'*-diacetylchitobiose

| | $A_{492}$ | |
|---|---|---|
| | Laminarin | *N,N'*-diacetylchitobiose |
| *A. caliginosa* | 0.596±0.081 | 0.072±0.026 |
| *A. icterica* | 0.681±0.075 | 0.065±0.031 |
| *A. longa* | 0.542±0.073 | 0.055±0.019 |
| *A. rosea* | 0.567±0.068 | 0.061±0.023 |
| *D. veneta* | 0.569±0.083 | 0.071±0.022 |
| *E. fetida* | 0.712±0.051 | 0.563±0.085 |
| *L. terrestris* | 0.643±0.090 | 0.068±0.034 |
| *L. rubellus* | 0.705±0.066 | 0.082±0.039 |

For each species, the coelomic fluid of 10 individual animals was tested. The level of activation is expressed as the difference between $A_{492}$ of the sample with and without serine protease inhibitor±SD.

Using specific primers designed from the nucleotide sequence of *E. fetida* CCF, approximately 1100 bp fragments were obtained in PCR from the cDNAs of all eight earthworm species (Fig. 1b). In order to assemble the full-length cDNA sequence of CCF-like genes, RACE amplifications of the 5′ and 3′ cDNA ends were performed. Resulting PCR products were cloned and sequenced. Deduced amino acid sequences of CCF-like molecules of all species are shown in Fig. 2a. CCF-like molecules display 80–90% sequence identity; they are nearly identical in the polysaccharide-binding motif (with an exception in *A. icterica* where histidine residue 150 is substituted by serine, and in *A. rosea* where histidine 150 is replaced by arginine and methionine 165 by leucine) and glucanase motif (except for one threonine residue 186 which is in endogeic earthworms replaced by either serine in *A. caliginosa* and *A. icterica* or by alanine in *A. rosea*). A high level of homology in this central part of CCF, which is considered as a lectin-like domain capable of binding β-1,3-glucans, is in coincidence with the ability of CCF and all CCF-like molecules to bind laminarin and initiate activation of the prophenoloxidase cascade. All molecules contain a high number of tryptophan residues (5–7%) located mainly in the C-terminal part of the molecules. However, this part of all CCF-like molecules is the most variable as compared to the C-terminal part of *E. fetida* CCF, which is in accordance with the fact that *E. fetida* CCF is the only one recognizing *N,N'*-diacetylchitobiose. Nucleotide sequences of CCF-like molecules were submitted to the GeneBank database and can be found under these accession numbers: *A. caliginosa* DQ017143; *A. icterica* DQ 018721; *A. longa* DQ018722; *A. rosea* DQ018723; *D. veneta* DQ018724 and *L. rubellus* DQ018725. We found that the sequence of CCF homologue described previously for *L. terrestris* [4] is, due to a taxonomic error, a sequence of *A. longa*, a species macroscopically similar.

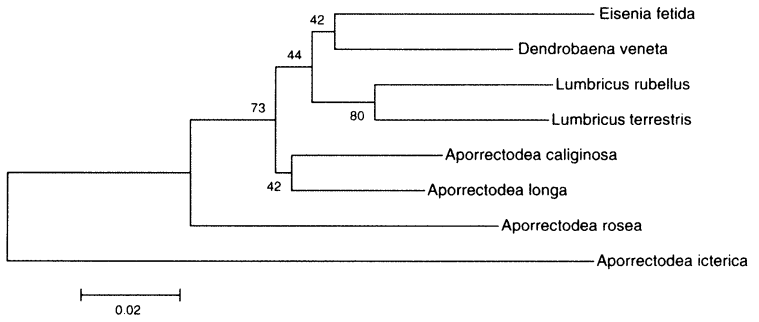
To gain more insight into the relationships among CCF-like genes, all putative CCF-like amino acid sequences were subjected to phylogenetic analysis. Based on both amino acid and nucleotide sequences of CCF and CCF-like molecules the neighbor-joining phylogenetic trees were constructed using MEGA version 3.0 software [21]. Both data sets resulted in trees with identical topologies; only the protein tree is therefore shown (Fig. 2b). The confidence in each node was assessed by 100 or 10,000 bootstrap replicates.

The amino acid sequences of CCF-like molecules of earthworms of genus *Lumbricus* are highly homologous (94% identity) and fall together into a well-supported group (bootstrap value 80%) having the same progenitor. On the other hand, CCF-like molecules of the genus *Aporrectodea* are more heterogeneous; they form a paraphyletic group although the identity between their CCF-like molecules is more than 80%. *E. fetida* CCF is closely related to the CCF-like molecule of *D. veneta* (bootstrap value 40%) although its saccharide recognition specificity differs.

Both *E. fetida* and *D. veneta* are epigeic earthworms which prefer the loose upper topsoil layer where they feed on decaying organic matter. They are adapted to the high predation pressure and the widely variable moisture and temperature conditions at the soil surface. The composition of microflora varies in different parts of the soil and can play an important role in molecular evolution of CCF and CCF-like molecules. It was documented that the soil microbial diversity is dependent on a vast number of environmental factors such as water and substrate availability, temperature, pH, mineral components as well as soil structure and pore size [22,23]. Thus each earthworm group living in a different part of the soil is subjected to different microbial conditions. The highest microbial activity is definitely found in the places where the organic matter is the most abundant. It is therefore obvious that *E. fetida* living in decaying organic matter, in compost and in mold appears to be best equipped to

```
E.fetida      MRWTLVVLCL LFGEG*FAFTD WDQYHIVWQD EFDYFDGAKW QHEVTATGGG NSEFQLYTQD   60
A.caliginosa  ----V----- -L---C----- ---------- ---F-E-G-- ---------- ----------   61
A.icterica    ----V----- -I---C---D- ---FE----- ---TL-DK-- ------S--- ----------   61
A.longa       ----A----- -LS--C----- ---H------ ---F---G-- ---------- ----------   61
A.rosea       ---AA----- -L--CCL---- ----Q----- ---F---G-- ---------- ----------   61
D.veneta      ----A----- -LS--*L---- ----Q----- ---FL-TG-- ---------- ----------   60
L.rubellus    MSLT-V----- -----CL---- ---------- ---FL-YG-- ---------- ----------   62
L.terretris   --L-V----- -L-G-CM---- ---HQ----- ---F---G-- ---------- ----------   60

E.fetida      GANSFVRDGK LFIKPTLLAD NINPQTGAPF GTDFMYNGVL DVWAMYGACT NTDNNGCYRT   120
A.caliginosa  S--------- ---------- TN-----Q-Y -----N---- ----N--T-- ----------   121
A.icterica    K--------- --------V- ----E-DK-Y -N---KS--- --K-N-ES-- -A-----S--   121
A.longa       SR-------- -----S---- -N-----Q-Y -----N---- ----N----- ----------   121
A.rosea       SR-------- -----S---- -N-----Q-Y --E--N---- --S-N----- ----------   121
D.veneta      T---Y----- ---------- -------Q-Y -----N---- ----N----- ----------   120
L.rubellus    SV-------- ---------- TN-----Q-Y -----N---- ----N----- -S--------   122
L.terretris   ----W----- ---------- TN-----Q-Y -----N--I- ----N----- -S--------   120

E.fetida      GAAGDIPPAM SARVRTFQKY SFTHGRVVVH AKMPVGDWLW PAIWMLPEDW VYGGWPRSGE   180
A.caliginosa  --------V- ---L------ ---------- ---------- --------N- ----------   181
A.icterica    --------V- ---L-SYE-F ---F----IS ---------- --------K- E--D------   181
A.longa       --------V- ---------F ---------- ---------- --------N- ----------   181
A.rosea       ----N---V- ---L-----F ---F-----R ---------- ----L---N- ----------   181
D.veneta      --------V- ---L--Y-R- ---------- ---------- --------A- ----------   180
L.rubellus    --------V- ---L----R- ---------- ---------- ---------- ----------   182
L.terretris   --------V- --------RF ------A--- ---------- ---------- ----------   180

E.fetida      IDIIETIGNR DFKNTGGEFL GIQKMGSTMH WGPGWDDNRY WLTSLPKHSD DWNYGDNFHT   240
A.caliginosa  -----S---- ---------- ---------- ---------- --------E- G---------   241
A.icterica    -----S---- ---D-N-G-I --------L- ---AP-E--F G--H-S-ND- GR--------   241
A.longa       ---------- ---S-S---- ---------- ---------- --------D- G---------   241
A.rosea       -----A---- ---S-S---- --H-----L- ---A-----F -K-------D- GR--------   241
D.veneta      ---------- --------- ---------- ---------- ---------F -------D- G---------   240
L.rubellus    ---------- -------M- ---------- ---------- --------E- S---------   242
L.terretris   ---------- ---S-S--M- ---------- ---------- --------D- G---------   240

E.fetida      FWFDWSPNGL RFFVDDENQA LLDVPYPLID ANPWWVDFWE WGKPWLPQYE NDNPWAGGTN   300
A.caliginosa  ---------- ---I------ ---------- ---------- -----K---T **-----AS-   299
A.icterica    -Y-------- ---------- ---------- K--D--N--- -----K-ETT **---ES---   299
A.longa       -Y-------- ---------- ---------- ---------- -----K-E-T **-----AS-   299
A.rosea       -Y-------- ---I------ ---------- K--S--N--- -----K---- ------R-S-   301
D.veneta      ---------- ---------- ---------- ---------- ---------- ------S---   300
L.rubellus    ---------- ---------- ---------- --D--IN--- -----K-E-P *---------   301
L.terretris   -Y-------- ---------- ---------- --D------- -----A---P *---------   299

E.fetida      LAPFDQNFHF ILNVAVGGTN GFIPDGCINR GGDPALQKPW SNGDWYNDAM RKFFDARGNW   360
A.caliginosa  ------A--- ---------- -------V-- --N------- ------VT-- Q--YN-----   359
A.icterica    -----KA--- --------L- ------AT-* --E***---- ----S-VS-- Q--Y------   355
A.longa       ------A--- ---------- ---------- ---------- ------VT-- QN-YN-----   359
A.rosea       ------A--- ---------- ---------- ---------- ------VT-- Q--YN--W--   361
D.veneta      ------A--- ---------- ---------- ---------- ------VD-- Q--FN-----   360
L.rubellus    ---------- --------L- ---------- ---------- ------VT-L Q--YN-----   361
L.terretris   ---------- ---------- ---------- ---------- ------VT-- Q--YN-----   359

E.fetida      KWTWDDEGDN NAMQVDYIRV YKRN                                        384
A.caliginosa  -----N---- ---------- ----                                        383
A.icterica    -G-------- ---------- ---K                                        379
A.longa       -----N---- --L------- ----                                        383
A.rosea       -----N---- ---------- --HI                                        385
D.veneta      -----N---- --L------- --HI                                        384
L.rubellus    ---------- --L------- ----                                        385
L.terretris   ---------- --L------- ---I                                        383
```

(a)




(b)

Fig 2. (a) Alignment of amino acid sequences of *E. fetida* (GeneBank accesion no. AF030028), *A. caliginosa* (DQ017143), *A. icterica* (DQ018721), *A. longa* (DQ018722), *A. rosea* (DQ018723), *D. veneta* (DQ018724) *L. rubellus* (DQ018725) and *L. terrestris* (AF395805) CCF and CCF-like molecules. Only mutated amino acids as compared with *E. fetida* CCF are indicated. Polysaccharide-binding motif is underlined; glucanase motif is double-underlined, signal peptide is dotted line underlined, asterisks indicate missing amino acids. (b) Neighbor-joining phylogenetic tree relating CCF and CCF-like proteins in selected Lumbricids. The numbers near the nodes represents bootstrap values.

resist microbial activity, as reflected by the broader CCF pattern recognition specificity. This can explain why the CCF-like molecule of *D. veneta* that lives in a similar but usually less aggressive environment is so similar to *E. fetida* CCF.

To sum up, we have identified the presence of CCF-like molecules in other different earthworm species both on mRNA and protein level. Furthermore, we found that the CF of *E. fetida* earthworms is particular—it is the only one that recognizes *N,N'*-diacetylchitobiose and has CCF-based cytolytic and trypanolytic activities. We suggest that the broad recognition repertoire of *E. fetida* CCF reflects a particular microbial environment this species lives in.

## Acknowledgements

This work, supported by the Czech Science Foundation (310/04/0806 and 310/03/4147), the Grant Agency of the Academy of Sciences (A5020208), an Institutional Research Concept (AV0Z50200510) and a bilateral international scientific and technological cooperation grant from the Ministry of the Flemish Community (BOF-BWS 03/06), was performed within the frames of an Interuniversity Attraction Pole Program. Authors are grateful to Václav Pižl for helpful discussion.

## References

[1] Janeway Jr CA. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989;54(Part 1):1–13.

[2] Bilej M, Brys L, Beschin A, Lucas R, Vercauteren E, Hanusova R, et al. Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms. Immunol Lett 1995;45(1–2):123–8.

[3] Beschin A, Bilej M, Hanssens F, Raymakers J, Van Dyck E, Revets H, et al. Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. J Biol Chem 1998;273(38):24948–54.

[4] Bilej M, De Baetselier P, Van Dijck E, Stijlemans B, Colige A, Beschin A. Distinct carbohydrate recognition domains of an invertebrate defense molecule recognize Gram-negative and Gram-positive bacteria. J Biol Chem 2001;276(49):45840–7.

[5] Cerenius L, Soderhall K. The prophenoloxidase-activating system in invertebrates. Immunol Rev 2004;198:116–26.

[6] Bilej M, Rossmann P, Sinkora M, Hanusova R, Beschin A, Raes G, et al. Cellular expression of the cytolytic factor in earthworms *Eisenia foetida*. Immunol Lett 1998;60(1):23–9.

[7] Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, et al. Human tumor necrosis factor. Production, purification, and characterization. J Biol Chem 1985;260(4):2345–54.

[8] Olson EJ, Standing JE, Griego-Harper N, Hoffman OA, Limper AH. Fungal beta-glucan interacts with vitronectin and stimulates tumor necrosis factor alpha release from macrophages. Infect Immun 1996;64(9):3548–54.

[9] Lucas R, Magez S, De Leys R, Fransen L, Scheerlinck JP, Rampelberg M, et al. Mapping the lectin-like activity of tumor necrosis factor. Science 1994;263(5148):814–7.

[10] Magez S, Geuskens M, Beschin A, del Favero H, Verschueren H, Lucas R, et al. Specific uptake of tumor necrosis factor-alpha is involved in growth control of *Trypanosoma brucei*. J Cell Biol 1997;137(3):715–27.

[11] Olivares Fontt EO, De Baetselier P, Heirman C, Thielemans K, Lucas R, Vray B. Effects of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha on *Trypanosoma cruzi* trypomastigotes. Infect Immun 1998;66(6):2722–7.

[12] Olivares Fontt E, Beschin A, Van Dijck E, Vercruysse V, Bilej M, Lucas R, et al. *Trypanosoma cruzi* is lysed by coelomic cytolytic factor-1, an invertebrate analogue of tumor necrosis factor, and induces phenoloxidase activity in the coelomic fluid of *Eisenia foetida foetida*. Dev Comp Immunol 2002;26(1):27–34.

[13] Beschin A, Bilej M, Brys L, Torreele E, Lucas R, Magez S, et al. Convergent evolution of cytokines. Nature 1999;400(6745):627–8.

[14] Tiho S, Josens G. Earthworm populations of Roosevelt Avenue (Brussels, Belgium): composition, density and biomass. Belg J Zool 2000;130(2):131–8.

[15] Stein E, Cooper EL. The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. Dev Comp Immunol 1981;5(3):415–25.

[16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680–5.

[18] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74(12):5463–7.

[19] Murray V. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Res 1989;17(21):8889.

[20] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22(22):4673–80.

[21] Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 2004;5:150–63.

[22] Ranjard L, Richaume A. Quantitative and qualitative microscale distribution of bacteria in soil. Res Microbiol 2001;152(8):707–16.

[23] Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H, Kandeler E. Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Appl Environ Microbiol 2001;67(9):4215–24.

## 4.3. Interaction of CCF with vertebrate cells

Coelomic cytolytic factor, a pattern recognition molecule of *E. fetida* earthworms, was shown to share functional analogies with the mammalian cytokine TNF that are based on similar saccharide recognition specificity. It was documented that CCF lyses TNF-sensitive tumor cell lines (Bilej *et al.* 1995). In addition, CCF is secreted by macrophage-like coelomocytes upon LPS stimulation, while TNF is produced by LPS-activated macrophages (Aggarwal *et al.* 1985; Bilej *et al.* 1998). Both TNF and CCF have opsonizing properties (Luo *et al.* 1993; Bilej *et al.* 1995) and bind β-1,3-glucans and *N*, *N'*-diacethylchitobiose via lectin-like interactions (Olson *et al.* 1996; Beschin *et al.* 1998). The lectin-like domain of TNF is involved in the killing of African and American trypanosomes (Lucas *et al.* 1994; Magez *et al.* 1997; Olivares Fontt *et al.* 1998) and so is the lectin-like domain of CCF (Olivares Fontt *et al.* 2002). Interestingly, the functional similarity of CCF and TNF is not based on a structural homology but rather represents a convergence of function based on a similar lectin-like activity (Beschin *et al.* 1999; Beschin *et al.* 2001).

More recently, the ability of the *N*, *N'*-diacetylchitobiose lectin-like domain of TNF to induce a pH-dependent increase of membrane conductance resulting in membrane depolarization of endothelial cell and macrophages was shown (Hribar *et al.* 1999). It was suggested that the membrane depolarization is due to the interaction of TNF with amiloride-sensitive ion channels, most likely sodium ion channels (van der Goot *et al.* 1999; Fukuda *et al.* 2001). Similarly, CCF was found to activate amiloride-sensitive ion channels in a TNF receptor (TNFR) independent manner via its *N*, *N'*-diacetylchitobiose lectin-like domain (Bloc *et al.* 2002). In this study, we wanted to investigate whether the interaction of CCF with the membrane of non-elicited adherent peritoneal cells (PECs) affects the activation of these cells.

The incubation of PECs isolated either from wild-type or TNFR 1 and 2 knock-out mice with CCF resulted in the production of TNF, IL-6 and NO in a dose-dependent

manner. Moreover, the secretion of cytokines and NO is completely inhibited by *N*, *N'*-diacetylchitobiose.

Membrane depolarization in PECs from wild-type, TNFR 1, TNFR 2 and TNF knock-out mice caused by CCF was evidenced using the fluorescent dye bis-oxonol by fluorescence microcopy and flow cytometry. CCF-induced membrane depolarization is inhibited by amiloride and *N*, *N'*-diacetylchitobiose confirming lectin-like saccharide interaction of CCF with a sodium channel or with some associated structure.

To further address the role of sodium channels in the activation of PECs, their three inhibitors were tested. Phenamil, inhibiting both ion-gated sodium channels and $Na^+/H^+$ exchangers, was found to be the most potent inhibitor, whereas amiloride, an inhibitor of epithelial sodium channels and $Na^+/H^+$ exchangers, is less potent.

To identify the intracellular signaling pathway involved in CCF-mediated PECs activation, three different inhibitors were tested and the results suggest that membrane depolarization leads to NF-κB activation but not on MAPKK activation.

In summary, we propose a model of macrophage activation where CCF binds via its *N*, *N'*-diacetylchitobiose domain to an $Na^+/H^+$ exchanger or an $Na^+/H^+$ exchanger associated molecule. The resulting membrane depolarization leads to NF-κB activation and subsequent production of TNF, IL-6 and NO. In turn, the secreted TNF interacts mainly via its *N*, *N'*-diacetylchitobiose lectin-like domain with the ion channel/ion channel-related structure, or 'classically' via its receptor-binding site with TNFR1 or TNFR2 on the macrophage surface thus boosting the activation signal provided by the lectin domains of CCF and TNF.

**Bilej M., Josková R., Van den Bergh R., Procházková P., Šilerová M., Ameloot P., De Baetselier P., Beschin A.: An invertebrate TNF functional analogue activates macrophages via lectin-saccharide interaction with ion channels. *Int. Immunol.* 18(12):1663-70 (2006).**

Bilej M., Josková R., Van den Bergh R., Procházková P., Šilerová M., Ameloot P., De Baetselier P., Beschin A.: An invertebrate TNF functional analogue activates macrophages via lectin-saccharide interaction with ion channels. *Int. Immunol.* 18(12):1663-70 (2006).

*International Immunology, Vol. 18, No. 12, pp. 1663–1670*
doi:10.1093/intimm/dxl100



# An invertebrate TNF functional analogue activates macrophages via lectin–saccharide interaction with ion channels

**Martin Bilej[1], Radka Josková[1], Rafael Van den Bergh[2], Petra Procházková[1], Marcela Šilerová[1], Paul Ameloot[3], Patrick De Baetselier[2]** and **Alain Beschin[2]**

[1]Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[2]Laboratory of Cellular and Molecular Immunology, Department of Cellular and Molecular Interactions, 'Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel', Brussels, Belgium
[3]Department for Molecular Biomedical Research, 'Vlaams Interuniversitair Instituut voor Biotechnologie', Ghent, Belgium

*Keywords*: invertebrate cytokine, membrane depolarization, NF-κB, pattern-recognition protein, TNF

**Abstract**

**The invertebrate pattern-recognition protein named coelomic cytolytic factor (CCF) and the mammalian cytokine tumor necrosis factor (TNF) share functional analogies that are based on a similar saccharide recognition specificity. In particular, CCF and TNF have been shown to interact with ion channels on the surface of vertebrate cells via *N,N′*-diacetylchitobiose lectin-like activity. In the present study, we show that CCF-induced membrane depolarization results in the release of TNF, IL-6 and nitric oxide (NO) by macrophages via nuclear factor-κB signaling. Interestingly, our data suggest that TNF contributes, through lectin–saccharide interaction, to the secretion of IL-6 and NO induced by CCF. This experimental non-physiological setting based on the interaction of an invertebrate defense lectin with vertebrate cells involved in the innate immune response may have highlighted an evolutionarily ancient mechanism of macrophage activation in vertebrates.**

## Introduction

Coelomic fluid of common brandling earthworms *Eisenia fetida* (*Oligochaeta*, *Annelida*) contains numerous molecules with different activities, including anti-bacterial, hemolytic and cytolytic [for review see (1)]. Previously we have isolated from the coelomic fluid a cytolytic factor named coelomic cytolytic factor (CCF) (GenBank accession no. AF030028) acting in earthworm defense as a pattern-recognition molecule (2, 3). Upon binding microbial pathogen-associated molecular patterns, namely the O-antigen of LPS, β-1,3-glucans, peptidoglycan constituents or *N,N′*-diacetylchitobiose, CCF triggers the prophenoloxidase-based anti-microbial defense mechanism in the earthworm coelomic fuid. In addition, CCF was shown to exhibit tumor necrosis factor (TNF)-like features. Indeed, CCF lyses TNF-sensitive tumor cell lines (4). CCF expression is up-regulated in macrophage-like coelomocytes upon LPS stimulation (5, 6) while TNF is produced by macrophages (7). Moreover, both TNF and CCF were suggested to interact with various pathogens via saccharide recognition (2, 8), for review see Lucas *et al.* (9), and to lyse African and American trypanosomes via a similar lectin-like activity with *N,N′*-diacetylchitobiose specificity (10–12). Interestingly, the functional similarity of CCF and TNF is not based on a structural homology but rather represents a convergence of function based on a similar lectin-like activity (11, 13).

More recently, the capacity of the *N,N′*-diacetylchitobiose lectin-like domain of TNF to induce a pH-dependent increase of membrane conductance resulting in membrane depolarization has been demonstrated in primary lung microvascular endothelial cells and in peritoneal macrophages (14). Since the insertion of TNF into the cell membrane is not sufficient to change the conductance, it was suggested that the membrane depolarization is due to the interaction of TNF with amiloride-sensitive, most likely sodium ion channels (15, 16). Similarly, CCF was found to activate amiloride-sensitive ion channels in lung endothelial cells and peritoneal macrophages in a TNF receptor (TNFR)-independent manner via its *N,N′*-diacetylchitobiose lectin-like domain (17). In the present study, we investigated whether the interaction of CCF lectin-like domain with the membrane of non-elicited adherent peritoneal cells (PECs), mainly macrophages, affects the activation of the cells.

*Correspondence to*: M. Bilej; E-mail: mbilej@biomed.cas.cz

*Transmitting editor*: A. Falus

*Received* 17 March 2006, *accepted* 11 September 2006

Advance Access publication 11 October 2006

We show that CCF-induced membrane depolarization via *N,N′*-diacetylchitobiose lectin-like activity results in the release of TNF, IL-6 and NO by macrophages via nuclear factor-κB (NF-κB) signaling. Further, we propose that the lectin-like activity of the invertebrate functional analogue of TNF divulges an ancient mechanism of macrophage activation in vertebrates.

## Methods

### *Animals and reagents*

Female wild-type C57BL/6 mice (8- to 10-weeks old) were purchased from Harlan. *tnf* knock-out C57BL/6 mice were a kind gift from K. Sekikawa (National Institute of Animal Health, Tsukuba City, Japan). *tnfr1* and *tnfr2* knock-out C57BL/6 mice were a kind gift of H. Bluethmann (Hoffman-La Roche). All knock-out mice were bred at our animal facility and were kept in filter-top cages. The mice care was in accordance with institutional guidelines.

*N,N′*-diacetylchitobiose, amiloride, benzamil and phenamil were obtained from Sigma. Bis-oxonol [bis-(1,3-dibutylbarbituric acid)-trimethine oxonol, $DiBAC_4$] was purchased from Molecular Probes.

Recombinant CCF (CCF) was expressed as described (2) and further purified to homogeneity on an anti-CCF-1 mAb column (4). CCF was re-suspended in PBS (pH 8.0) and LPS contamination was excluded using QCL LAL test (Bio-Whittaker Europe, Verviers, Belgium).

### *Cells*

Resident peritoneal cells were collected in 0.34 M sucrose and washed in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, $5 \times 10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine, 100 IU $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin and 0.1 mM non-essential amino acids; all from Invitrogen Life Technologies). Adherent cells were recovered after plastic adherence as described previously (18). Collected cells [peritoneal exudate cells (PECs)] had a viability >95%, as determined by trypan blue exclusion, and were F4/80$^+$ from 75%, as determined by flow cytometry analysis (data not shown).

### *Quantification of cytokines and nitrite*

PECs ($2 \times 10^5$ per 200 μl) from individual mice were cultured in triplicate in humidified atmosphere containing 5% $CO_2$ in complete medium in the absence or presence of CCF at the indicated concentration for 24 h at 37°C. Cytokines were quantified using sandwich ELISAs for TNF and IL-6, performed as recommended by the supplier (R&D Systems and PharMingen, respectively). NO in culture supernatants was estimated by quantifying $NO_2$ using Griess reagent as described previously (19). When required, PECs were incubated with amiloride, benzamil or phenamil at the indicated concentrations for 30 min before CCF activation. Alternatively, CCF was pre-incubated with the indicated concentrations of *N,N′*-diacetylchitobiose before addition to the cell cultures.

### *Analysis of membrane depolarization*

For fluorescence microscopy, PECs ($10^6$ $ml^{-1}$) were adhered overnight on microscope cover slips and extensively washed (1% BSA in PBS). Cells were then incubated with 100 μl serum-free RPMI 1640 alone or 100 μl CCF (10 μg $ml^{-1}$) in serum-free RPMI 1640 for 1 h at room temperature and pulsed with bis-oxonol at a final concentration of 1 μM for a further 30 min. For inhibition experiments, cells were incubated with 100 μM amiloride for 30 min before CCF activation. Alternatively, CCF was pre-incubated with *N,N′*-diacetylchitobiose (5 μg $ml^{-1}$) before addition to the cell cultures. Samples were washed three times with 3 ml 1% BSA in PBS and examined under an Olympus Provis (Olympus Optical) fluorescence microscope.

For flow cytometric analysis, PECs were seeded in polystyrene FACS tubes, washed and then treated with CCF, inhibitors and bis-oxonol as described above. After the final washing, cells were re-suspended in 200 μl of FACS buffer and analyzed immediately using a Becton Dickinson FACSVantage SE system. Relative fluorescence unit values were calculated by dividing the percentage of positive cells in treated samples by that in untreated samples.

### *RNA isolation, cDNA synthesis and PCR*

Total RNA was prepared from PECs by using 1 ml of Trizol reagent according to the manufacturer's protocol (Invitrogen). DNAse I-treated total RNA (2 μg) was reverse transcribed with $Oligo(dT)_{12-18}$ (Invitrogen) and SUPERSCRIPT™ II Rnase H$^-$ Reverse Transcriptase (Invitrogen). Resulting cDNAs were used in PCR with specific primers for $Na^+/H^+$ exchanger (NHE-1, GenBank accession no. U51112)—sense primer 5′-CTT CTG TTG GCC AGT TCT AC-3′ and anti-sense primer 5′-TAC ATG GTT GTC GAT GTC AC-3′. PCRs were performed with cDNA templates (0.5 μl of the RT reaction product), to which 2 U of *Taq* polymerase (Invitrogen), 0.2 mM deoxynucleoside triphosphate, 1× company-supplied buffer, 1.5 mM $MgCl_2$ and 0.4 μM primer pairs were added. PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 40 s, 72°C for 90 s and 72°C for 7 min.

The identity of PCR products was confirmed by sequencing.

### *Statistical analysis*

All comparisons were tested for statistical significance ($P < 0.05$) via the unpaired *t*-test, using GraphPad Prism 3.0 software. Data were expressed as the mean ± SD of three individual mice and are representative of at least three independent experiments.

## Results

### *The effect of CCF on cytokine and NO production by PECs*

The *in vitro* activation of non-elicited adherent PECs (75% F4/80$^+$ cells, data not shown) isolated from wild-type and TNFR (1 and 2) knock-out mice with CCF resulted in the production of TNF in a dose-dependent manner in the range of 0.5–5 μg $ml^{-1}$ (Fig. 1). A higher CCF concentration did not further enhance the cytokine release. The differences in TNF production by PECs isolated from wild-type and TNFR knock-out mice were negligible, thus excluding the autocrine effect of TNF via surface TNFRs. Expectedly, PECs from TNF knock-out mice did not produce TNF upon activation with CCF. CCF

triggered similar production of IL-6 and of NO in PECs from wild-type and TNFR knock-out mice (Fig. 1). IL-6 and NO production in PECs from TNF knock-out mice was <40% of the value obtained in PECs from wild-type or TNFR knock-out mice suggesting the cumulative effect of CCF and TNF on IL-6 and NO release.

Increased TNF, IL-6 and NO production by PECs was likely not due to LPS contamination since CCF samples were below the detection limit of LAL test. Moreover, comparable cytokine and NO secretion levels were observed with CCF samples treated or not treated with polymixin. Finally, the digestion of CCF with pronase abolished its ability to induce cytokine and NO release (data not shown).

### *The effect of ion channel inhibitors on CCF-mediated PECs activation*

In agreement with our previous patch-clamp experiments (17), CCF caused a membrane depolarization in PECs that was evidenced using the fluorescent anionic dye bis-oxonol in fluorescence microscopy and flow cytometry. As shown in Fig. 2, CCF-induced membrane depolarization was inhibited by amiloride and by *N,N'*-diacetylchitobiose, confirming a

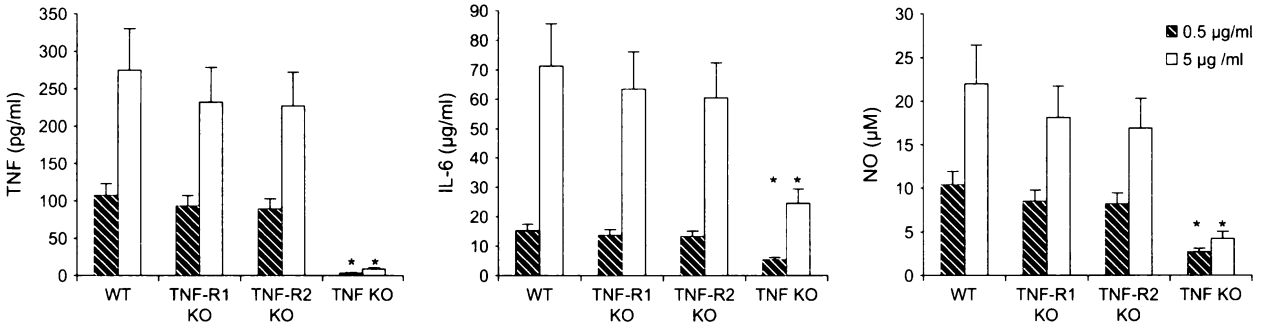



**Fig. 1.** CCF triggers TNF, IL-6 and NO production in adherent PECs. Non-elicited adherent PECs isolated from wild-type (WT), TNFR1, TNFR2 and TNF knock-out (KO) mice were stimulated with two doses of CCF for 24 h. TNF, IL-6 and NO production was then determined in cell supernatants. Data are expressed as the mean ± SD of three individual mice and are representative of at least three independent experiments. (*$P < 0.05$).

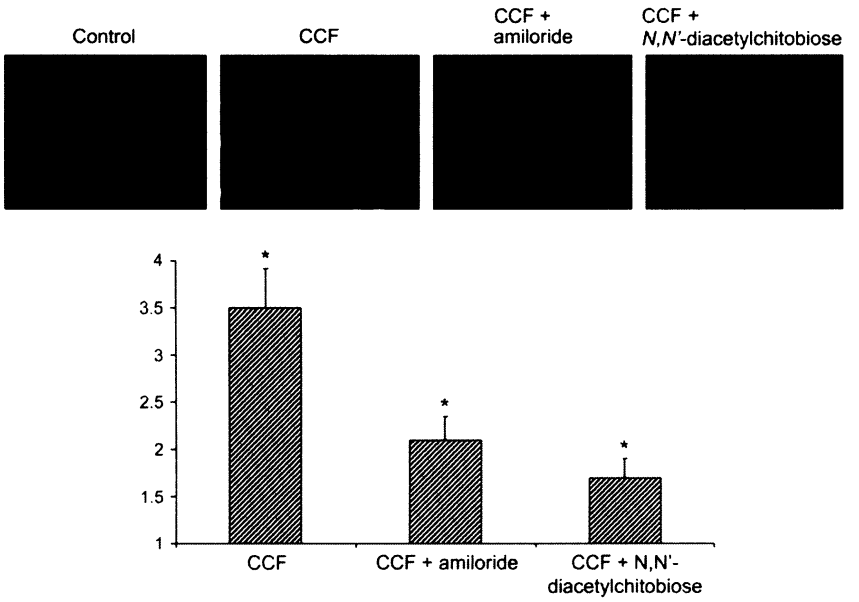



**Fig. 2.** Depolarization induced by CCF is inhibited by amiloride and *N,N'*-diacetylchitobiose. Non-elicited adherent PECs isolated from wild-type mice were incubated with CCF for 1 h. Cells were then pulsed with bis-oxonol for a further 30 min and examined under fluorescence microscope (upper panel) or analyzed by flow cytometry (lower panel). For inhibition experiments, cells were incubated with amiloride for 30 min before CCF activation (CCF + amiloride) or CCF was pre-incubated with *N,N'*-diacetylchitobiose before addition to the cell cultures (CCF + *N,N'*-diacetylchitobiose). Flow cytometry data representative of at least three independent experiments are shown as relative fluorescence units (mean ± SD of three individual mice) as compared with non-stimulated (control) cells. (*$P < 0.05$).

lectin-like saccharide interaction of CCF with a sodium channel or a sodium channel-associated structure. Similar data were observed in PECs from TNFR1 knock-out, TNFR2 knock-out and TNF knock-out mice (data not shown).

To further address the role of sodium channels in the induction of TNF, IL-6 and NO production by CCF-activated macrophages, three amiloride-related molecules were tested. Amiloride is a potent inhibitor of epithelial sodium channels as well as $Na^+/H^+$ exchangers. Yet, benzamil and phenamil are amiloride derivatives that have higher specificity for the cell type and the type of ion channel, inhibiting both ion-gated sodium channels and $Na^+/H^+$ exchanger. The three inhibitors did not exert any toxic activity on PECs up to a concentration of 200 μM as assessed by the MTT test (data not shown). On this basis, PECs from the four mouse strains (wild-type, TNFR1 knock-out, TNFR2 knock-out and TNF knock-out) were stimulated with the sub-optimal CCF concentration of 0.5 μg $ml^{-1}$ in the presence of inhibitors in the range of 6.25–100 μM. Phenamil was the most efficient inhibitor in the four cell types, causing already 50% inhibition of TNF, IL-6 and NO secretion at the lowest concentration tested (6.25 μM; Fig. 3). Amiloride and benzamil, given in the concentration of 25–50 μM, caused ~50–60% inhibition. Yet, amiloride, the inhibitor of epithelial sodium channels and $Na^+/H^+$ exchangers, was a less potent inhibitor of CCF-mediated macrophage activation as compared with its derivatives with specificity for ion-gated sodium

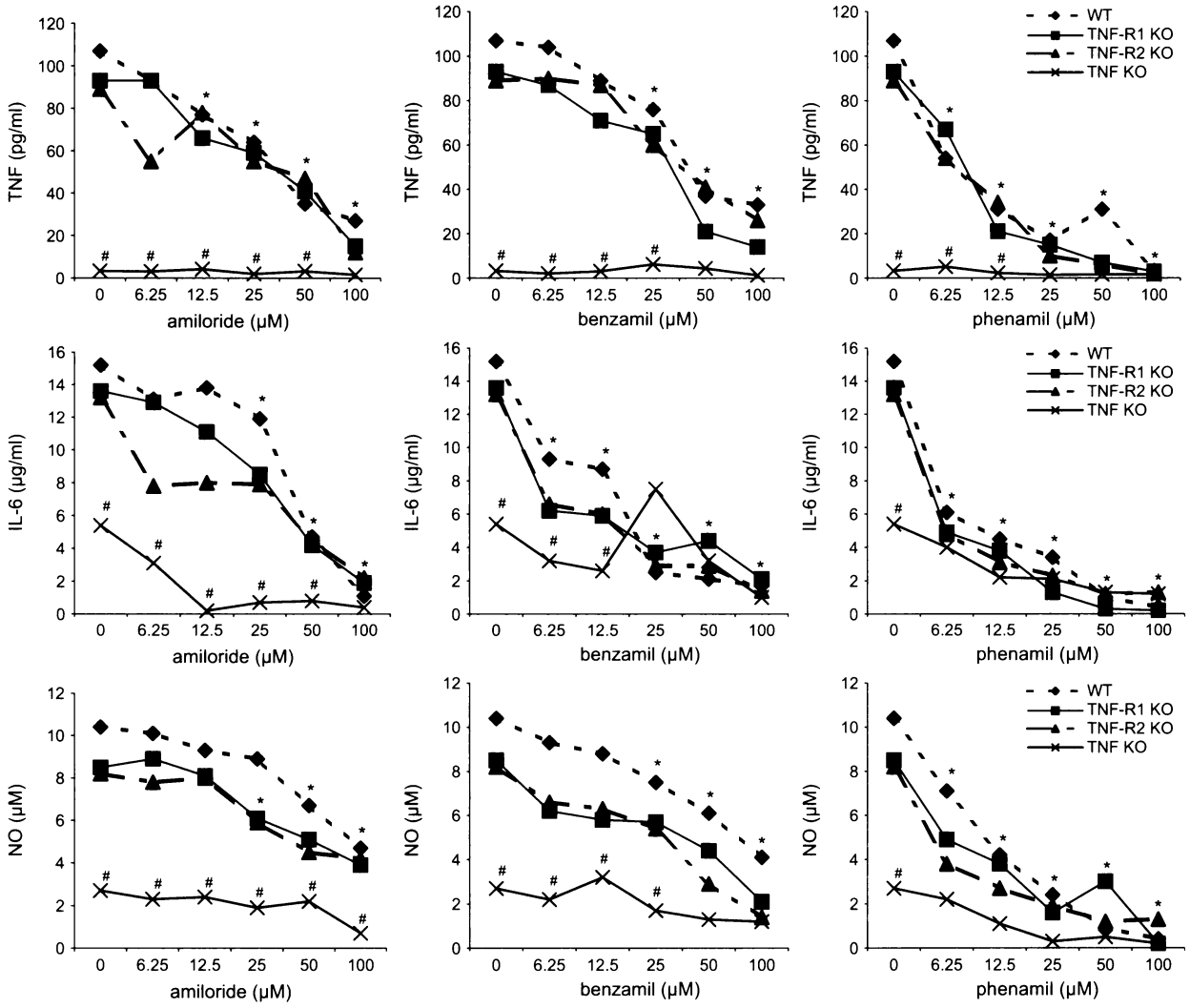



**Fig. 3.** Amiloride derivatives differentially affect the ion gating effect of CCF. Non-elicited adherent PECs isolated from wild-type (WT), TNFR1, TNFR2 and TNF knock-out (KO) mice were pre-incubated for 30 min with different concentrations of amiloride, benzamil or phenamil before stimulation with 0.5 μg $ml^{-1}$ CCF for 24 h. TNF, IL-6 and NO production was then determined in cell supernatants. Data are expressed as the mean of three individual mice and are representative of at least three independent experiments. SDs were below 15% of the mean. (*$P < 0.05$, comparing WT, TNFR1 or TNFR2 KO in presence and absence of inhibitor; #$P < 0.05$, comparing TNF KO versus WT, TNFR1 or TNFR2 KO).

channels or $Na^+/H^+$ exchanger. Furthermore, PCR analysis using specific primers followed by sequencing of the amplified products revealed the expression of α, β and γ subunits of epithelial sodium channel (GenBank accession nos NM_011324, NM_011325, NM_011326, respectively) in cells of mouse lung and kidney but not in PECs (data not shown). On the other hand, when specific primers for $Na^+/H^+$ exchanger (NHE-1, GenBank accession no. U51112) were tested, gene expression was detected in the absence of CCF stimulation in PECs from all mouse strains used in the present study (Fig. 4). These data indicate that a $Na^+/H^+$ exchanger could be involved in CCF-mediated activation of macrophage, resulting in the secretion of TNF, IL-6 and NO.

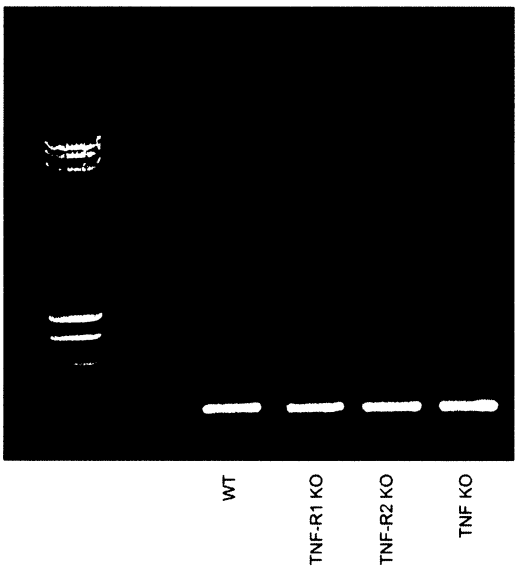



**Fig. 4.** $Na^+/H^+$ exchanger gene is expressed in adherent PECs. Constitutive (i.e. in the absence of CCF stimulation) $Na^+/H^+$ exchanger gene expression (GenBank accession no. U51112) was addressed in non-elicited adherent PECs isolated from wild-type (WT), TNFR1, TNFR2 and TNF knock-out (KO) mice by PCR using specific primers.

*The effect of N,N′-diacetylchitobiose on CCF-mediated PECs activation*

Since CCF interacts with ion channels via its lectin-like domain (Fig. 2), the inhibitory capacity of *N,N′*-diacetylchitobiose on PECs activation was tested in the range of 25–100 μg $ml^{-1}$ as described above for ion channel inhibitors. It was found that this saccharide efficiently inhibits cytokine and NO production in CCF-activated PECs from wild-type as well as TNFR1 knock-out, TNFR2 knock-out and TNF knock-out mouse strains (Fig. 5).

*The effect of signaling pathway inhibitors on CCF-mediated PECs activation*

Three different inhibitors were tested in order to identify the intracellular signaling pathway involved in CCF-mediated PECs activation. PD 98,059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase (MAPKK), SB 203580 inhibits the activation of p38 mitogen-activated protein kinase (MAPK) and MAPKAP kinase-2, but not JNK or p42 MAPK, and finally MG132 blocks I-κB degradation and thus NF-κB activation. The inhibitors did not exert toxic activity on PECs up to a concentration of 200 μM for PD 98,059 and SB 203580 or up to 20 μM for MG132 as assessed by MTT test (data not shown). As shown in Fig. 6, the inhibition of MAPKK or of p38 MAPK and MAPKAP kinase-2 had no significant effect on PECs activation triggered by CCF in the four mouse strains used in the present study. In contrast, the inhibition of NF-κB activation already reduced the cytokine and NO produced by PECs from the four mouse strains by as much as 50–60% at concentration of MG132 as low as 1.25 μM.

## Discussion

The invertebrate pattern-recognition protein CCF displays functional analogy with the mammalian cytokine TNF that is

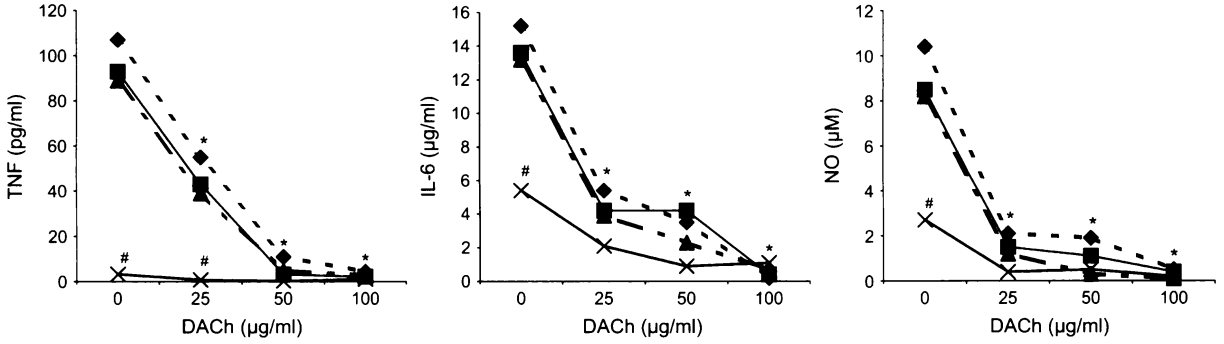



**Fig. 5.** Production of TNF, IL-6 and NO induced by CCF is inhibited by *N,N′*-diacetylchitobiose. CCF was pre-incubated for 30 min with different concentrations of *N,N′*-diacetylchitobiose before being added at 0.5 μg $ml^{-1}$ to non-elicited adherent PECs isolated from wild-type (WT), TNFR1, TNFR2 and TNF knock-out (KO) mice. Twenty-four hours later, TNF, IL-6 and NO production was determined in cell supernatants. Data are expressed as the mean of three individual mice and are representative of at least three independent experiments. SDs were below 15% of the mean. (*$P < 0.05$, comparing WT, TNFR1 or TNFR2 KO in presence and absence of inhibitor; #$P < 0.05$, comparing TNF KO versus WT, TNFR1 or TNFR2 KO).

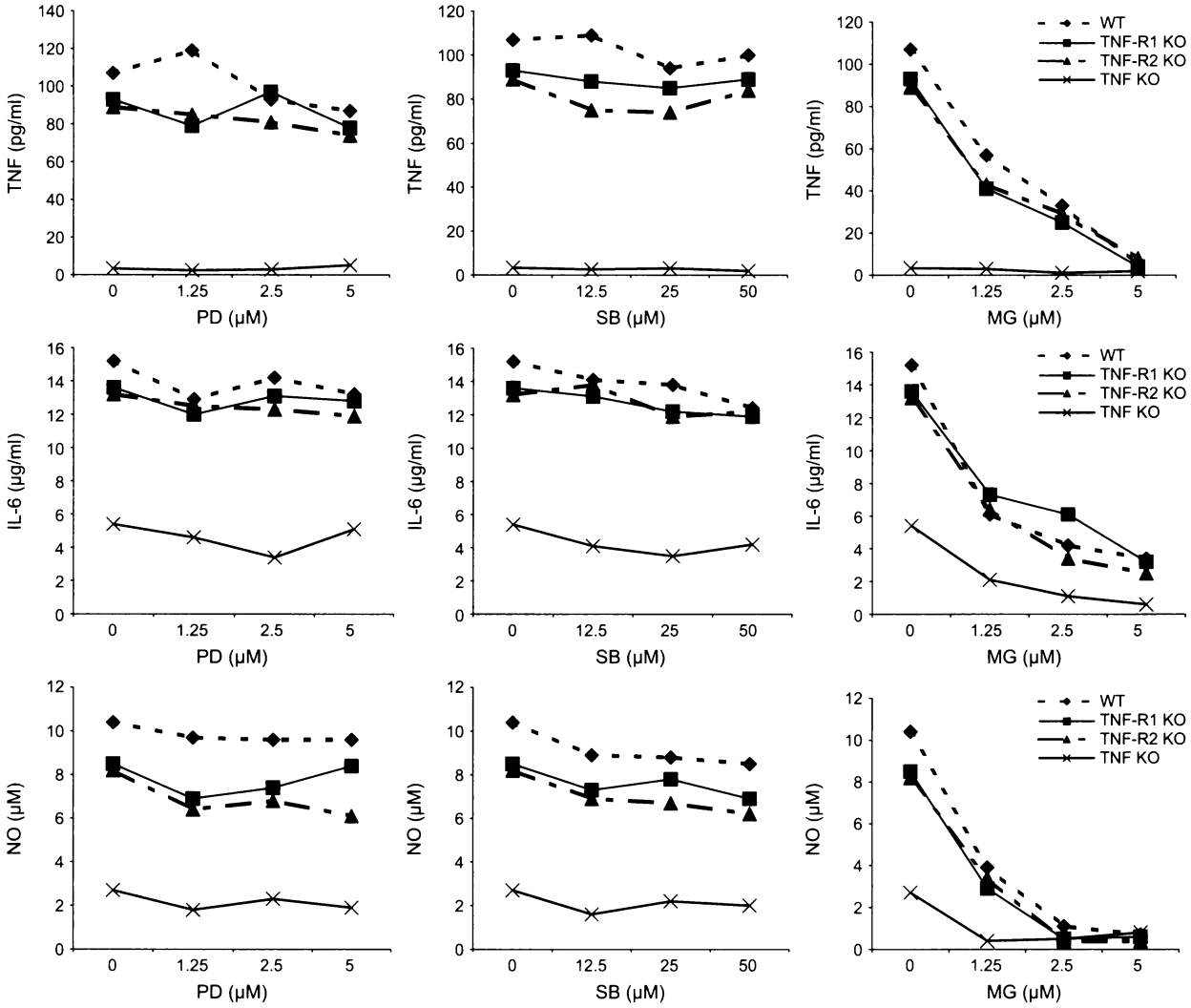



**Fig. 6.** CCF triggers TNF, IL-6 and NO production via NF-κB signaling. Non-elicited adherent PECs isolated from wild-type (WT), TNFR1, TNFR2 and TNF knock-out (KO) mice were pre-incubated for 30 min with different concentrations of PD 98,059 (PD), SB 203580 (SB) or MG132 (MG) before stimulation with 0.5 μg $ml^{-1}$ CCF for 24 h. TNF, IL-6 and NO production was then determined in cell supernatants. Data are expressed as the mean of three individual mice and are representative of at least three independent experiments. SDs were below 15% of the mean. (*$P < 0.05$, comparing WT, TNFR1 or TNFR2 KO in presence and absence of inhibitor).

based on a similar lectin-like *N,N'*-diacetylchitobiose specificity [for review see (13)]. Beside cytolytic and trypanolytic activities (4, 11), the ability of CCF to interact with ion channels on mammalian cell surface has been demonstrated using the whole-cell patch-clamp technique (17). Here we show that the membrane depolarization caused by CCF via a lectin–saccharide interaction results in the activation of non-elicited adherent peritoneal cells leading to the production of cytokines and NO. The composition of PECs (75% $F4/80^+$ cells) and our unpublished observation that CCF triggers membrane depolarization and TNF, IL-6 and NO production in the 2C11-12 macrophage cell line (20) support the idea that CCF is mainly interacting with macrophages. CCF-induced depolarization is not due to the interaction of autocrine TNF with TNFRs since the depolarization was not affected in PECs from TNFR1 and TNFR2 knock-out mice, and as reported previously in microvascular endothelial cells that do not produce TNF (17).

Bloc *et al.* (17) have suggested that CCF interacts with amiloride-sensitive ion channels on the surface of lung microvascular endothelial cells. Yet, the exact type of these channels was not determined. Amiloride is described as a potent inhibitor of epithelial sodium channels. Inhibition experiments using the amiloride derivatives benzamil and phenamil indicate that other sodium channels, most likely ion-gated sodium channels or the $Na^+/H^+$ exchanger are involved in the interaction of CCF with murine macrophages. Moreover, PCR analysis evidencing the $Na^+/H^+$ exchanger expression in

peritoneal macrophages, but not epithelial sodium channels expression, further supports this possibility. Finally, additional experiments with inhibitors of other types of ion channels [namely, quinine, paxilline, tetraethylammonium for potassium channels, ouabain for $Na^+$, $K^+$ ATPase, 5-nitro-2(3-phenylpropylamino)benzoic acid for chloride channels] rule out the direct involvement of potassium and chloride ions in CCF-mediated activation of macrophages (data not shown). Although we provide evidence that CCF interacts directly with the macrophage surface through its *N,N'*-diacetylchitobiose lectin-like domain, we cannot conclude whether CCF interacts directly with the saccharide moiety of an endogenous ion channel or rather with an as yet unknown receptor which in turn can activate an ion channel downstream of CCF binding.

CCF triggers the release of TNF, IL-6 and NO by macrophages likely by interacting with cell-surface ion channels considering that ion channel inhibitors completely abrogate the expression/production of these immune mediators as revealed by real-time PCR (data not shown). Moreover, this macrophage activation potential of CCF partially depends on the production of TNF since the levels of IL-6 and NO are significantly impaired in the absence of TNF, that is, in TNF knock-out mice. Furthermore, the secretion of IL-6 and NO induced by CCF in TNF knock-out mice is completely inhibited by *N,N'*-diacetylchitobiose. Although contention still exists as to whether or not the receptor-binding domains of TNF are involved in membrane depolarization (14, 16, 21), our data suggest that the autocrine effect of TNF is mediated mainly by its lectin-like domain in this experimental set-up. Accordingly, since CCF-mediated cytokine and NO production is similar in wild-type and in TNFR1 or TNFR2 knock-out mice, the interaction of TNF with TNFR may be less important in CCF-activated macrophages.

The induction of cytokine and NO secretion by macrophages activated by CCF depends on NF-κB activation, but not on MAPKK activation. Moreover, while the p38 activation pathway contributes to the activation of cells by inflammatory molecules (22), it does not seem to be involved in CCF-induced intracellular signaling. Thus, the signaling pathway triggered by CCF in macrophages converges downstream of p38 with the one of inflammatory cytokines including TNF. This again suggests that the autocrine effect of TNF on CCF-induced macrophage activation occurs via its lectin-like domain.

In summary, we propose a model of macrophage activation (Fig. 7) where CCF binds via its *N,N'*-diacetylchitobiose domain to an $Na^+/H^+$ exchanger or an $Na^+/H^+$ exchanger-associated molecule (a). The resulting membrane depolarization leads to NF-κB activation (b) and subsequent production of TNF (c), IL-6 and NO. In turn (the secreted), TNF interacts mainly via its *N,N'*-diacetylchitobiose lectin-like domain with the ion channel/ion channel-related structure (d), or 'classically' via its receptor-binding site with TNFR1 or TNFR2 on the macrophage surface (e) thus boosting the activation signal provided by the lectin domains of CCF and TNF (f). This artificial setting of macrophage activation triggered by an invertebrate defense molecule mimicking the lectin-like activity of TNF may reveal an ancient mechanism of cell activation that has evolved in parallel with the receptor-based network currently prevailing in vertebrates.

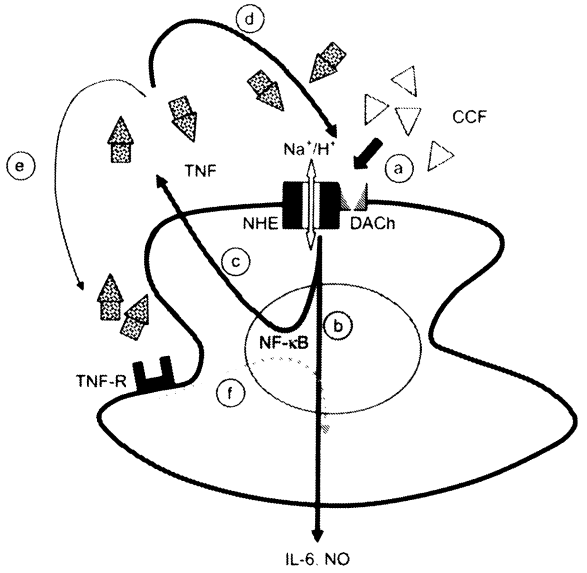



**Fig. 7.** Proposed model of activation of vertebrate macrophages by CCF. CCF binds via its *N,N'*-diacetylchitobiose domain to an $Na^+/H^+$ exchanger or an $Na^+/H^+$ exchanger-associated molecule (a) that results in membrane depolarization and subsequent NF-κB activation that contribute to the production of TNF, IL-6 and NO (b, c). The secreted TNF interacts mainly via its *N,N'*-diacetylchitobiose lectin-like domain with the ion channel/ion channel-related structure (bold line d), or to lesser extent 'classically' via its receptor-binding site with TNFR1 or TNFR2 on the macrophage surface (thin line e) thus boosting the activation signal provided by the lectin domains of CCF and TNF (dashed line f). NHE: $Na^+/H^+$ exchanger; DACh: *N,N'*-diacetylchitobiose.

## Acknowledgements

This work, supported by the Czech Science Foundation (310/02/1437, 310/04/0806 and 310/03/H147), an Institutional Research Concept (AV0Z50200510) and a bilateral international scientific and technological cooperation grant from the Ministry of the Flemish Community (BOF-BWS 03/06), was performed within the frames of an Interuniversity Attraction Pole Program. R.Vd.B. is supported by a grant from the Institute for Promotion of Innovation by Science and Technology in Flanders (IWT—Vlaanderen). Authors are grateful to Rudolf Lucas for helpful discussion.

## Abbreviations

| | |
|---|---|
| CCF | coelomic cytolytic factor |
| MAPKK | mitogen-activated protein kinase kinase |
| MAPK | mitogen-activated protein kinase |
| NF-κB | nuclear factor-κB |
| NO | nitric oxide |
| PECs | peritoneal exudate cells |
| TNF | tumor necrosis factor |
| TNFR | TNF receptor |

## References

1 Bilej, M., De Baetselier, P. and Beschin, A. 2000. Antimicrobial defense of the earthworm. *Folia Microbiol. (Praha)* 45:283.
2 Beschin, A., Bilej, M., Hanssens, F. *et al.* 1998. Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. *J. Biol. Chem.* 273:24948.

3 Bilej, M., De Baetselier, P., Van Dijck, E., Stijlemans, B., Colige, A. and Beschin, A. 2001. Distinct carbohydrate recognition domains of an invertebrate defense molecule recognize Gram-negative and Gram-positive bacteria. *J. Biol. Chem.* 276:45840.
4 Bilej, M., Brys, L., Beschin, A. *et al.* 1995. Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms. *Immunol. Lett.* 45:123.
5 Bilej, M., Rossmann, P., Sinkora, M. *et al.* 1998. Cellular expression of the cytolytic factor in earthworms *Eisenia foetida*. *Immunol. Lett.* 60:23.
6 Köhlerova, P., Beschin, A., Silerova, M., De Baetselier, P. and Bilej, M. 2004. Effect of experimental microbial challenge on the expression of defense molecules in *Eisenia foetida* earthworm. *Dev. Comp. Immunol.* 28:701.
7 Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamson, B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl Acad. Sci. USA* 72:3666.
8 Luo, G., Niesel, D. W., Shaban, R. A., Grimm, E. A. and Klimpel, G. R. 1993. Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. *Infect. Immun.* 61:830.
9 Lucas, R., Magez, S., De Leys, R. and De Baetselier, P. 1994. Cytokines: multifunctional pathogen-specific lectins?. In Van Driessche, E., Fischer, J., Beeckmans, S. and Bøg-Hansen, T. C., eds., *Lectins: Biology, Biochemistry, Clinical Biochemistry*, p. 244. Textop, Hellerup, Denmark.
10 Lucas, R., Magez, S., De Leys, R. *et al.* 1994. Mapping the lectin-like activity of tumor necrosis factor. *Science* 263:814.
11 Beschin, A., Bilej, M., Brys, L. *et al.* 1999. Convergent evolution of cytokines. *Nature* 400:627.
12 Olivares Fontt, E., Beschin, A., Van Dijck, E. *et al.* 2002. *Trypanosoma cruzi* is lysed by coelomic cytolytic factor-1, an invertebrate analogue of tumor necrosis factor, and induces phenoloxidase activity in the coelomic fluid of *Eisenia foetida foetida*. *Dev. Comp. Immunol.* 26:27.
13 Beschin, A., Bilej, M., Torreele, E. and De Baetselier, P. 2001. On the existence of cytokines in invertebrates. *Cell. Mol. Life Sci.* 58:801.
14 Hribar, M., Bloc, A., van der Goot, F. G. *et al.* 1999. The lectin-like domain of tumor necrosis factor-alpha increases membrane conductance in microvascular endothelial cells and peritoneal macrophages. *Eur. J. Immunol.* 29:3105.
15 van der Goot, F. G., Pugin, J., Hribar, M. *et al.* 1999. Membrane interaction of TNF is not sufficient to trigger increase in membrane conductance in mammalian cells. *FEBS Lett.* 460:107.
16 Fukuda, N., Jayr, C., Lazrak, A. *et al.* 2001. Mechanisms of TNF-alpha stimulation of amiloride-sensitive sodium transport across alveolar epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 280:L1258.
17 Bloc, A., Lucas, R., Van Dijck, E. *et al.* 2002. An invertebrate defense molecule activates membrane conductance in mammalian cells by means of its lectin-like domain. *Dev. Comp. Immunol.* 26:35.
18 Rodriguez-Sosa, M., Satoskar, A. R., Calderon, R. *et al.* 2002. Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low interleukin-12 production and Th2-biasing ability. *Infect. Immun.* 70:3656.
19 Brys, L., Beschin, A., Raes, G. *et al.* 2005. Reactive oxygen species and 12/15-lipoxygenase contribute to the antiproliferative capacity of alternatively activated myeloid cells elicited during helminth infection. *J. Immunol.* 174:6095.
20 De Baetselier, P. and Schram, E. 1986. Luminescent bioassays based on macrophage cell lines. *Methods Enzymol.* 133:507.
21 Berthiaume, Y. 2003. Tumor necrosis factor and lung edema clearance: the tip of the iceberg? *Am. J. Respir. Crit. Care Med.* 168:1022.
22 Vanden Berghe, W., Plaisance, S., Boone, E. *et al.* 1998. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.* 273:3285.

## 4.4. Identification and characterization of calreticulin

Calreticulin is a highly conserved calcium-protein-binding protein affecting many cellular processes. In the endoplasmic reticulum lumen, calreticulin performs two major functions: chaperoning (Trombetta 2003) and regulation of $Ca^{2+}$ homeostasis by modulation of endoplasmic reticulum $Ca^{2+}$ storage and transport (Michalak *et al.* 2002). As a molecular chaperon, calreticulin participates in the synthesis of many molecules, including ion-channel proteins, surface and nuclear receptors, integrins and, importantly, perforin (Helenius *et al.* 1997; Andrin *et al.* 1998). Furthermore, calreticulin was shown to control osmotic lysis mediated by perforin (Fraser *et al.* 2000).

Calreticulin is a 46-kDa protein with an N-terminal amino acid signal sequence and C-terminal KDEL endoplasmic reticulum retrieval signal (Fliegel *et al.* 1989; Denning *et al.* 1997). Structural predictions of calreticulin suggest that the protein has three domains – a highly conserved globular N-domain, central proline-rich P domain and an acidic C-domain, which contains a high-capacity $Ca^{2+}$-binding site (Baksh and Michalak 1991).

The first piece of evidence on the existence of calreticulin in *E. fetida* earthworms was reported by Kauschke et al. Analyzing a MALDI-MS peptide map of perforin-like lytic protein of *E. fetida* coelomocyte lysate, they found similarity to calreticulin. This finding is not surprising since the interaction of calreticulin N-domain with perforin as well as copurification of these two proteins was reported (Andrin *et al.* 1998).

The aim of our study was to identify and describe calreticulin in *E. fetida* earthworms and to show its phylogenetic relationships with calreticulin molecules of other species and determine its expression in different organs.

To assign the cDNA sequence of *E. fetida* calreticulin, we designed sets of degenerated primers based on known calreticulin sequences of invertebrates. Subsequent PCR reaction resulted in a specific PCR product and its sequencing revealed homology to calreticulin molecules. In order to assemble the full length cDNA

sequence, rapid amplifications of the 5' and 3' cDNA ends were performed. The full length cDNA of *E. fetida* calreticulin has an open reading frame coding for 428 amino acids. The primary structure of the protein contains specific sequences that are responsible for targeting and retention of calreticulin in the endoplasmic reticulum lumen, i. e. a signal peptide and C-terminal KDEL sequence. The central P-domain comprises two sets of amino acid repeats.

Furthermore, we found that *E. fetida* calreticulin shares significant homology (65-75 %) with calreticulin molecules of invertebrates. To gain more insight into the relationship of calreticulin molecules, their amino acid sequences were subjected to phylogenetic analysis, which suggests the common origin of *E. fetida* calreticulin and that of the mollusk *Aplysia californica*.

We used a polyclonal rabbit anti-calreticulin antibody to localize calreticulin in coelomocytes and cryo-sections of *E. fetida*. Antibody labeling was detected in the mesenchymal lining of the coelomic cavity, i. e. a site from where the coelomocytes are derived and proliferate, and in granular intracellular bodies of certain coelomocytes.

Both *in situ* hybridization and quantitative real-time PCR revealed calreticulin expression in various cells and tissues of *E. fetida*; it is dominantly expressed in the epidermis, neurons of ventral nerve cord and in sperm cells, but also in the epithelial cells of the intestinal tract, in the cells of body wall muscles, in the cells of mesenchymal lining of the coelomic cavity and in coelomocytes.

To conclude, we proved the presence of calreticulin in *E. fetida* earthworms and specified the sequence of calreticulin-coding cDNA. Phylogenetic analysis revealed the common origin of *E. fetida* calreticulin and calreticulins of mollusks. Calreticulin expression was detected in different tissues by using immunohistochemistry, *in situ* hybridization and real-time PCR.

**Šilerová M., Kauschke E., Procházková P., Josková R., Tučková L., Bilej M.: Characterization, molecular cloning and localization of calreticulin in *Eisenia fetida* earthworms. *Gene* 397: 169-177 (2007).**

Šilerová M., Kauschke E., Procházková P., Josková R., Tučková L., Bilej M.: Characterization, molecular cloning and localization of calreticulin in *Eisenia fetida* earthworms. *Gene* 397: 169-177 (2007).

Available online at www.sciencedirect.com
ScienceDirect
Gene 397 (2007) 169–177
GENE
www.elsevier.com/locate/gene

# Characterization, molecular cloning and localization of calreticulin in *Eisenia fetida* earthworms

Marcela Šilerová [a,*], Ellen Kauschke [b], Petra Procházková [a], Radka Josková [a], Ludmila Tučková [a], Martin Bilej [a]

[a] *Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic*
[b] *Institute of Zoology, Justus Liebig University Giessen, Stephanstraße 23, 35390 Giessen, Germany*

Received 23 January 2007; received in revised form 23 March 2007; accepted 25 April 2007
Available online 10 May 2007
Received by M. Di Giulio

**Abstract**

Calreticulin is a highly conserved calcium-binding protein affecting many cellular processes inside and outside of the endoplasmic reticulum (ER). It participates in the regulation of $Ca^{2+}$ homeostasis, acts as a chaperone and modulates gene transcription, integrin-mediated cell signalling as well as cell adhesion.

Here we report on the sequence characterization of a calreticulin-coding cDNA of *Eisenia fetida* earthworms. The neighbor-joining phylogeny tree constructed based on the deduced amino acid sequence indicates a common origin of the *E. fetida* calreticulin molecule and that of mollusks. A polyclonal anti-calreticulin antibody used for immunocytochemistry and immunohistochemistry localized the protein in the mesenchymal lining of the coelomic cavity and in coelomocytes of *E. fetida*. *In situ* hybridization revealed high expression of *E. fetida* calreticulin in various cells and tissues, namely epidermis, neurons of the ventral nerve cord, intestine, sperms, body wall muscles and some coelomocytes. Real-time PCR confirmed the strong expression of calreticulin in the nervous system, particularly in cerebral ganglia, in body wall muscles and in seminal vesicles. Moreover, a high calreticulin expression was measured in the muscular pharynx.


*Keywords:* Earthworms; Annelids; Invertebrates; *In situ* hybridization; Molecular chaperone

## 1. Introduction

Numerous antimicrobial factors including lysozyme, proteins with hemolytic activity and cytolytic factors have been identified and characterized in *Eisenia fetida* earthworms (Bilej et al., 2000; Cooper et al., 2002). These secreted lytic molecules require molecular chaperones that prevent their autolysis and enable proper protein folding and assembly. Calreticulin was described to be the main lectin-like chaperone necessary for the maturation of glycoproteins in the eukaryotic cells.

Calreticulin was first isolated as $Ca^{2+}$-binding protein from the sarcoplasmic reticulum of rabbit muscle (Ostwald and MacLennan, 1974) and the isolation of coding cDNA followed (Fliegel et al., 1989; Smith and Koch, 1989). Calreticulin is a major protein in the endoplasmic reticulum (ER) lumen that was identified in all mammalian cells, except erythrocytes, as well as in a variety of other vertebrates, invertebrates and higher plants (Michalak et al., 1999). It is involved in the regulation of diverse cellular functions (Corbett and Michalak, 2000; Johnson et al., 2001; Nakamura et al., 2001) and remained highly conserved during evolution.

In the ER lumen, calreticulin performs two major functions: chaperoning (Trombetta 2003) and regulation of $Ca^{2+}$ homeostasis by modulation of ER $Ca^{2+}$ storage and transport (Michalak et al., 2002). As a molecular chaperone it is binding to monoglycosylated high mannose-type oligosaccharides, thus preventing the aggregation of partially folded proteins and increasing the yield of correctly folded ones (Helenius et al., 1997; Johnson et al., 2001; Michalak et al., 1999). It participates

*Abbreviations:* BSA, bovine serum albumin; ER, endoplasmic reticulum; ORF, open reading frame; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +420 2 4106 2367; fax: +420 2 4172 1143.
*E-mail address:* marcelka@biomed.cas.cz (M. Šilerová).

0378-1119/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2007.04.035

in the synthesis of many molecules, including ion channel proteins, surface and nuclear receptors, integrins and transporters (Helenius et al., 1997). Importantly, calreticulin controls osmotic lysis mediated by perforin (Fraser et al., 2000).

Calreticulin is a 46-kDa protein, which contains an N-terminal amino acid signal sequence (Denning et al., 1997; Fliegel et al., 1989) and C-terminal KDEL/HDEL ER retrieval signal. These specific amino acid sequences are responsible for targeting and retention of calreticulin in the ER lumen. The protein has three structural and functional domains — a highly conserved globular *N*-domain, central proline-rich *P*-domain which binds $Ca^{2+}$ with high affinity (Baksh and Michalak, 1991) and an acidic *C*-domain, which contains a high-capacity $Ca^{2+}$-binding site (Baksh and Michalak, 1991).

Here we report on the sequence characterization of a calreticulin-coding cDNA in *E. fetida* earthworms isolated

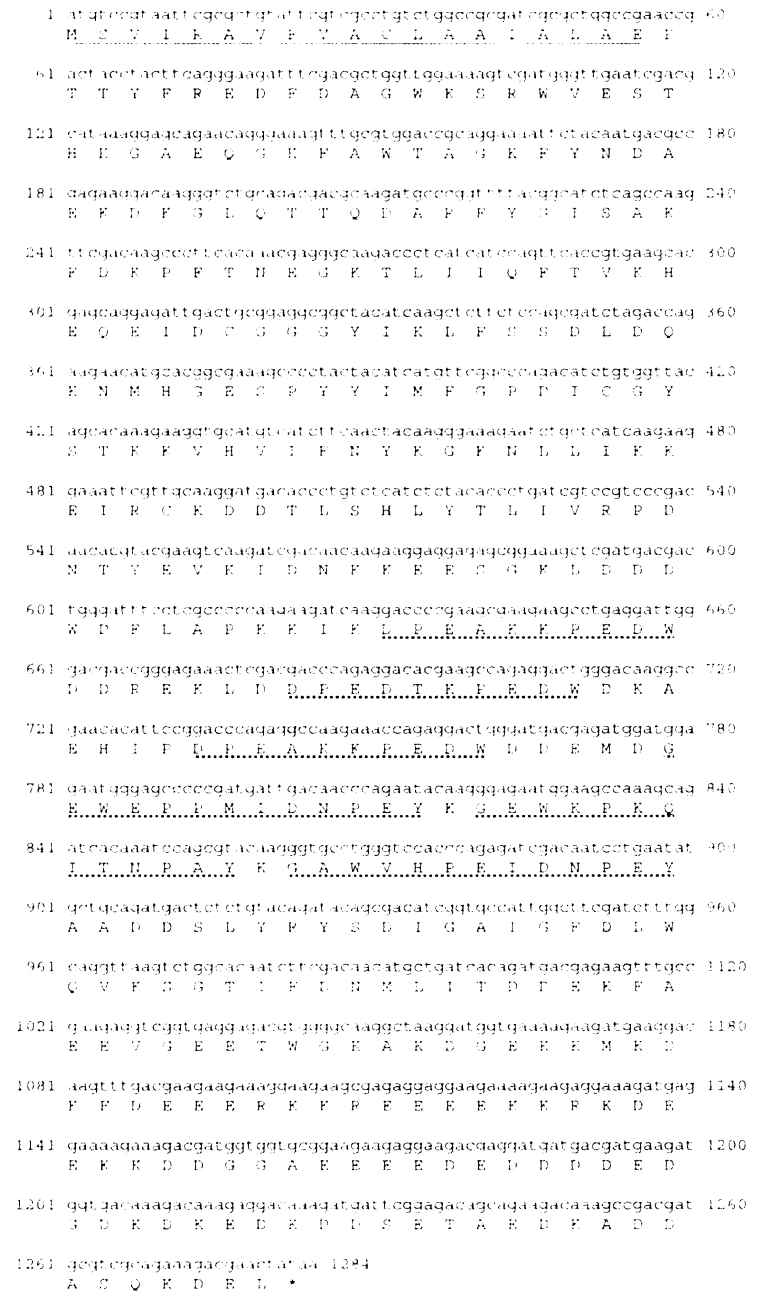


Fig. 1. Nucleotide and deduced amino acid sequence of *E. fetida* calreticulin (GenBank accession no. DQ887090). Signal peptide is underlined. An asterisk marks a TAA stop codon. The two repeats in *P*-domain are underlined with dotted line.

by RT-PCR using degenerated primers. The neighbor-joining phylogeny tree supports a common origin of *E. fetida* calreticulin molecule and that of mollusks. Calreticulin expression was detected in different tissues by using immunohistochemistry, *in situ* hybridization and real time PCR.

## 2. Materials and methods

### *2.1. Isolation of coelomic fluid and coelomocytes*

Adult *E. fetida* Sav. (*Oligochaeta, Annelida*) earthworms were maintained on moist paper towels without food for 2 days to lower gut load and avoid sample contamination during coelomocyte collection. Coelomic fluid containing free coelomocytes was harvested by puncturing post-clitellum segments of the coelomic cavity with a Pasteur micropipette. Coelomocytes were isolated by centrifugation (500 g, 10 min, 4 °C) and washed twice with *Lumbricus* balanced salt solution (71.5 mM NaCl, 4.8 mM KCl, 3.8 $CaCl_2$, 1.1 mM $MgSO_4$, 0.4 mM $KH_2PO_4$, 0.4 mM $NaH_2PO_4$, 4.2 mM $NaHCO_3$; pH 7.3; (Stein and Cooper, 1981)) before being used in further experiments.

### *2.2. RNA isolation, cDNA synthesis, PCR and rapid amplification of cDNA ends (RACE)*

Total RNA was isolated from coelomocytes using TRIZOL reagent (Gibco BRL) according to the manufacturer's protocol. Two micrograms of DNAse I-treated total RNA were reverse-

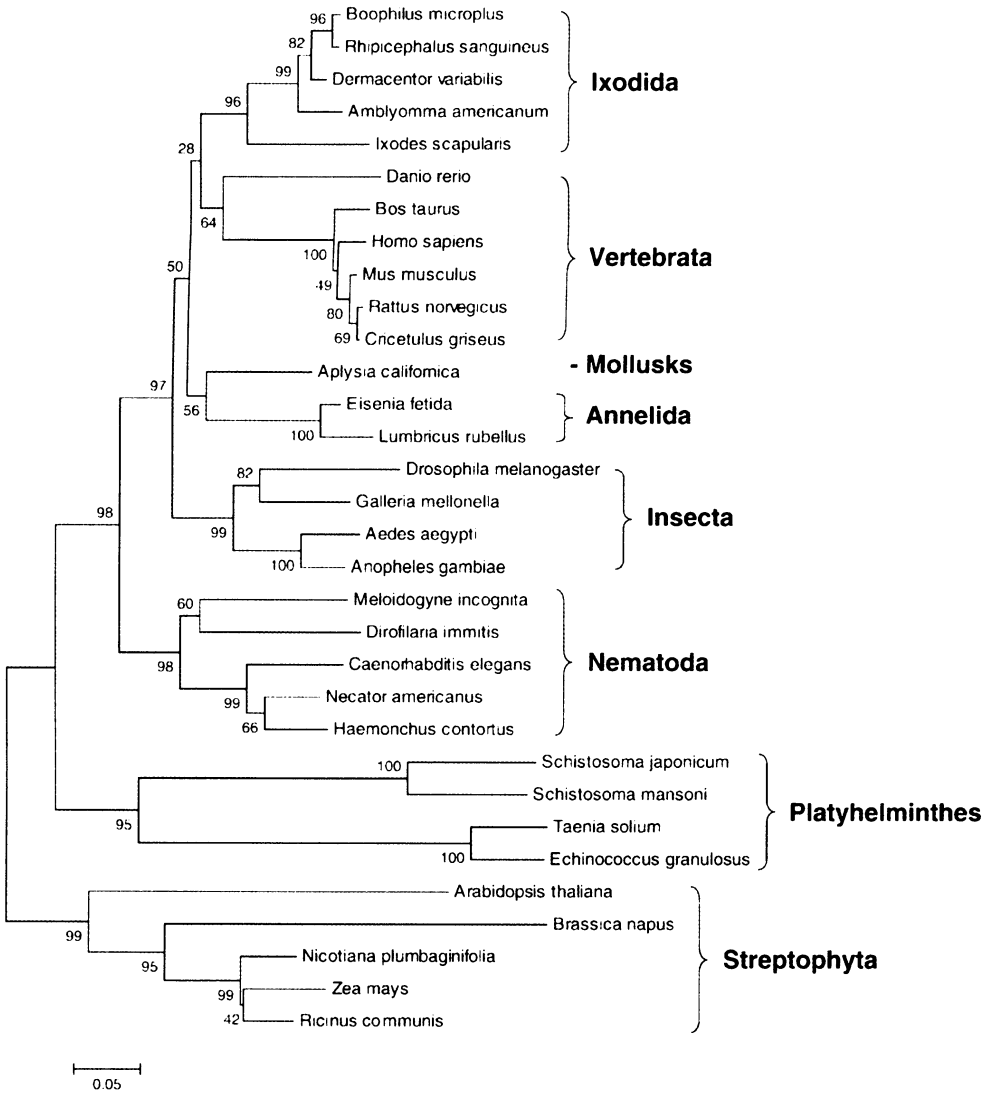



Fig. 2. Neighbor-joining phylogenetic tree relating amino acid sequences of *E. fetida* calreticulin and calreticulins of selected species. The numbers near the nodes represent bootstrap values after 10,000 resampling efforts.

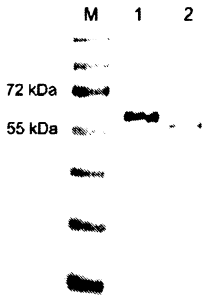



Fig. 3. Western blot analysis. Recognition of calreticulin in *E. fetida* coelomocyte lysate (lane 1) and recombinant calreticulin (lane 2) by a polyclonal anti-human calreticulin antibody. Protein markers are on the left margin (M).

transcribed using Oligo(dT)$_{12\ 18}$ primer and Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen) and then used in PCR reaction. A set of degenerated primers was designed based on all known invertebrate calreticulin sequences using the CODEHOP program (Rose et al., 1998). Combination of the primers 5′-GCACGAGCAGAACATCgaytgyggngg-3′ and 3′-tgnta-daarctGTTGAAGTACTAGTGGCT-5′ resulted in a specific PCR product of about 700 bp which was amplified using the following cycling parameters: 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 40 s at 55 °C and 120 s at 72 °C and a final extension for 10 min at 72 °C. The PCR product was ligated in pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The 3′ end of calreticulin cDNA was obtained using 3′ RACE System (Invitrogen). Total RNA (2 μg) was reverse-transcribed using an adapter primer (5′-GGCCACGCGTCGACTAGTAC-TTTTTTTTTTTTTTTT-3′). Universal amplification primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′) and calreticulin-specific internal sense primer (5′-AAG-GAAATTCGTTGCAAGGATGATGC-3′) were used in subsequent PCR reaction. Similarly, the 5′ end of calreticulin cDNA was obtained using a 5′ RACE System (Invitrogen). Reverse transcription was carried out using 2 μg of total RNA and an antisense calreticulin internal primer (5′-CGAAGCCGATAG-CACCGATGTC-3′). Terminal deoxynucleotidyl transferase was used to add homopolymeric oligo-dC tails to the 3′ end of purified cDNA. Primers used in subsequent PCR were a sense abridged anchor primer (5′-GGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG-3′) and an antisense calreticulin-specific primer (5′-GGGCTCCCATTCACCATCCA-3′). Both 3′ and 5′ RACE products were cloned in pCR2.1-TOPO and sequenced as described below.

### 2.3. Sequencing

Isolated and purified plasmid DNA was sequenced with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The chain termination reaction (Sanger et al., 1977) was performed by cycle sequencing technique (Murray 1989) according to the manufacturer's protocol. Finally, sequences were determined using an ABI PRISM 3100 DNA sequencer (Applied Biosystems).

### 2.4. Phylogenetic analysis

The amino acid sequences of *E. fetida* calreticulin and calreticulin molecules of other species were aligned using the CLUSTALW program (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.0 software (Kumar et al., 2004). The reliability of the branching pattern was assessed by bootstrap analysis (100 or 10,000 replications).

### 2.5. Preparation of recombinant E. fetida calreticulin

A DNA fragment containing the entire coding region for *E. fetida* calreticulin was generated by PCR using calreticulin cDNA as a template and was cloned into the pET-28a(+) vector containing an N-terminal 6× His-affinity tag (Novagen). The *E. coli* BL21(DE3) strain was transformed, grown in the presence of kanamycin (25 μg/ml) and the expression of calreticulin was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The His-tagged-fused protein from bacterial lysates was purified to homogeneity by affinity chromatography using Ni-CAM HC Resin (Sigma Aldrich) and eluted with 500 mM imidazole.

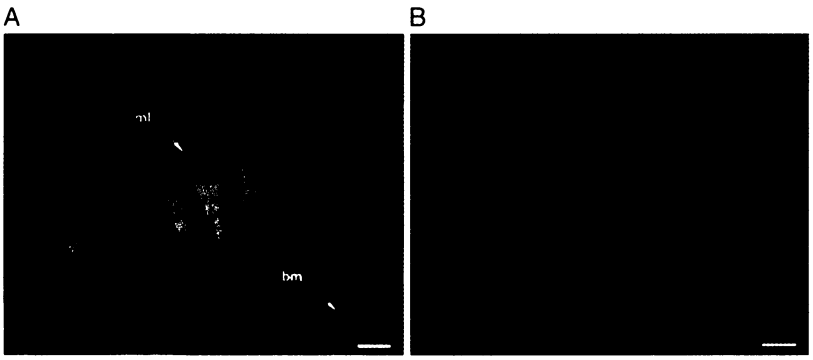



Fig. 4. Immunohistochemistry. (A) Detection of calreticulin in the mesenchymal lining (ml) of the coelomic cavity of *E. fetida* using a polyclonal rabbit anti-human CRT antibody. (B) Negative control with normal rabbit serum. Nuclei were stained with Hoechst 33342. (bm)-body wall muscles. Bar corresponds to 20 μm.

### 2.6. Polyacrylamide gel electrophoresis and western blot analysis

Gel electrophoresis of *E. fetida* coelomocyte lysate (obtained by five cycles of freezing and thawing and treated with a proteinase inhibitor Pefabloc; Boehringer Mannheim) and of recombinant calreticulin was performed in 12% SDS-polyacrylamide gel. Proteins were transferred on a nitrocellulose membrane and 2% bovine serum albumin was used for blocking. A polyclonal rabbit anti-human calreticulin antibody was added (1:50) and, after repeated washings, peroxidase labeled anti-rabbit IgG antibody (1:1000; Sigma Aldrich) was used for visualization (4-chloro-1-naphtol as substrate).

### 2.7. Immunocytochemistry

Coelomocytes were fixed on slides using 4% paraformaldehyde for 20 min and cytoplasmic membranes were permeabilized by 0.1% Triton X-100. After incubation in PBS containing 2% BSA (1 h), the samples were incubated with polyclonal rabbit anti-human calreticulin antibody (1:30) for 2 h at room temperature. After washing in PBS, anti-rabbit-IgG-peroxidase conjugate (1:1000; Sigma Aldrich) was added for 30 min. The antibody binding was visualized by using the 3,3′-diaminobenzidine-HCl (DAB)-$H_2O_2$ reagent.

### 2.8. Immunohistochemistry

Transversal and longitudinal cryo-sections (6 μm) of *E. fetida* were fixed by 3.7% formaldehyde and treated with 0.1% Triton X-100. Sections were incubated in PBS containing 2% BSA for 30 min before the polyclonal rabbit anti-human calreticulin antibody (1:30) was added for 2 h. After washing in PBS, samples were incubated with an anti-rabbit-IgG-FITC antibody (1:100; Sigma Aldrich) for 30 min, washed again in PBS and nuclei were stained with Hoechst 33342 (1:100, Molecular Probes) for 5 min.

### 2.9. Preparation of probes for in situ hybridization

Antisense and sense digoxigenin(DIG)-labeled RNA probes were prepared from linearized pBluescript II SK (+) plasmid containing cDNA of *E. fetida* calreticulin central *P*-domain. The probes were synthesized by *in vitro* transcription using the DIG RNA Labeling Kit (Roche) and either SP6 or T7 RNA polymerases.

### 2.10. In situ hybridization

Cryo-sections (8 μm) of different earthworm body parts (first five anterior body segments, genital region, clitellum region,

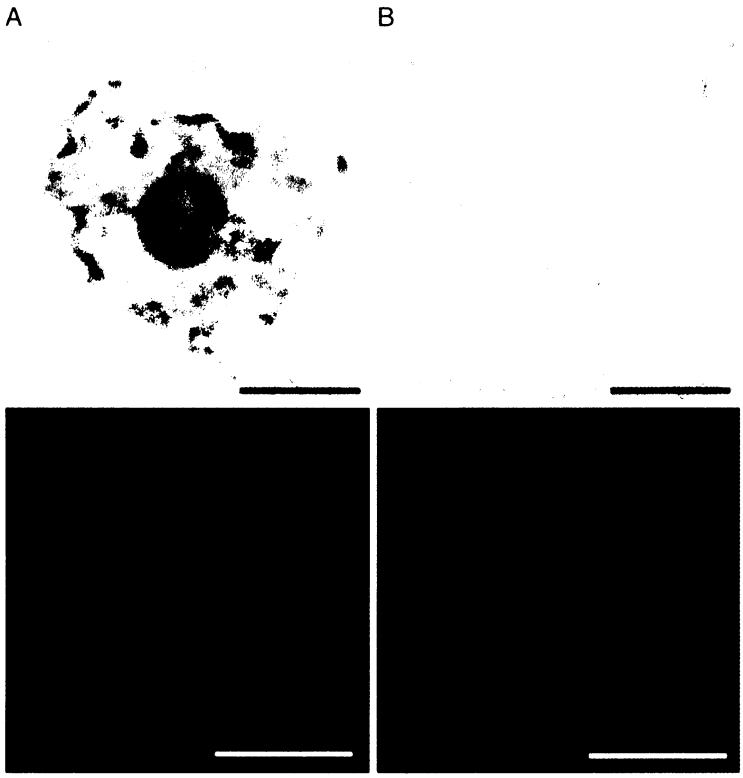
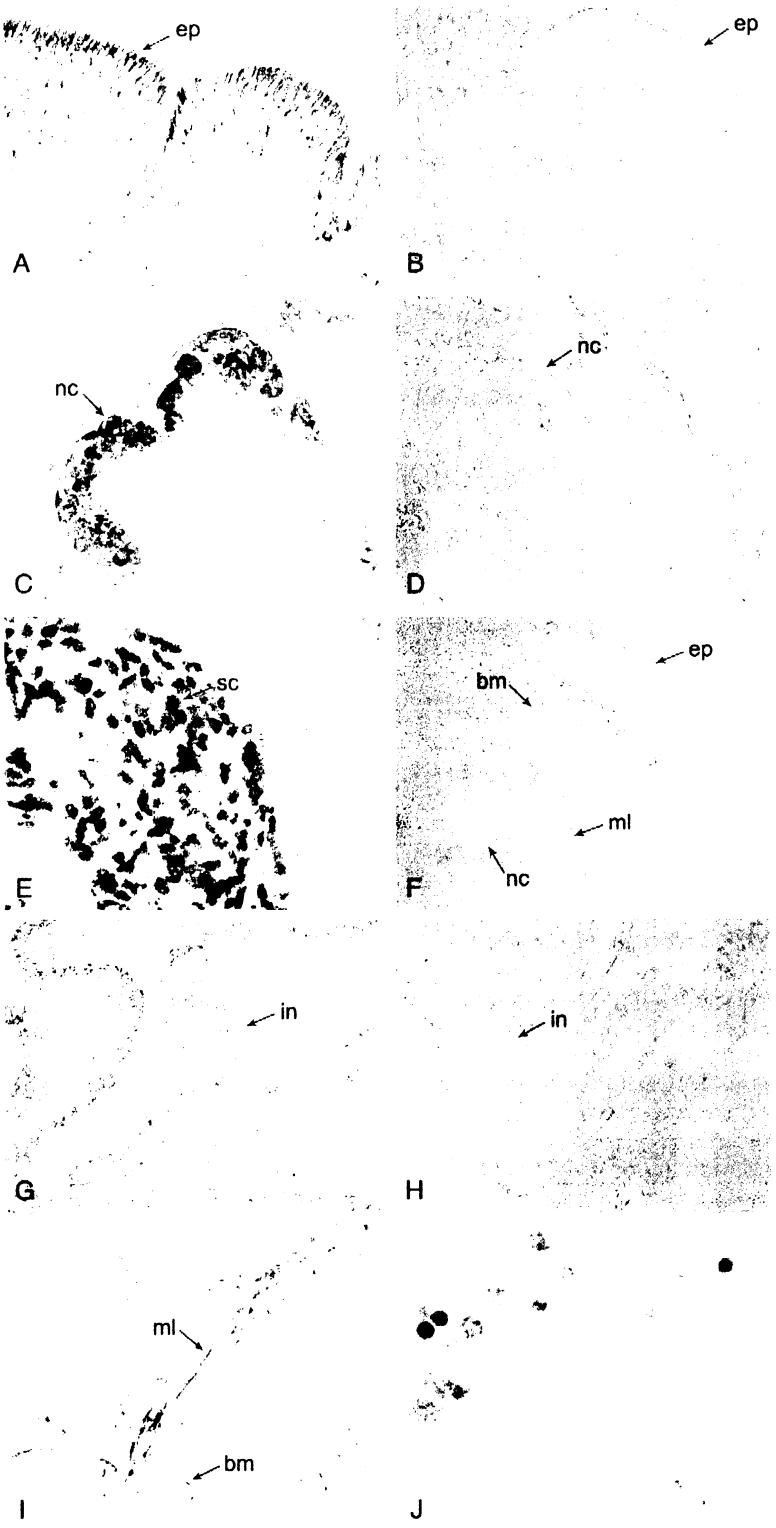



Fig. 5. Immunocytochemistry. (A) Detection of calreticulin in granular intracellular bodies of free coelomocytes using a polyclonal rabbit anti-human calreticulin antibody and both anti-rabbit-IgG-peroxidase and anti-rabbit-IgG-FITC secondary antibodies. (B) Negative control with normal rabbit serum. Nuclei were stained with Hoechst 33342 (lower pictures). Bar corresponds to 10 μm.


ep
A
ep
B
nc
C
nc
D
sc
E
ep
bm
ml
nc
F
in
G
in
H
ml
bm
I
J

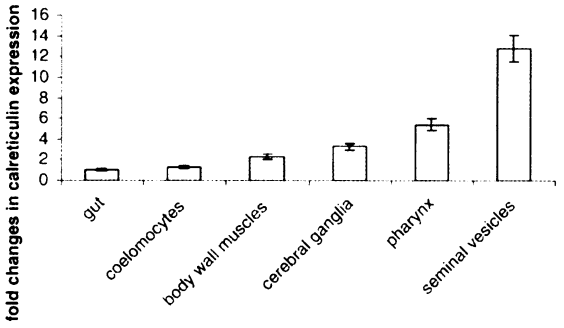



Fig. 7. Gene expression levels of calreticulin in different parts of *E. fetida* were determined by real-time PCR and normalized for the housekeeping gene actin. Fold changes in gene expression are relative to calreticulin expression in the gut. The values are means of three experiments (±SD) performed in triplicates.

midgut, last five posterior body segments) were fixed in RNase-free 4% paraformaldehyde, washed twice in PBS, dehydrated sequentially using 70%–100% ethanol and stored in 100% ethanol at 4 °C for later use. Sections were then rehydrated, digested with 20 μg/ml proteinase K (Sigma) in PBS for 3 min and fixed again with paraformaldehyde. After acetylation with 0.25% acetic anhydride in triethanolamine (pH 8) for 10 min, samples were washed with PBS, dehydrated through ethanol series and prehybridized for 2 h at 42 °C in prehybridization buffer (10 mg/ml tRNA, 5× SSC, 1× Denhardt's solution, 50% formamide, 10% dextran sulphate). Sections were then hybridized overnight at 42 °C in 30 μl of prehybridization solution containing a 1:100 dilution of the DIG-labeled RNA probe heat denaturated for 10 min at 65 °C. Repeated washing in 4× SSC was followed by an RNase A treatment (20 μg/ml, Sigma) and washes in 2× SSC, 1× SSC, 0.5× SSC at RT and in 0.1× SSC at 52 °C. Sections were treated with Blocking reagent (Boehringer) for 1 h and then incubated with anti-DIG-alkaline phosphatase antibody (1:500) for 2 h. The labeling was visualized using nitroblue tetrazolium/bromochloroindoyl phosphate (NBT/BCIP, Boehringer).

### 2.11. Real-time PCR

Two micrograms of total RNAs isolated from coelomocytes and from different earthworm tissues (gut, body wall muscles, cerebral ganglia, pharynx and seminal vesicles) were reverse-transcribed as previously indicated. Real-time PCR was carried out with an iQ™ Real-Time PCR Detection System (Biorad), using iQ SYBR Green Supermix (BioRad) to monitor dsDNA synthesis. Each reaction was performed in a volume of 25 μl containing 4 μl of a 1:20 dilution of each cDNA preparation, 10 μl of SYBR Green and 0.2 μM of each primer (calreticulin forward primer 5′-ACACTCTTATCGTCCGTCCTG-3′ and calreticulin reverse primer 5′-CCTCTGGCTTCTTCGCTTC-3′). Controls containing no template were included in all experiments. The amplification protocol was as follows: 3 min template denaturation step at 95 °C, 40 cycles of 94 °C for 30 s, 60 °C (annealing for calreticulin primers) for 30 s, 72 °C for 20 s, plus an extension at 72 °C for 70 s. The temperature was then gradually increased to 95 °C to obtain the melting curves of the amplified fragments. Quantitative measurements were normalized using *E. fetida* actin mRNA levels as a housekeeping gene. The actin primers were as follows: forward 5′-GATGAAGCCCAGAGCAAGAG-3′, and reverse 5′-TGTGAGCAGAACGGGATGT-3′. The differences in the $C_T$ values of calreticulin and the internal control actin were calculated. Relative expression of calreticulin was determined as fold expression relative to calreticulin expression in the gut. The SYBR green assay was done in triplicate and the mean was used for further calculations.

## 3. Results and discussion

### 3.1. Sequence characterization and phylogenetic analysis

Calreticulin is a multi-functional protein, the main functions of which are associated with $Ca^{2+}$ binding and chaperoning (Michalak et al., 2002; Trombetta 2003). Calreticulin genes isolated so far showed a strong evolutionary conservation. The first piece of evidence about calreticulin in *E. fetida* earthworms was reported by Kauschke et al., (2001). Analyzing a MALDI-MS peptide map for perforin-like lytic proteins of *E. fetida* coelomic fluid, they found similarity to *Brassica napus* (*Brassicaceae*, *Streptophyta*) calreticulin. This finding is not surprising as it was reported that calreticulin interacts with perforin through *N*-domain and moreover these molecules are often copurified (Andrin et al., 1998). We therefore decided to confirm this finding and designed degenerated primer sets based on all known invertebrate calreticulin nucleotide sequences using CODEHOP program (Rose et al., 1998). Subsequent RT-PCR reactions with these primer sets resulted in a specific PCR product corresponding to a calreticulin fragment of approximately 700 bp. Following cloning and sequencing revealed its homology to already known calreticulin molecules.

In order to assemble the full length cDNA sequence of calreticulin, RACE amplifications of the 5′ and 3′cDNA ends were performed. Resulting PCR products were cloned and sequenced. The full length cDNA of *E. fetida* calreticulin has an ORF coding for 428 amino acids. Analysis of both the nucleotide sequence and the deduced amino acid sequence of earthworm calreticulin (as shown in Fig. 1) identified it as a member of the highly conserved calreticulin family. The signal

Fig. 6. *In situ* hybridization experiments for earthworm calreticulin on cryo-sections (8 μm) of different body parts of the earthworm *E. fetida*. Earthworm calreticulin was expressed in epithelial cells of the epidermis (ep; A) as well as of the intestinal tract (in; G), in neurons of ventral nerve cord (nc; C), in sperm cells in seminal vesicles (sc; E), cells of the mesenchymal lining (ml; I) as well as in body wall muscle cells (bm; I) and coelomocytes (J). The reaction for sense probes (controls) is shown for epithelial cells (epidermis: B, F; intestine: H), for nerve cord (D, F) and for the mesenchymal lining and body wall muscle (F). Figures C–F and I show transversal sections of the genital region, A, G, H a longitudinal section of the midgut region and B a transversal section from the clitellum region of *E. fetida*. (A–D) magnification ×200, (E–I) ×100, (J) ×400.

peptide of *E. fetida* calreticulin comprises 18 amino acids as predicted by using SignalP 3.0 software (Bendtsen et al., 2004; Nielsen et al., 1997). The central *P*-domain contains three repeats of the amino acid sequence DPEXXKPEDWD followed by three repeats of the sequence GXWXXXXIXNPXY (Fig. 1). The ER retention signal KDEL is located prior to the stop codon. Moreover, the molecule contains three cysteine residues at conserved positions 88, 120 and 146 and a histidine at position 153, which is essential for the chaperone function of calreticulin (Guo et al., 2003). However, there is no potential N-linked glycosylation site within the amino acid sequence. The nucleotide sequence of the *E. fetida* calreticulin molecule was submitted to the GenBank database (accession number: DQ887090).

We found that *E. fetida* calreticulin shares significant homology (65–75%) with previously characterized calreticulin molecules of invertebrates. To gain more insight into the relationship of calreticulin molecules, their amino acid sequences were subjected to phylogenetic analysis. The neighbor-joining phylogenetic trees were constructed based on both amino acid and nucleotide sequences of calreticulin using MEGA version 3.0 software (Kumar et al., 2004). Both data sets resulted in trees with identical topologies; only the protein tree is therefore shown (Fig. 2). The confidence in each node was assessed by 100 or 10,000 bootstrap replicates. Similar topology of the phylogenetic tree was obtained when maximum likelihood method was used for the phylogenetic analysis (data not shown).

Calreticulin of *E. fetida* is the only one fully-sequenced, described and characterized calreticulin molecule of *Oligochaeta* phylum; however, cDNA libraries of other earthworms *Lumbricus rubellus* and *Eisenia andrei* are available (LumbriBASE, EandreiBASE; http://www.earthworms.org). Searching these libraries we found partial putative calreticulin sequences (accession numbers: LRC04082_1, LRC02602_1, EAC00116). *L. rubellus* putative calreticulin sequence was included in the phylogenetic analysis. Unfortunately, *E. andrei* library contains only short segments matching to calreticulin and therefore was not found suitable for the analysis. Not surprisingly *E. fetida* and *L. rubellus* calreticulin molecules have the same origin.

Since *E. fetida* calreticulin and that of the mollusk *Aplysia californica* are clustered together we presume their common origin. Moreover, these molecules share very high identity among amino acid sequences, i. e. 75%. Calreticulins of *Insecta* as well as *Ixodida* have less homology with *E. fetida* calreticulin and they form different branches, while the *Nematode* sequences were found to be in a separate cluster.

### 3.2. Production of recombinant calreticulin, western blot analysis

Successful expression of recombinant *E. fetida* calreticulin was achieved in *E. coli* BL21(DE3) cells transformed with pET-28a(+) vector. The recombinant calreticulin was used in further experiments.

We documented that a polyclonal anti-human calreticulin antibody specifically recognizes recombinant calreticulin of *E. fetida* as well as calreticulin in *E. fetida* coelomocyte lysate (Fig. 3). Western blot analysis revealed that apparent molecular weight of recombinant calreticulin is approximately 59 kDa while the calculated theoretical value is 51.5 kDa including His-tag. The discrepancy is not surprising since the apparent molecular weight of calreticulins is often higher than the theoretical values due to high negative charge of the protein mainly at the C-terminus as well as folding and posttranslational modifications.

Since the polyclonal anti-human calreticulin antibody was specific, we found this antibody appropriate for immunocytochemistry and immunohistochemistry assays.

### 3.3. Immunocytochemistry, immunohistochemistry

Calreticulin is found in many different locations of various eukaryotic cells. The major cellular location of calreticulin is the ER. However, it was detected also in cytosolic granules, in perinuclear areas and at the cell surface (Johnson et al., 2001; Krause and Michalak, 1997; Michalak et al., 1999).

We used a polyclonal rabbit anti-calreticulin antibody to localize calreticulin in coelomocytes and on transversal as well as longitudinal cryo-sections of *E. fetida*. Antibody labeling was detected in the mesenchymal lining of the coelomic cavity, i.e. a site from where the coelomocytes are derived and proliferate (Bilej et al., 1992) (Fig. 4A), and in granular intracellular bodies of certain coelomocytes (Fig. 5A). This observation might reflect the presence of calreticulin in the ER of secreting cells. No labeling occurred in negative controls, where serum of nonimmunized rabbits was used (Figs. 4B, 5B).

### 3.4. In situ hybridization and real-time PCR

*In situ* hybridization revealed calreticulin expression in various cells and tissues of *E. fetida* in all analyzed body parts (Fig. 6A–J). Calreticulin was dominantly expressed in cells of the epidermis (Fig. 6A), in neurons of the ventral nerve cord (Fig. 6C) and in sperm cells (Fig. 6E), but also in epithelial cells of the intestinal tract (Fig. 6G), in cells of body wall muscles (Fig. 6I), in cells of the mesenchymal lining of the coelomic cavity (Fig. 6I) and in coelomocytes (Fig. 6J). Sense probes used as controls did not result in any labeling (Fig. 6B, D, F, H).

These results were confirmed by real-time PCR, which quantified calreticulin mRNA levels. Real-time PCR experiments revealed high-level expression of calreticulin in the body wall muscles, cerebral ganglia, pharynx and seminal vesicles (Fig. 7).

Both *in situ* hybridization and quantitative PCR revealed calreticulin expression in various cells and tissues of *E. fetida*. Since cellular signalling, especially in muscle cells and neurons, is strongly dependent on dynamic changes of $Ca^{2+}$ levels, a regulatory function of calreticulin as previously shown for *B. mori* (Goo et al., 2005) might be considered also for earthworms. Calreticulin expression in cells of the mesenchymal lining and in coelomocytes suggests its chaperoning function as well, presumably with importance to stress conditions as demonstrated in *C. elegans* (Park et al., 2001).

In view of previous data, the functional role of calreticulin in earthworms should involve innate immune mechanisms like pore formation, phagocytosis and encapsulation, since participation of calreticulin in non self recognition and cellular defence has been shown for insects (Asgari and Schmidt, 2003; Choi et al., 2002; Zhang et al., 2006).

To sum up, we proved the presence of calreticulin in *E. fetida* earthworms and specified the sequence of calreticulin-coding cDNA. We proved its high expression in various tissues such as epidermis, body wall muscles, ventral nerve cord, sperm cells and mesenchymal lining. Considering its chaperoning function, calreticulin can be a useful tool for identification and affinity purification of novel cytolytic factors.

## Acknowledgements

This work was supported by the Grant Agency of the Academy of Sciences (A5020208), the Czech Science Foundation (310/03/H147), the Ministry of Education, Youth and Sports (2B06155) and an Institutional Research Concept (AV0Z50200510). Authors are very grateful to Tomáš Mrkvan for helpful discussion.

## References

Andrin, C., et al., 1998. Interaction between a Ca2+-binding protein calreticulin and perforin, a component of the cytotoxic T-cell granules. Biochemistry 37, 10386–10394.

Asgari, S., Schmidt, O., 2003. Is cell surface calreticulin involved in phagocytosis by insect hemocytes? J. Insect Physiol. 49, 545–550.

Baksh, S., Michalak, M., 1991. Expression of calreticulin in Escherichia coli and identification of its Ca2+ binding domains. J. Biol. Chem. 266, 21458–21465.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Bilej, M., De Baetselier, P., Beschin, A., 2000. Antimicrobial defense of the earthworm. Folia Microbiol. (Praha) 45, 283–300.

Bilej, M., Sima, P., Slipka, J., 1992. Repeated antigenic challenge induces earthworm coelomocyte proliferation. Immunol. Lett. 32, 181–184.

Choi, J.Y., et al., 2002. Calreticulin enriched as an early-stage encapsulation protein in wax moth *Galleria mellonella* larvae. Dev. Comp. Immunol. 26, 335–343.

Cooper, E.L., Kauschke, E., Cossarizza, A., 2002. Digging for innate immunity since Darwin and Metchnikoff. Bioessays 24, 319–333.

Corbett, E.F., Michalak, M., 2000. Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem. Sci. 25, 307–311.

Denning, G.M., et al., 1997. Calreticulin biosynthesis and processing in human myeloid cells: demonstration of signal peptide cleavage and n-glycosylation. Blood 90, 372–381.

Fliegel, L., Burns, K., MacLennan, D.H., Reithmeier, R.A., Michalak, M., 1989. Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 264, 21522–21528.

Fraser, S.A., Karimi, R., Michalak, M., Hudig, D., 2000. Perforin lytic activity is controlled by calreticulin. J. Immunol. 164, 4150–4155.

Goo, T.W., et al., 2005. Endoplasmic reticulum stress response of *Bombyx mori* calreticulin. Mol. Biol. Rep. 32, 133–139.

Guo, L., et al., 2003. Identification of an N-domain histidine essential for chaperone function in calreticulin. J. Biol. Chem. 278, 50645–50653.

Helenius, A., Trombetta, E.S., Herbert, D.N., Simons, J.F., 1997. Calnexin, calreticulin and the folding of glycoproteins. Trends Cell. Biol. 7, 193–200.

Johnson, S., Michalak, M., Opas, M., Eggleton, P., 2001. The ins and outs of calreticulin: from the ER lumen to the extracellular space. Trends Cell. Biol. 11, 122–129.

Kauschke, E., Komiyama, K., Moro, I., Eue, I., Konig, S., Cooper, E.L., 2001. Evidence for perforin-like activity associated with earthworm leukocytes. Zoology (Jena) 104, 13–24.

Krause, K.H., Michalak, M., 1997. Calreticulin. Cell 88, 439–443.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

Michalak, M., Corbett, E.F., Mesaeli, N., Nakamura, K., Opas, M., 1999. Calreticulin: one protein, one gene, many functions. Biochem. J. 344 (Pt 2), 281–292.

Michalak, M., Robert Parker, J.M., Opas, M., 2002. Ca2+ signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium 32, 269–278.

Murray, V., 1989. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Res. 17, 8889.

Nakamura, K., et al., 2001. Functional specialization of calreticulin domains. J. Cell Biol. 154, 961–972.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Ostwald, T.J., MacLennan, D.H., 1974. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. J. Biol. Chem. 249, 974–979.

Park, B.J., et al., 2001. Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in *Caenorhabditis elegans*. Mol. Biol. Cell 12, 2835–2845.

Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M., Henikoff, S., 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res. 26, 1628–1635.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

Smith, M.J., Koch, G.L., 1989. Multiple zones in the sequence of calreticulin (CRP55, calregulin, HACBP), a major calcium binding ER/SR protein. Embo J. 8, 3581–3586.

Stein, E., Cooper, E.L., 1981. The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. Dev. Comp. Immunol. 5, 415–425.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Trombetta, E.S., 2003. The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis. Glycobiology 13, 77R–91R.

Zhang, G., Schmidt, O., Asgari, S., 2006. A calreticulin-like protein from endoparasitoid venom fluid is involved in host hemocyte inactivation. Dev. Comp. Immunol. 30, 756–764.

## 4.5. Modulation of defense molecules during immune response

Currently, several antimicrobial factors, including lysozyme-like molecule (Cotuk and Dales 1984) and factors with hemolytic activity (Roch 1979; Roch *et al.* 1981; Lange *et al.* 1997; Lassegues *et al.* 1997; Sekizawa *et al.* 1997; Eue *et al.* 1998; Yamaji *et al.* 1998; Lange *et al.* 1999) have been identified in *E. fetida* earthworms. Furthermore, a pattern recognition molecule named CCF has been described (Bilej *et al.* 1995; Beschin *et al.* 1998). However, the modulation of these molecules during an ongoing *in vivo* immune response has not yet been clarified. Therefore, we monitored the effect of an experimental microbial infection on the expression of defense molecules CCF and fetidin in coelomocytes and, moreover, on the defense activity of the coelomic fluid, i. e. lysozyme-like activity, hemolytic activity and the production of CCF.

Earthworms were injected with Gram-positive and Gram-negative bacteria and β-1,3-glucan and the changes in expression as well as coelomic fluid activities were followed up to 13 days after the injection. As it is known that earthworms respond not only to the antigenic stimulation but also to the body injury caused by the injection by a non-specific increase in protein concentration of the coelomic fluid, we used isotonic salt solution (LBSS) stimulated earthworms as sham controls. LBSS stimulated earthworms were found to have an increased protein concentration within two days after the injection and then it reached the levels of non-injected animals. Administration of bacteria and microbial polysaccharides additionally modulated the coelomic fluid protein concentration.

Infection with Gram-positive and Gram-negative bacteria resulted in an increased lysozyme-like activity as early as 6 h post-infection as compared to sham-stimulated animals. The enhanced activity was sustained for 4 days post-infection while β-1,3-glucan injection caused increased activity 1-3 days after the injection. Since lysozyme degrades peptidoglycan and hence its activity is considered to be directed mainly against Gram-positive bacteria, it was surprising that

we did not observe a principal difference in the maximum lysozyme-like activity in Gram-positive bacteria injected animals as compared to those injected either with Gram-negative bacteria or β-1,3-glucan.

Hemolytic activity of the coelomic fluid was followed up to 6 days after the injection. It was expressed as the protein concentration that causes the lysis of 50 % of erythrocytes (LD50). Thus, an increase in protein concentration causing 50 % hemolysis reflects a relative decrease of the hemolytic activity. Gram-positive bacteria injection led to a decrease of the hemolytic activity 2 days after the challenge while Gram-positive bacteria injection caused an additional transient decrease 12 h post-injection. No modulation of hemolytic activity was observed after the injection of β-1,3-glucan as compared to sham stimulated controls. Since the decrease in hemolytic activity in all experimental groups including LBSS injected animals correlated with synchronously increased protein concentration in the coelomic fluid, it can be assumed that hemolytic factor levels did not change upon challenge while the concentration of other proteins increased. This idea is supported by the fact that mRNA levels for fetidin determined by semi-quantitative RT-PCR were not significantly modulated by microbial stimulation.

Concentration of CCF in the coelomic fluid as determined by ELISA assay was found to be increased within the first 24 h post-injection regardless of the microbial particles used for stimulation. Yet, since CCF acts as an agglutinin strongly binding pathogen-associated molecular patterns, the increase in CCF concentrations may represent only the free molecules and thus may be underestimated.

The increase in CCF concentration in the coelomic fluid correlated with an increase at the transcriptional level. The maximum levels of CCF mRNA were reached 17 h after the injection of both types of bacteria or β-1,3-glucan, i. e. when humoral CCF levels started to decrease. This may suggest that early after the stimulation CCF is released from intracellular stocks and then its *de novo* synthesis is required to refill the deposit in cellular granules.

To conclude, parenteral administration of live bacteria or microbial polysaccharides results in increased CCF levels in the coelomic fluid, in increased lysozyme-like activity and decreased hemolytic activity of the coelomic fluid. The biosynthesis of CCF, but not fetidin, is up-regulated upon microbial stimulation.

**Köhlerová P., Beschin A., Šilerová M., De Baetselier P., Bilej M.: Effect of experimental microbial challenge on the expression of defense molecules in *Eisenia foetida* earthworm. *Dev. Comp. Immunol.* 28: 701-711 (2004).**

Köhlerová P., Beschin A., Šilerová M., De Baetselier P., Bilej M.: Effect of experimental microbial challenge on the expression of defense molecules in *Eisenia foetida* earthworm. *Dev. Comp. Immunol.* 28: 701-711 (2004).

Available online at www.sciencedirect.com
SCIENCE DIRECT
Developmental and Comparative Immunology 28 (2004) 701–711
Developmental & Comparative Immunology
www.elsevier.com/locate/devcompimm

# Effect of experimental microbial challenge on the expression of defense molecules in *Eisenia foetida* earthworm

Petra Köhlerová[a], Alain Beschin[b], Marcela Šilerová[a], Patrick De Baetselier[b], Martin Bilej[a,*]

[a]*Department of Immunology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic*
[b]*Department of Cellular and Molecular Interactions, Flemish Interuniversity Institute for Biotechnology, Free University Brussels (VUB), Pleinlaan 2, B-1050 Brussels, Belgium*

Received 23 July 2003; revised 3 December 2003; accepted 15 January 2004

**Abstract**

Earthworms are able to protect themselves against invading pathogens due to efficient innate defense mechanisms. Currently, two types of antimicrobial factors including lysozyme-like molecule and factors with hemolytic activity, as well as a pattern recognition protein named coelomic cytolytic factor (CCF) have been identified in *Eisenia foetida* earthworms. However, the modulations of these defense molecules during in vivo immune response have not been addressed. In this study, we investigated the effect of experimental challenge with live Gram-negative and Gram-positive bacteria and with β-1,3-glucan on the expression of CCF and the hemolytic factor fetidin. In parallel, we followed levels of hemolytic activity and lysozyme-like activity in the coelomic fluid of challenged earthworms. We show that the biosynthesis of CCF, but not fetidin, is up-regulated upon microbial stimulation. Parenteral administration of bacteria or microbial polysaccharides in earthworms results, in the coelomic fluid, in augmented level of CCF, increased lysozyme-like activity and decreased hemolytic activity. The decreased hemolytic activity of the coelomic fluid reflects the increase of the whole protein content in the absence of synthesis of hemolytic proteins.


*Keywords:* Innate immunity; Earthworm; *Eisenia*; Coelomic cytolytic factor; Lysenin; Fetidin; Lysozyme

## 1. Introduction

Invertebrates have evolved a variety of active defense pathways efficiently recognizing and responding to non-self substances despite the absence of an adaptive immune system based on antibodies or lymphocytes. Invertebrates rely on innate defense strategies, which are based on pattern recognition receptors recognizing surface determinants common to potential pathogens, and on antimicrobial factors [1]. Currently, one pattern recognition protein named coelomic cytolytic factor (CCF) [2,3] and two main groups of antimicrobial factors—including lysozyme-like molecule [4] and factors with hemolytic activity [5–12]—have been identified in *Eisenia foetida* earthworms.

*Abbreviations:* CCF, coelomic cytolytic factor; CF, coelomic fluid; LPS, lipopolysaccharide; PCR, polymerase chain reaction; LBSS, *Lumbricus* balanced salt solution; PBS, phosphate buffered solution.

* Corresponding author. Tel.: +420-2-4106-2367; fax: +420-2-4172-1143.
*E-mail address:* mbilej@biomed.cas.cz (M. Bilej).

0145-305X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2004.01.001

Lysozyme provides an efficient protection by hydrolyzing glycosidic bonds of peptidoglycan in bacterial cell walls. Recently, a lysozyme-like active protein from *E. foetida* earthworm was partially sequenced and characterized [13]. On the basis of the N-terminal sequence, it was suggested to form a novel class of lysozymes shared by mollusks, echinoderms and the nematode *Caenorhabditis elegans*.

In *E. foetida*, hemolytic activity is connected to the antimicrobial activity of the coelomic fluid. Accordingly, numerous hemolytic factors that exert agglutination and antibacterial activities against pathogenic soil bacteria have been described [7,14–22]. Du Pasquier and Duprat [17] were the first to evidence a hemolytic activity against erythrocytes of various vertebrates in the coelomic fluid of *E. foetida andrei*. Further experiments revealed that the hemolytic factors constitute a polymorphic system [7]. Roch et al. [8,18] described an *E. foetida* hemolytic factor, with two isoforms of 40 and 45 kDa differing in the extent of glycosylation. These molecules are secreted by chloragocytes or eleocytes and exhibit antibacterial activity. *E. foetida* hemolytic factors were named fetidins and one of them was later on cloned [6,19]. Independently, a 41 kDa hemolytic protein, produced by coelomocytes and causing the contraction of rat vascular smooth muscle, was cloned and described as lysenin by Sekizawa et al. [11,20]. Simultaneously, two 42 kDa lysenin-related proteins were identified. Lysenin and lysenin-related proteins reveal a high sequence homology with fetidins, suggesting that these molecules are isoforms [11,21,22].

We have characterized a 42 kDa lectin named CCF acting as a pattern recognition molecule in *E. foetida* earthworms [2,3]. CCF binds efficiently different pathogen-associated molecular patterns [2,23,24] hereby triggering the activation of the prophenoloxidase (proPO) cascade, which is an important invertebrate defense mechanism. Besides the coelomic fluid, CCF is localized in the cells of chloragogenous tissue adjacent to the gut wall, and in free large coelomocytes exhibiting macrophage-like function [25].

Although antibacterial activity based on lysozyme-like protein, fetidin-related proteins and CCF has been evidenced in vitro in earthworms, modulation of this activity during an ongoing immune response [4] has not been demonstrated yet. In the present study, the effect of experimental infection with live Gram-negative and Gram-positive bacteria, and of administration of yeast β-1,3-glucan, on the expression of mRNA coding for CCF and fetidin was investigated in *E. foetida*. In parallel, lysozyme-like activity, hemolytic activity, and protein level of CCF in the coelomic fluid were analyzed. We show that coelomic fluid level of CCF and lysozyme-like activity increase after in vivo microbial stimulation, while the hemolytic activity rather decreases. Increased humoral level of CCF parallels with the up-regulation of CCF gene transcription in coelomocytes. In contrast, expression of fetidin is not affected upon experimental administration of microbial compounds in *E. foetida* earthworms.

## 2. Materials and methods

### 2.1. Animals and their stimulation

Adult *E. foetida* earthworms (Oligochaeta; Annelida) kept at 20 °C in compost were transferred 2 days before experiments on filter paper soaked with isotonic *Lumbricus* balanced salt solution (LBSS) [26]. They were injected post-clitellum with insoluble β-1,3-glucan preparation from *Saccharomyces cerevisiae* (kindly provided by Dr Novak, Institute of Microbiology, Prague), live Gram-negative bacteria *Escherichia coli* K12 and live Gram-positive bacteria *Bacillus subtilis* W23. Fifty micrograms of β-1,3-glucan were given in 25 μl of LBSS. Cultures of bacteria at OD of 1 (50 ml of culture in LB broth, Fluka, Buchs, Switzerland) were centrifuged (3000*g*, 20 min) and resuspended in 10 ml LBSS. Twenty-five microliters (approximately $10^7$ CFU) of resuspended cultures were injected into earthworms. Non-stimulated earthworms and earthworms injected with LBSS were used as controls.

### 2.2. Harvesting of the coelomic fluid and coelomocytes

Coelomic fluid was obtained by puncturing post-clitellum segments of the coelomic cavity with a Pasteur micropipette and kept at 4 °C. At different time intervals post-challenge (0, 6, 12, 18, and 24 h and 2, 3, 4 and 6 days after the stimulation) and for each experimental group (non-stimulated animals, animals injected with LBSS only, animals

stimulated with *E. coli*, *B. subtilis* and β-1,3-glucan), equal volumes of coelomic fluid suspension from 15 earthworms were pooled and centrifuged (500*g*, 10 min, 4 °C). Supernatant was centrifuged again (7000*g*, 10 min, 4 °C), and stored at − 20 °C.

To isolate coelomocytes, pooled coelomic fluid diluted (1:2 vol/vol) into pre-cooled anticoagulant buffer (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6; [27]) was centrifuged (500*g*, 10 min, 4 °C). The coelomocyte pellet was washed twice with anticoagulant buffer before isolation of RNA.

### 2.3. Evaluation of protein concentration

Protein concentration of coelomic fluid was assayed according to Bradford [28] using Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, USA) and bovine serum albumin as a standard.

### 2.4. Evaluation of lysozyme-like activity

To evaluate lysozyme-like activity, 100 μl of coelomic fluid diluted (1:10) in PBS were mixed in microtiter plates with 100 μl of freeze-dried cell walls of *Micrococcus lysodeikticus* suspension (0.7 mg/ml; Sigma-Aldrich, Steinheim, Germany) resuspended in 0.1 M phosphate buffer (pH 6.4). After incubation at 37 °C for 30 min, OD was measured at 450 nm with spectrophotometer (Titertek Multiscan MCC/340 ELISA Reader). Chicken-egg white lysozyme (Grade III; Sigma-Aldrich) was used as a standard and its activity (reflected by decrease in OD) was linear in the range 10–5000 U/ml.

### 2.5. Evaluation of hemolytic activity

To evaluate hemolytic activity, 100 μl of coelomic fluid serially diluted (1:1000–1:64,000) in 145 mM NaCl (pH 7.4) in microtiter plates were incubated with 100 μl of sheep erythrocyte suspension (3% in 145 mM NaCl, pH 7.4) for 2 h at room temperature. Plates were centrifuged (100*g*, 10 min, 4 °C) and OD of transferred supernatants was measured at 405 nm. The coelomic fluid protein concentration causing 50% hemolysis (LD50) was estimated for each sample by linear regression (maximum lysis was determined in a sample treated with 1% SDS).

### 2.6. Determination of CCF level

Level of CCF in coelomic fluid was determined by ELISA using anti-CCF monoclonal antibody 12C9 as previously described [3]. Briefly, wells were coated overnight at 4 °C with 100 μl coelomic fluid diluted (1:100) in PBS. Plates were washed with PBS containing 0.05% Tween-20 (T-PBS) and saturated with 200 μl bovine serum albumin (2% in PBS, 1 h, 37 °C). After washing wells were incubated with 100 μl 12C9 antibody (20 μg/ml in PBS, 1 h, 37 °C), washed, and 100 μl alkaline phosphatase-labeled rabbit anti-mouse IgG antibody (Sigma-Aldrich, 1:1000 in PBS, 1 h, 37 °C) were added. Bound antibody was revealed using 100 μl substrate buffer containing 1 mM 4-nitrophenyl phosphate disodium salt hexahydrate and 2% ethanamine ethanol (pH 10.2, SERVA, Heidelberg, Germany). OD was assessed at 405 nm. CCF concentration in the samples was inferred from a standard curve (linear in the range of 1–400 μg/ml) generated with recombinant CCF [2].

### 2.7. Statistical analysis

Three independent experiments were performed. In each experiment, all parameters were measured in triplicates. Data were expressed as mean ± SD of the values obtained in all three experiments. Paired Student's *t*-test using GraphPad Prism software was performed to evaluate the significance of the data. Differences were considered significant when $P < 0.05$.

### 2.8. RNA isolation and cDNA synthesis

For each experimental group and for each time interval post-challenge (2, 5, 17, 40, and 70 h), total RNA was prepared from coelomocytes pooled from 15 animals using 800 μl of Trizol reagent as recommended by the supplier (Gibco BRL, Grand Island, NY, USA). Three micrograms of DNAse I-treated total RNA were reversed-transcribed with $Oligo(dT)_{12-18}$ (Gibco-BRL) and SUPERSCRIPT™ II Rnase $H^-$ Reverse Transcriptase (Gibco-BRL).

### 2.9. Semi-quantitative PCR

Each PCR cycle consisted of denaturation at 94 °C for 60 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 90 s. PCR primer pairs were: for CCF (accession number AF030028), sense (5′-ATCAAT ATCACATCGTCTGGCAG-3′) and anti-sense (5′-GTCGATCTCGCCCGATCGAG-3′) giving an amplicon of 0.510 kb; for fetidin (accession number UO2710), sense (5′-AAGCATGCGGACAGGAAGG AGTAT-3′) and anti-sense (5′-GCGGAAGCAAA TGTGTAATGGTG-3′) giving an amplicon of 0.443 kb; for myosin (accession number AF537290), sense (5′-GCGAAATCGAGGATGTTCGT-3′) and anti-sense (5′-CTACTTCTCGGGAGGTCCAGC-3′) giving an amplicon of 0.453 kb. Amplifications were performed for 26 cycles. The amount of template cDNA and number of PCR cycles were optimized so that the analysis of the PCR products could be carried out within the linear range of amplification. Myosin was used as a control to ensure that differences in the expression levels of each gene in different samples were not due to differences in the amounts of template cDNA.

Amplicons were separated on a 1.1% agarose gel (Gibco-BRL) containing ethidium bromide in 1 × TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.3) at 90 V for 2 h and documented by Image Reader FUJIFILM LAS-1000. Gel images were analyzed using the Aida 1D software. Levels of expression of CCF and fetidin were related to the expression of myosin and expressed as a relative value setting the control animals as one (non-stimulated earthworm).

## 3. Results

### 3.1. Protein level in the coelomic fluid of E. foetida challenged with bacteria and β-1,3-glucan

Earthworms respond to body injury by non-specific changes in the protein concentration of the coelomic fluid [29]. Therefore, protein level was determined in the coelomic fluid of *E. foetida* parenterally injected with LBSS or β-1,3-glucan as well as in the coelomic fluid of animals infected with live Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria (Fig. 1). Over the period of time investigated (up to 6 days) and as compared to non-injected animals, a significant yet transient increase (up to 2 × ) in protein concentration was observed at 6, 18 and 48 h post-stimulation in LBSS-treated (sham-stimulated) animals. Administration of bacteria and microbial polysaccharides further modulated coelomic fluid protein concentration. In particular, as compared to LBSS stimulation, increased protein concentration was found 6 h, 18 h, 2 days and 4 days post-infection in *E. coli*-infected animals, 12 h post-administration in *B. subtilis*-infected earthworms, and 24 h post-inoculation in β-1,3-glucan-injected individuals.

### 3.2. Lysozyme-like activity in the coelomic fluid of E. foetida challenged with bacteria and β-1,3-glucan

Modulation of lysozyme-like activity was determined in *E. foetida* injected with LBSS, β-1,3-glucan, Gram-negative and Gram-positive bacteria (Fig. 2). A significant increase in the lysozyme-like activity occurred between 1 and 3 days post-injection in sham-stimulated earthworms, returning to values observed in non-stimulated worms 4 days after the challenge. Infection with Gram-negative and Gram-positive bacteria resulted in increased lysozyme-like activity as early as 6 h post-infection as compared to sham-stimulated animals. This enhanced activity was sustained up to 4 days post-infection in both experimental groups, with the highest increase occurring 2 days post-infection. No significant difference was observed between animals infected with Gram-negative or Gram-positive bacteria. As compared to LBSS-injected animals, significantly increased lysozyme-like activity occurred 1–3 days post-injection in *E. foetida* triggered with β-1,3-glucan.

### 3.3. Hemolytic activity in the coelomic fluid of E. foetida challenged with bacteria and β-1,3-glucan

Hemolytic activity was investigated in coelomic fluids of *E. foetida* up to 6 days post-LBSS, β-1,3-glucan, Gram-negative and Gram-positive bacteria administration. This activity was expressed as the protein concentration of the coelomic fluid that lyses

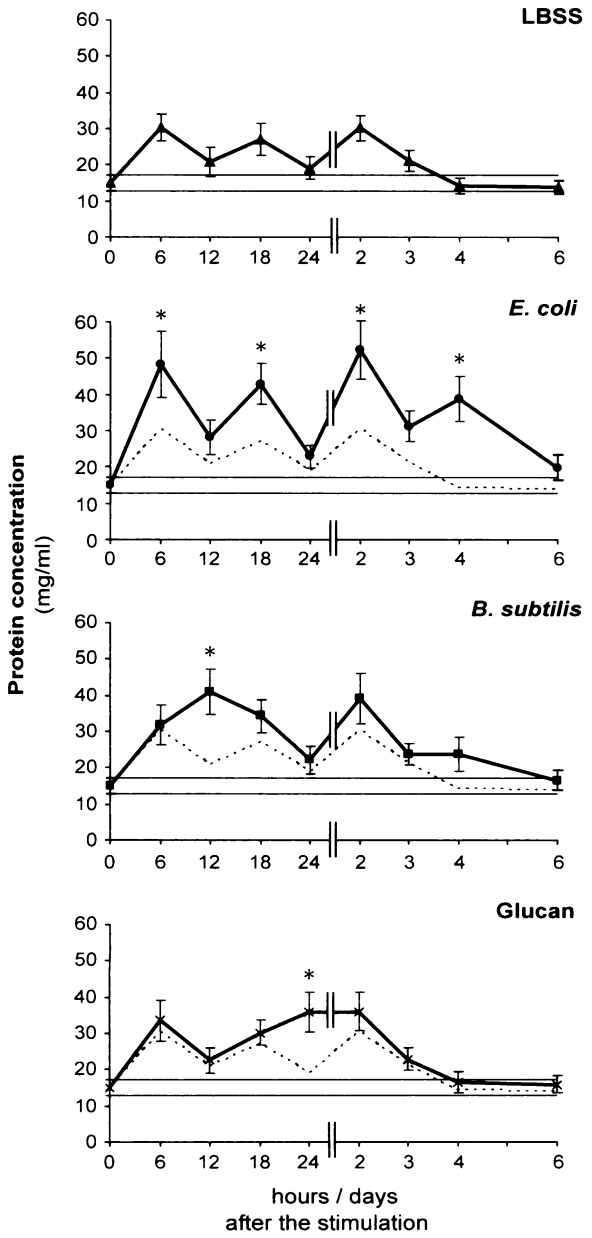



Fig. 1. Protein concentration in the coelomic fluid of *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with live Gram-negative bacteria *E. coli*, live Gram-positive bacteria *B. subtilis* and β-1,3-glucan. Sham-stimulated earthworms received LBSS. At different time intervals post-administration, protein concentration (mg/ml) in the coelomic fluid was determined. Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of protein concentration in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control ($P < 0.05$).

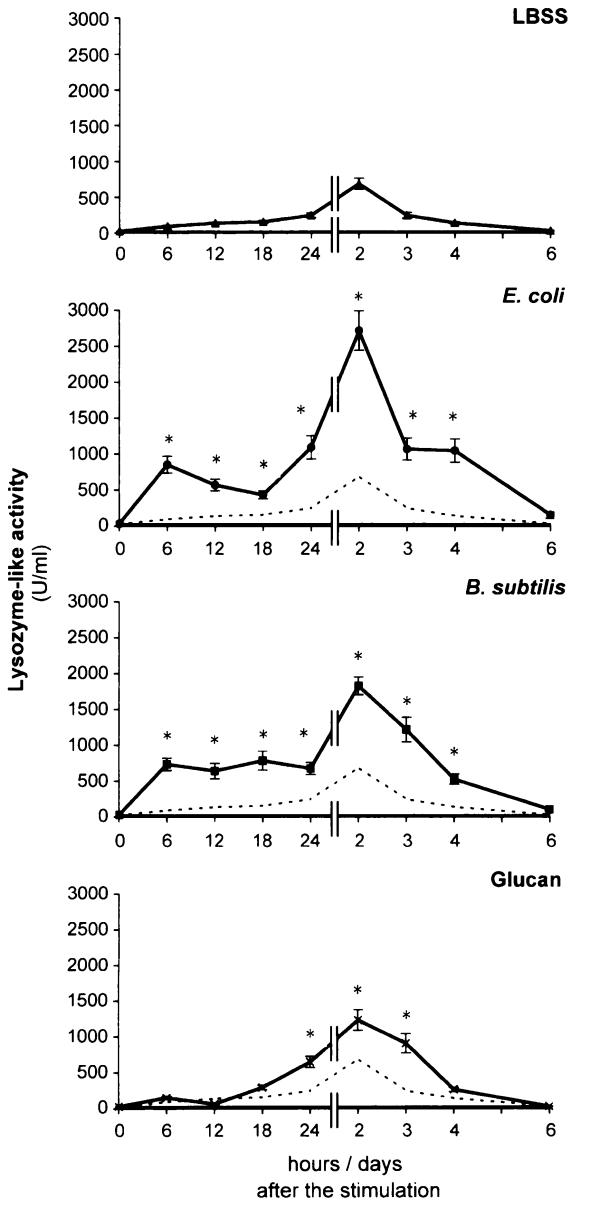



Fig. 2. Lysozyme-like activity in the coelomic fluid of *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with LBSS, live Gram-negative bacteria *E. coli*, live Gram-positive bacteria *B. subtilis* and β-1,3-glucan. At different time intervals post-administration, lysozyme-like activity was determined and expressed (U/ml) using chicken-egg white lysozyme as a standard. Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of lysozyme-like activity in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control ($P < 0.05$).

50% of erythrocytes (LD50). Thus, an increase in protein concentration causing 50% hemolysis reflects a relative decrease in the hemolytic activity of the coelomic fluid.

As compared to non-injected animals, a significant decrease in hemolytic activity occurred in the coelomic fluid 2 days post-LBSS treatment (Fig. 3). Injection of Gram-negative bacteria caused a transient decrease of hemolytic activity 12 h and 2 days after infection as compared to sham-stimulated worms. The hemolytic activity decreased in the coelomic fluid of Gram-positive bacteria-infected individuals 2 days post-stimulation. No significant modulation of the hemolytic activity was observed in β-1,3-glucan-injected individuals as compared to sham-stimulated animals.

### *3.4. Protein level of CCF in the coelomic fluid of E. foetida challenged with bacteria and β-1,3-glucan*

The concentration of CCF was determined up to 6 days post-injection in the coelomic fluid of *E. foetida* injected with LBSS, β-1,3-glucan, Gram-negative and Gram-positive bacteria. CCF protein level increased (3–5 times) in the coelomic fluid of LBSS-treated animals between 6 h and 3 days post-injection, then returning to values observed in non-injected worms (Fig. 4). Significant increase in CCF protein level (approximately 2–5 times) was observed both in Gram-negative and Gram-positive bacteria-infected worms as compared to LBSS-treated animals between 6 and 24 h post-infection. As compared to LBSS-treated animals, CCF protein level further increased in the coelomic fluid between 6 and 12 h following parenteral administration of β-1,3-glucan.

### *3.5. mRNA level of CCF and fetidin in E. foetida challenged with bacteria and β-1,3-glucan*

Levels of mRNA coding for CCF and fetidin in coelomocytes of *E. foetida* injected with LBSS, β-1,3-glucan, Gram-negative and Gram-positive bacteria were determined relatively to myosin mRNA level by semi-quantitative RT-PCR 2, 5, 17, 40 and 70 h post-injection.

We found that the level of CCF mRNA significantly increased from 2 to 40 h after all microbial

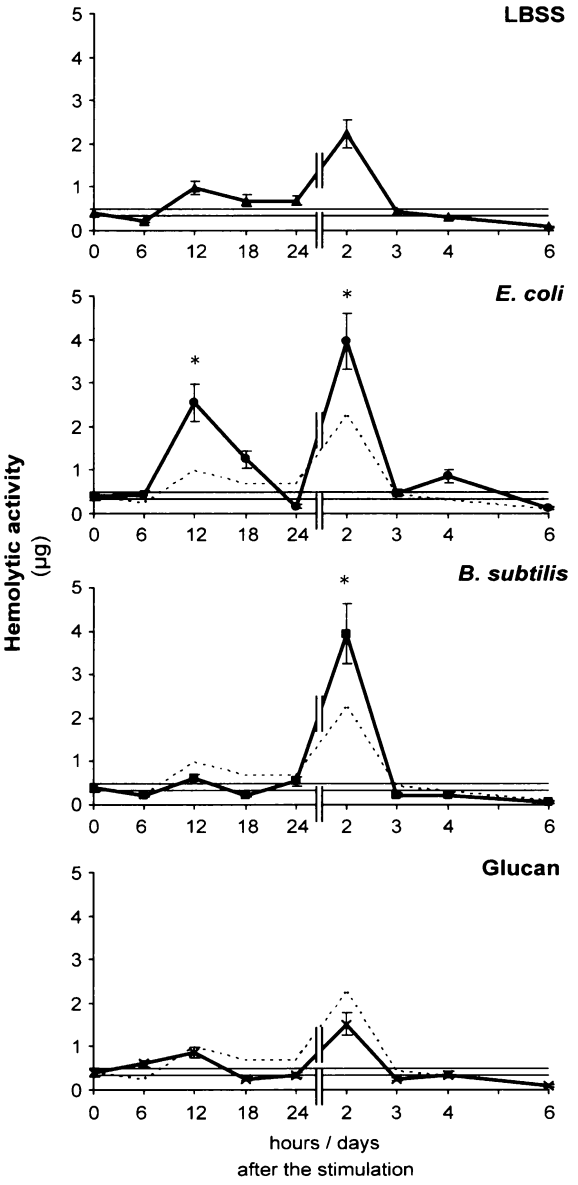



Fig. 3. Hemolytic activity in the coelomic fluid of *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with live Gram-negative bacteria *E. coli*, live Gram-positive bacteria *B. subtilis* and β-1,3-glucan. Sham-stimulated earthworms received LBSS. At different time intervals post-administration, hemolytic activity of coelomic fluid, was expressed as protein concentration (μg) causing 50% hemolysis (LD50). Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of hemolytic activity in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control ($P < 0.05$).

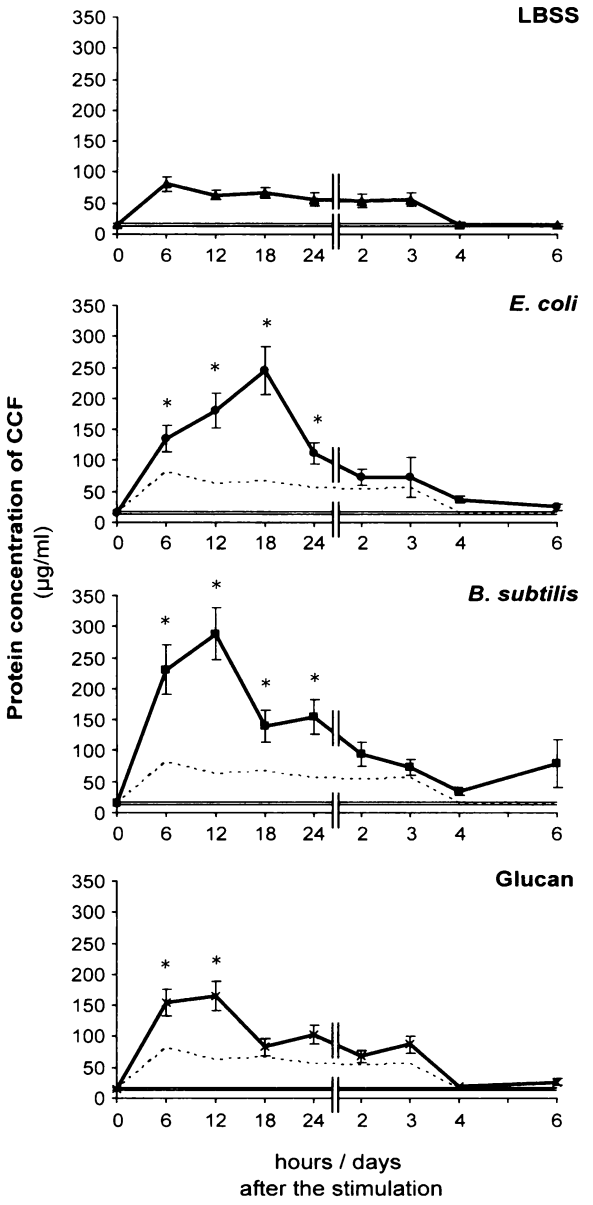


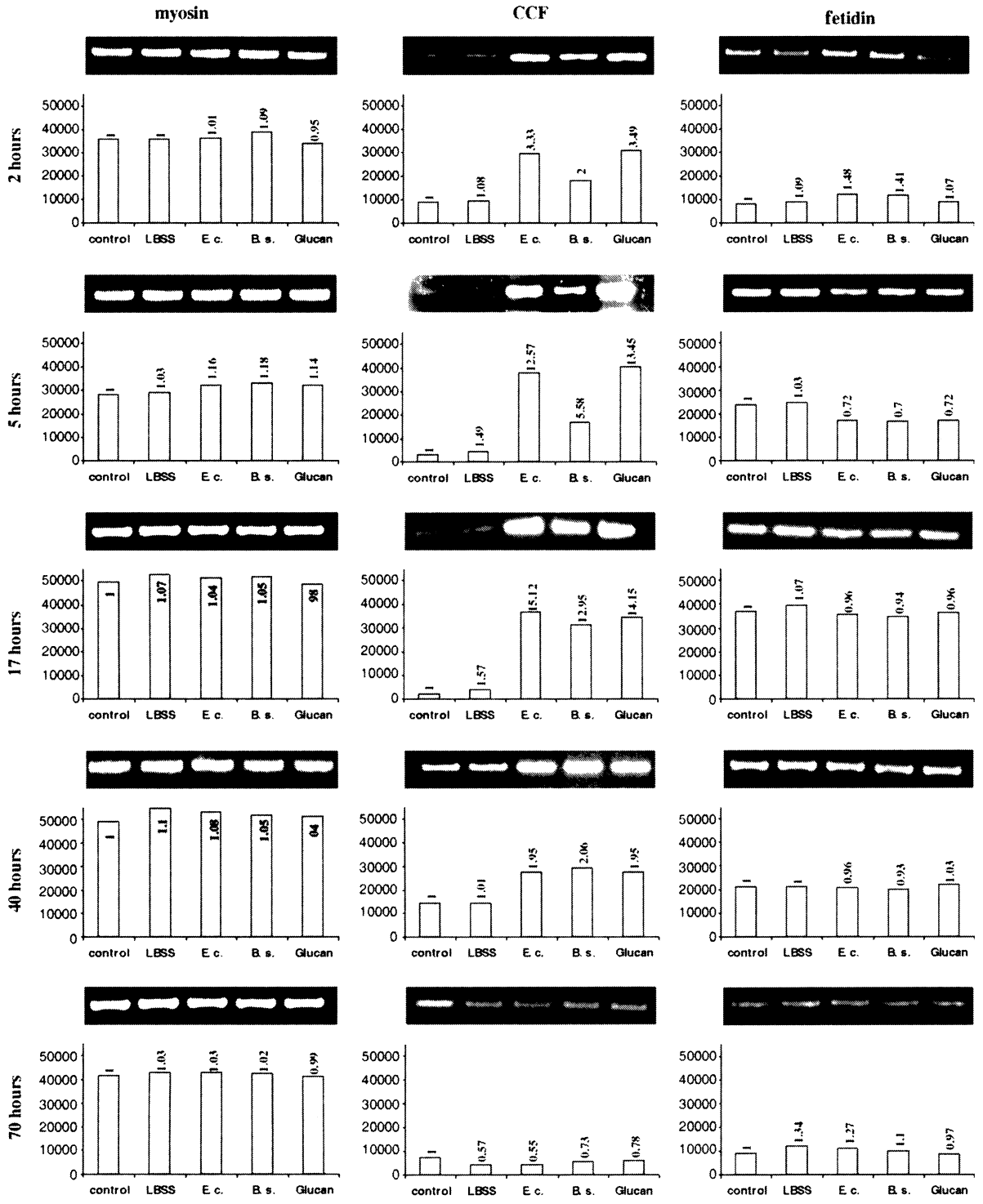

Fig. 4. Protein level of CCF in the coelomic fluid of *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with LBSS, live Gram-negative bacteria *E. coli*, live Gram-positive bacteria *B. subtilis* and β-1,3-glucan. At different time intervals post-administration, protein level of CCF (µg/ml) in the coelomic fluid was determined by ELISA using recombinant CCF as a standard. Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of CCF protein level in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control ($P < 0.05$).

stimulations as compared to non-stimulated and sham-stimulated earthworms, maximum level being observed 17 h post-injection (Fig. 5). The induction of CCF mRNA was lower in *B. subtilis*-infected individuals as compared to *E. coli*-infected or β-1,3-glucan-treated animals, in particular 2 and 5 h post-stimulation. At 40 h post-inoculation, CCF expression level was still twice as high in *E. coli*, *B. subtilis* and β-1,3-glucan-injected worms as compared to non-injected and LBSS-injected *E. foetida*. Finally, it can be mentioned that LBSS-treatment marginally increased CCF mRNA level at 5 and 17 h post-injection as compared with untreated animals. Seventy hours post-treatment, CCF mRNA level in the four experimental groups dropped below the levels observed in non-injected worms.

On the other hand, as compared to non-stimulated and sham-stimulated earthworms, level of mRNA coding for fetidin was not significantly modulated at all time intervals investigated in *E. foetida* injected with Gram-negative and Gram-positive bacteria, as well as with β-1,3-glucan (Fig. 5).

## 4. Discussion

The coelomic cavity of annelids is not aseptic and typically contains bacteria, protozoans and fungi entering from the outer environment mainly via dorsal pores. Dales and Kalac [30] reported that the concentration of naturally occurring bacteria was commonly of the order of $6 \times 10^5$/ml of coelomic fluid, or $0.9 \times 10^5$ per worm of an average size. Because the number of potentially phagocytic cells in the coelomic fluid is more than 10 times higher than the number of naturally occurring bacteria, the excess of phagocytic cells and humoral factors can prevent the detrimental multiplication of coelomic microflora. The amount of bacteria injected in the present study exceeded approximately by 10 times the number of naturally occurring phagocytic cells, yet it did not affect the viability of *E. foetida* and was sufficient to activate defense mechanisms. We did not estimate in vitro the number of colony forming units in the collected coelomic fluids in the course of infection with live bacteria for several reasons. First, it is worthwhile remembering that the coelomic cavity of annelids is metameric, segments being separated by


myosin
CCF
fetidin
2 hours
5 hours
17 hours
40 hours
70 hours
50000
40000
30000
20000
10000
0
control
LBSS
E. c.
B. s.
Glucan
1
1
1.01
1.09
0.95
1
1.08
3.33
2
3.49
1
1.09
1.48
1.41
1.07
1
1.03
1.16
1.18
1.14
1
1.49
12.57
5.58
13.45
1
1.03
0.72
0.7
0.72
1
1.07
1.04
1.05
98
1
1.57
15.12
12.95
14.15
1
1.07
0.96
0.94
0.96
1
1.1
1.08
1.05
04
1
1.01
1.95
2.06
1.95
1
1
0.96
0.93
1.03
1
1.03
1.03
1.02
0.99
1
0.57
0.55
0.73
0.78
1
1.34
1.27
1.1
0.97

transversal septa. The transport of coelomic fluid and cells between neighboring segments is regulated by sphincters within the septa [31]. As such, the metamerization restrains the homogeneous distribution of bacteria and other corpuscular antigens, but not of soluble factors, in the coelomic cavity in the early stage of infection. Second, agglutination occurring in the coelomic fluid of *E. foetida* [2,32], may further impair the spreading of bacteria through the coelomic cavity via channels of the septa. Therefore, the precise assessment of bacteria number through puncturing the coelomic cavity is not obvious.

Earthworms respond to antigenic stimulation, body injury or stress conditions by a marked non-specific increase in coelomic fluid protein concentration [29]. Accordingly, we have documented that coelomic fluid protein level increased within the first 2 days post LBSS-injection (sham-stimulated earthworms), probably as a result of wounding. Further transient changes in comparison with sham-stimulated control group occurred in β-1,3-glucan- and bacteria-injected earthworms within the first 4 days post-challenge. On day 6, coelomic fluid protein concentration returned back to the level of non-injected *E. foetida* in the four experimental groups. It should be mentioned that from day 7 up to 13 days post-challenge, all assessed parameters did not differ in the four experimental groups from those of non-stimulated earthworms (not shown).

Cotuc and Dales [4] have reported that the lysozyme-like activity in coelomic fluid of *E. foetida* is low, yet the enzyme may be released from coelomocytes as a part of an internal defense mechanism against particular bacterial species. Accordingly, we have shown that the in vitro induction of phenoloxidase activity in *E. foetida* coelomic fluid by Gram-positive bacteria required lysozyme-like activity [24]. Therefore, it was of interest to investigate the modulation of lysozyme-like activity in earthworms challenged in vivo with Gram-positive bacteria as well as Gram-negative bacteria and β-1,3-glucan. Maximum increase of lysozyme-like activity in the coelomic fluid was observed 2 days after microbial stimulation, returning back to control levels on day 6. A significant increase in lysozyme-like activity was also recorded on day 2 in sham-stimulated as compared to non-stimulated earthworms, but did not reach the values observed in animals challenged by microbial injections. Lysozyme degrades peptidoglycan, an activity mainly considered to be directed against Gram-positive bacteria. Surprisingly, we did not observe a principal difference in the maximum lysozyme-like activity in Gram-positive bacteria infected worms as compared to animals stimulated with Gram-negative bacteria or β-1,3-glucan raising the question of the specificity of the increased lysozyme-like activity recorded in Gram-negative bacteria and β-1,3-glucan-challenged earthworms.

Fetidins are involved in the hemolytic activity of *E. foetida* coelomic fluid [19]. Although these proteins were suggested to participate in the antimicrobial activity of the coelomic fluid [6], we did not observe significant modulation of fetidin mRNA levels in coelomocytes of earthworms injected with LBSS, both types of bacteria and β-1,3-glucan. On the other hand, by evaluating LD50, i.e. the amount of coelomic fluid proteins required to induce 50% hemolysis, we found that the hemolytic activity significantly decreased in LBSS-treated animals as well as in animals challenged with microbial particles the second day post-stimulation. Considering that decrease of hemolytic activity in all experimental groups correlated with concomitant increased protein concentration in the coelomic fluid, it can be assumed that hemolytic factor levels did not change upon challenge while the concentration of other proteins increased.

CCF from *E. foetida* earthworms represents a pattern recognition molecule that binds microbial compounds including the O-antigen of LPS on Gram-negative bacteria, muramyl dipeptide of Gram-positive bacteria peptidoglycan, or the β-1,3-glucan of yeast cell walls [2,24]. Bilej et al. [25] have reported

Fig. 5. mRNA levels of CCF and fetidin in *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. CCF and fetidin mRNA expression in coelomocytes of non-challenged earthworms and of earthworms challenged with LBSS, live Gram-negative bacteria *E. coli (E.c.)*, live Gram-positive bacteria *B. subtilis (B.s.)* and β-1,3-glucan was analyzed by semi-quantitative RT-PCR. At different time intervals post-administration, levels of expression of CCF and fetidin genes were normalized to the expression of myosin and expressed as fold induction as compared to non-stimulated earthworms. Data are representative of two independent experiments.

that both cellular and humoral levels of CCF significantly increased within 24 h after parenteral injection of LPS, then dropped down to control values.

The coelomic fluid of non-stimulated earthworms contained a rather low level of CCF (15.0 ± 2.5 μg/ml, i.e. 0.1% of the coelomic fluid protein content). Upon sham-stimulation, CCF protein level increased approximately 3–5 times between 6 h and 3 days post-challenge. Injection of bacteria or β-1,3-glucan particles led to a further increase of CCF protein level within first 24 h post-injection. On day 6, the CCF concentration was comparable with that of non-injected controls. Yet, since CCF acts as agglutinin strongly binding pathogen-associated molecular patterns [2,23,24], the increase in CCF concentrations observed in coelomic fluids of *E. foetida* challenged with microbial particles may represent only the free molecules and thus may be underestimated.

The increase in CCF concentration in the coelomic fluid correlated increase at the transcriptional level. The mRNA level in coelomocytes from bacteria- and β-1,3-glucan-injected earthworms increased as early as 2 h post-challenge, reaching maximum after 17 h. In *B. subtilis*-infected worms, maximum mRNA level was lower or reached later than in *E. coli* or β-1,3-glucan-injected animals. This might reflect the requirement of lysozyme pre-digestion for recognition of peptidoglycan compounds by CCF [24]. mRNA synthesis reached maximum when humoral CCF level started to decrease, in particular upon *E. coli* and β-1,3-glucan stimulation. This could mean that early after challenge, CCF was released from intracellular stocks and de novo synthesis was required to refill the deposit in granules. In this regard, FACS analysis revealed the in vitro degranulation of coelomocytes incubated with bacteria or β-1,3-glucan as detected by a shift in Side Scatter (data not shown).

It can be concluded from the present study that lysozyme-like activity and synthesis of CCF are up-regulated upon in vivo microbial stimulation in *E. foetida* while fetidin expression is not modulated. CCF is released in the coelomic fluid within the first 24 h following microbial challenge, i.e. earlier than lysozyme-like activity which appears 1–3 days after challenge. This strengthens the idea that CCF as a pattern recognition molecule is an important initiator of the innate immune reaction in earthworms.

## Acknowledgements

This work supported by the Czech Science Foundation (310/02/1437), by the Grant Agency of the Academy of Sciences of the Czech Republic (A5020208), by Institutional Research Concept (AV0Z5020903), and by the Foundation for Scientific Research-Flanders (G.0169.01) was performed in frame of an Interuniversity Attraction Pole Program.

## References

[1] Janeway Jr. CA. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989;54(Pt 1):1–13.

[2] Beschin A, Bilej M, Hanssens F, Raymakers J, Van Dyck E, Revets H, Brys L, Gomez J, De Baetselier P, Timmermans M. Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. J Biol Chem 1998;273(38):24948–54.

[3] Bilej M, Brys L, Beschin A, Lucas R, Vercauteren E, Hanusova R, De Baetselier P. Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms. Immunol Lett 1995;45(1–2):123–8.

[4] Cotuk A, Dales RP. Lysozyme activity in the coelomic fluid and coelomocytes of the earthworm *Eisenia foetida* SAV in relation to bacterial infection. Comp Biochem Physiol 1984; 78A:469–74.

[5] Eue I, Kauschke E, Mohrig W, Cooper EL. Isolation and characterization of earthworm hemolysins and agglutinins. Dev Comp Immunol 1998;22(1):13–25.

[6] Lassegues M, Milochau A, Doignon F, Du Pasquier L, Valembois P. Sequence and expression of an *Eisenia fetida*-derived cDNA clone that encodes the 40-kDa fetidin antibacterial protein. Eur J Biochem 1997;246(3):756–62.

[7] Roch P. Protein analysis of earthworm coelomic fluid: (1) polymorphic system of the natural hemolysin of *Eisenia fetida andrei*. Dev Comp Immunol 1979;3(4):599–608.

[8] Roch P, Valembois P, Davant N, Lassegues M. Protein analysis of earthworm coelomic fluid—II. Isolation and biochemical characterization of the *Eisenia fetida andrei* factor (EFAF). Comp Biochem Physiol 1981;69B:829–36.

[9] Lange S, Nussler F, Kauschke E, Lutsch G, Cooper EL, Herrmann A. Interaction of earthworm hemolysin with lipid membranes requires sphingolipids. J Biol Chem 1997; 272(33):20884–92.

[10] Lange S, Kauschke E, Mohrig W, Cooper EL. Biochemical characteristics of Eiseniapore, a pore-forming protein in the coelomic fluid of earthworms. Eur J Biochem 1999;262(2): 547–56.

[11] Sekizawa Y, Kubo T, Kobayashi H, Nakajima T, Natori S. Molecular cloning of cDNA for lysenin, a novel protein in

the earthworm *Eisenia foetida* that causes contraction of rat vascular smooth muscle. Gene 1997;191(1):97–102.

[12] Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, Umeda M. Lysenin, a novel sphingomyelin-specific binding protein. J Biol Chem 1998;273(9):5300–6.

[13] Ito Y, Yoshikawa A, Hotani T, Fukuda S, Sugimura K, Imoto T. Amino acid sequences of lysozymes newly purified from invertebrates imply wide distribution of a novel class in the lysozyme family. Eur J Biochem 1999;259(1–2):456–61.

[14] Valembois P, Roch P, Lassegues M, Cassand P. Antibacterial activity of the haemolytic system from the earthworm *Eisenia fetida andrei*. J Invertebr Pathol 1982;40:21–7.

[15] Valembois P, Roch P, Lassegues M. Antibacterial molecules in annelids. In: Brehelin M, editor. Immunity in invertebrates. Berlin: Springer; 1986. p. 74–93.

[16] Roch P, Lassegues M, Valembois P. Antibacterial activity of *Eisenia fetida andrei* coelomic fluid: III. Relationship within the polymorphic hemolysins. Dev Comp Immunol 1991; 15(1–2):27–32.

[17] Du Pasquier L, Duprat P. Humoral and cellular aspects of a non-specific natural immunity in the oligochetee *Eisenia foetida Sav. (Lumbricinae)*. CR Acad Sci Hebd Seances Acad Sci D 1968;266(5):538–41.

[18] Roch P, Davant N, Lassegues M. Isolation of agglutinins and lysins from earthworm coelomic fluid by gel filtration followed by chromatofocussing. J Chromatogr 1984;290: 213–35.

[19] Milochau A, Lassegues M, Valembois P. Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei*. Biochim Biophys Acta 1997;1337(1):123–32.

[20] Sekizawa Y, Hagiwara K, Nakajima T, Kobayashi H. A novel protein, lysenin, that causes contraction of the isolated rat aorta: its purification from the coelomic fluid of the earthworm, *Eisenia foetida*. Biomed Res 1996;17:197–203.

[21] Bilej M, De Baetselier P, Beschin A. Antimicrobial defense of the earthworm. Folia Microbiol (Praha) 2000;45(4):283–300.

[22] Koenig S, Wagner F, Kauschke E, Peter-Katalinic J, Cooper EL, Eue I. Mass spectrometric analyses of CL(39), CL(41) and H(1), H(2), H(3) confirm identity with fetidin and lysenin produced by earthworm leukocytes. Dev Comp Immunol 2003;27(6–7):513–20.

[23] Beschin A, Bilej M, Brys L, Torreele E, Lucas R, Magez S, De Baetselier P. Convergent evolution of cytokines. Nature 1999; 400(6745):627–8.

[24] Bilej M, De Baetselier P, Van Dijck E, Stijlemans B, Colige A, Beschin A. Distinct carbohydrate recognition domains of an invertebrate defense molecule recognize Gram-negative and Gram-positive bacteria. J Biol Chem 2001;276(49):45840–7.

[25] Bilej M, Rossmann P, Sinkora M, Hanusova R, Beschin A, Raes G, De Baetselier P. Cellular expression of the cytolytic factor in earthworms *Eisenia foetida*. Immunol Lett 1998; 60(1):23–9.

[26] Stein E, Cooper EL. The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. Dev Comp Immunol 1981;5(3):415–25.

[27] Söderhäll K, Smith VJ. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. Dev Comp Immunol 1983; 7(2):229–39.

[28] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72: 248–54.

[29] Tuckova L, Rejnek J, Sima P. Response to parenteral stimulation in earthworms *L. terrestris* and *E. foetida*. Dev Comp Immunol 1988;12(2):287–96.

[30] Dales RP, Kalac Y. Phagocytic defence by the earthworm *Eisenia foetida* against certain pathogenic bacteria. Comp Biochem Physiol 1992;101A(3):487–90.

[31] Vetvicka V. Physiology of annelids. In: Vetvicka V, Sima P, Cooper EL, Bilej M, Roch P, editors. Immunology of annelids. Boca Raton, FL: CRC Press; 1994. p. 42–102.

[32] Wojdani A, Stein EA, Lemmi CA, Cooper EL. Agglutinins and proteins in the earthworm, *Lumbricus terrestris*, before and after injection of erythrocytes, carbohydrates, and other materials. Dev Comp Immunol 1982;6(4):613–24.

## 4.6. Characterization of prophenoloxidase activating cascade

The prophenoloxidase (proPO) activating cascade represents one of the most important defense mechanisms of invertebrates. It is activated by recognition of non-self molecules by pattern recognition molecules and results in the production of a key enzyme responsible for the catalysis of melanization reaction. Melanin as a final product of the cascade has fungistatic, bacteriostatic and antiviral properties and thus is involved in innate immune mechanisms (Söderhäll *et al.* 1994; Söderhäll and Cerenius 1998).

The presence of proPO activating cascade was indicated by Beschin *et al.* (Beschin *et al.* 1998) by the finding that incubation of the coelomic fluid with constituents of microorganisms, like saccharides or LPS, leads to the oxidation of L-DOPA (L-β-3,4-dihydroxyphenylalanine), a known substrate of PO. However, neither the effector compound, i.e. the PO enzyme, nor the genes coding for proPO/PO have been characterized or identified in any annelid species. Accordingly, the aim of our study was to evidence the presence of PO in *E. fetida* earthworms.

We found out that the coelomic fluid causes a spontaneous oxidation of L-DOPA in 8 hours. However, in the presence of an activator such as LPS or β-1,3-glucan, the L-DOPA oxidation starts much earlier – after 2 hours – reaching a maximum level between 6 and 10 hours of incubation. As it was documented that PO activity in arthropods is detectable within minutes (Cerenius and Söderhäll 2004), the level of PO in earthworms seems to be very low.

To determine the substrate specificity of PO and to test non-specific activation of proPO, we tested various substrates (L-DOPA, dopamine, N-acyldopamine, 4-methylcatechol, tyrosine) and various activators (sodium dodecyl sulphate (SDS), cetylpiridium chloride (CPC), 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). The highest activation of the proPO was obtained by combining L-DOPA as a substrate and CPC as an activator.

To evaluate the PO activity observed, we separated coelomic fluid on native SDS-free PAGE and incubated it with L-DOPA and CPS. In these conditions only one band showing oxidase activity appeared. This oxidizing activity was completely blocked when the coelomic fluid was boiled before the separation or when it was preincubated with irreversible proteinase inhibitor Pefabloc. This suggests an enzymatic nature of the compound exhibiting PO activity.

Bands showing oxidation were eluted, separated in SDS-PAGE and stained. The eluted material revealed a high molecular weight band, which is probably formed by precipitated substances, a strong band of approximately 90 kDa and several bands of molecular weight lower than 40 kDa. Furthermore, the 90-kDa protein band was subjected to amino acid sequence analysis. However, Edman degradation revealed the sequence of 8 N-terminal amino acids only. This peptide was found to be homologous with the endogenous inhibitor of PO from the housefly *Musca domestica*. Sequencing of internal peptides yielded four peptide sequences. Two of them share partial homology with the phenoloxidase and hemocyanin of different invertebrate species.

In summary, these data suggest that phenoloxidase and its inhibitors exist in *E. fetida* earthworms but the level of PO activity is lower as compared to other invertebrates and, moreover, PO activation is slower.

**Procházková P., Šilerová M., Stijlemans B., Dieu M., Halada P., Josková R., Beschin A., De Baetselier P., Bilej M.: Evidence for proteins involved in prophenoloxidase cascade in *Eisenia fetida* earthworms. *J. Comp. Physiol. [B]*. 176: 581-587 (2006).**

Procházková P., Šilerová M., Stijlemans B., Dieu M., Halada P., Josková R., Beschin A., De Baetselier P., Bilej M.: Evidence for proteins involved in prophenoloxidase cascade in *Eisenia fetida* earthworms. *J. Comp. Physiol. [B]*. 176: 581-587 (2006).

J Comp Physiol B (2006) 176: 581–587
DOI 10.1007/s00360-006-0081-z

ORIGINAL PAPER

**Petra Procházková · Marcela Šilerová**
**Benoit Stijlemans · Marc Dieu · Petr Halada**
**Radka Josková · Alain Beschin · Patrick De Baetselier**
**Martin Bilej**

# Evidence for proteins involved in prophenoloxidase cascade *Eisenia fetida* earthworms

Received: 17 January 2006 / Revised: 16 March 2006 / Accepted: 23 March 2006 / Published online: 25 April 2006


**Abstract** The prophenoloxidase cascade represents one of the most important defense mechanisms in many invertebrates. Following the recognition of microbial saccharides by pattern recognition molecules, proteinases cleave inactive prophenoloxidase to its active form, phenoloxidase. Phenoloxidase is a key enzyme responsible for the catalysis of the melanization reaction. Final product melanin is involved in wound healing and immune responses. Prophenoloxidase cascade has been widely described in arthropods; data in other invertebrate groups are less frequent. Here we show detectable phenoloxidase activity in 90-kDa fraction of the coelomic fluid of earthworms *Eisenia fetida*. Amino acid sequencing of peptides from the active fraction revealed a partial homology with invertebrate phenoloxidases and hemocyanins. Moreover, the level of phenoloxidase activity is lower and the activation slower as compared to other invertebrates.

Communicated by G. Heldmaier

P. Procházková (✉) · M. Šilerová · R. Josková · M. Bilej
Department of Immunology, Institute of Microbiology,
Academy of Sciences of the Czech Republic, Videnska 1083,
142 20 Prague 4, Czech Republic
E-mail: kohler@biomed.cas.cz
Tel.: +420-2-41062367
Fax: +420-2-41721143

B. Stijlemans · A. Beschin · P. De Baetselier
Department of Cellular and Molecular Interactions,
Vlaams Interuniversitair Instituut voor Biotechnologie,
Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

P. Halada
Department of Biogenesis and Biotechnology of Natural Compounds, Institute of Microbiology,
Academy of Sciences of the Czech Republic,
Videnska 1083, 142 20 Prague 4, Czech Republic

M. Dieu
Facultés Universitaires Notre-Dame de la Paix,
Unité de Recherche en Biologie Cellulaire,
Spectrométrie de Masse,
rue de Bruxelles 61, 5000 Namur, Belgium

**Keywords** Innate immunity · Phenoloxidase · *Eisenia* · Earthworm · L-DOPA

**Abbreviations** CF: Coelomic fluid · LBSS: *Lumbricus* balanced salt solution · PBS: Phosphate buffered solution · proPO: Prophenoloxidase · PO: Phenoloxidase · L-DOPA: L-β-3,4-Dihydroxyphenyl alanine · ppA: Prophenoloxidase-activating enzyme · CPC: Cetylpyridium chloride · Hc: Hemocyanin

## Introduction

The prophenoloxidase-activating system is a sensitive non-self-recognizing cascade triggered by components of microbial cell walls such as lipopolysaccharides, peptidoglycans and β-1,3-glucan (Söderhäll and Cerenius 1998; Cerenius and Söderhäll 2004). One component of the system is phenoloxidase (PO; EC 1.14.18.1, EC 1.10.3.1.), an enzyme that is usually present in an inactive form, prophenoloxidase (proPO), in cells or body fluid of different invertebrate species. Current evidence suggests that proPO is stored in the granules of certain blood cells, from which it is released into body fluids and eventually activated.

Conversion of proPO to its active state is achieved by proteolytic cleavage that depends on a cascade of serine proteinases, the so-called prophenoloxidase-activating enzymes (ppA) and other factors. The resulting PO catalyzes both the *o*-hydroxylation of monophenols and the oxidation of diphenols to quinones. Then the quinones non-enzymatically polymerize into melanin (Ashida and Yamazaki 1990; Söderhäll et al. 1994). Melanin, as a final product of proPO cascade, has fungistatic, bacteriostatic and antiviral properties and together with its intermediates is involved in the innate immune response of certain invertebrates, especially arthropods. Melanin also serves as a structural component in wound healing and is important for encapsulation of foreign materials,

Besides the natural activators of proPO in vitro, which are components of cell walls of bacteria like LPS, peptidoglycans or glucans, proPOs can be activated also with several detergents, salts or lipids (Ashida and Yamazaki 1990; Sugumaran and Kanost 1993).

ProPO and PO were characterized in numerous arthropod groups, namely crustaceans and insects. Data on PO activity in other invertebrates are less frequent suggesting that proPO-activating cascade is not their most important defense mechanism. Indeed, PO activity was recorded in mollusks [for review see (Smith and Soderhall 1991; Cerenius and Söderhäll 2004)] and more recently in earthworms (Field et al. 2004).

Molecular weight of proPO and PO differs among species, and varies usually between 70 and 90 kDa. The primary structure of known proPOs reveals that they contain two functional copper-binding sites. Actually, the sequence of proPO in different species within and adjacent to the copper-binding sites shows 60–70% of similarity. Another conserved peptide motif, GCGEQNM (Dodds and Law 1998; Armstrong and Qiuigley 1999), which is present in α2-macroglobulins both in vertebrates and invertebrates, can be found in the sequence of proPOs as well. This motif is present also in the vertebrate thiolester-containing complement proteins (C3, C4) and in the complement-related proteins of some invertebrates (Marino et al. 2002; Levashina et al. 2003; Dishaw et al. 2005; Zhu et al. 2005).

The hemolymph of several arthropods and mollusks contains another copper-binding protein hemocyanin (Hc), having a molecular weight of approximately 90 kDa. Its physiological function is to transport oxygen, which is facilitated by its capability to reversibly bind dioxygen to a dinuclear copper site (Solomon et al. 1992). Thus, Hc and POs are equipped with structurally similar oxygen-binding centers. The potential copper-binding sites of proPO are highly homologous to the corresponding sites of Hc in arthropods like the tarantula *Eurypelma californicum*, the horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus*, the crab *Cancer magister* and the crayfish *Pacifastacus leniusculus* (Decker and Rimke 1998; Decker and Terwilliger 2000; Nagai and Kawabata 2000; Decker et al. 2001; Lee et al. 2004). Interestingly, the latter Hc have been reported to exert PO activity after proteolytic cleavage. In addition, PO activities have been documented for molluscan Hc (Zlateva et al. 1996; Salvato et al. 1998).

Phenoloxidase catalyzes early steps in the pathway to melanin formation. In annelids, melanization reactions proceed like cellular defense reactions of the host through the formation of brown bodies around encapsulation invading pathogens (Dales 1983). The origin and function of the brown pigment in nodules were initially described in *Nereis diversicolor* and it was suggested that the brown color is due to melanin (Porchet-Henneré and Vernet 1992). Formation of brown bodies containing bacteria, parasites or altered self-structures and the oxidizing activity of the coelomic fluid (CF) have been described in *Eisenia fetida* as well (Valembois et al. 1991, 1992). Yet, PO activity was revealed in *E. fetida* by incubating its CF with constituents of microorganisms, like saccharides or LPS, and measuring the oxidation of L-DOPA, a known substrate of PO. Although these data indicate the presence of proPO-activating cascade in *E. fetida* (Beschin et al. 1998), neither the effector compound involved in the PO defense pathway, i.e., the PO enzyme, nor the gene coding for proPO/PO has been characterized or identified in any annelid species. Evidence provided here suggests the presence of PO in *E. fetida* earthworms.

## Material and methods

### Earthworms and harvesting of the CF and coelomocytes

Adult *E. fetida* (*Oligochaeta; Annelida*) were kept in compost and 3 days prior to experiments were transferred onto filter paper soaked with isotonic *Lumbricus*-balanced salt solution (LBSS) (Stein and Cooper 1981). CF containing coelomocytes was obtained by puncturing postclitellum segments of the earthworm coelomic cavity with a Pasteur micropipette and kept on ice. Samples were centrifuged (500*g*, 10 min, 4°C). The supernatant containing the cell-free CF was centrifuged again (7,000*g*, 10 min, 4°C) and the supernatant stored at −20°C.

### Activation of proPO cascade

The level of proPO activation was assessed as described previously (Beschin et al. 1998; Bilej et al. 2001). Briefly, 10 µl of the CF [with or without 1 mM serine proteinase inhibitor Pefabloc (Boehringer Mannheim), 90 µl of 100 mM Tris, pH 8, containing 50 mM $Ca^{2+}$ and 10 µl L-β-3,4-dihydroxyphenylalanine (L-DOPA; Fluka); final concentration 1.5 mM] was incubated at room temperature up to 12 h in the absence or presence of LPS (*E. coli* 055:B5 S strain, Sigma) or β-1,3-glucan (laminarin, Sigma), at final concentration of 1 µg/ml. The oxidation of L-DOPA to dopachrome was measured every 2 h at 475 nm and evaluated as the difference between the $A_{475}$ values with or without Pefabloc.

To determine the substrate specificity of PO and to test non-specific activation of proPO, different activators of PO and substrates were tested. CF (10 µl) was diluted in 50 mM sodium phosphate buffer (pH 6.5) in a total volume of 100 µl containing one of the substrates for PO (final concentration 1 mM): L-DOPA (Fluka), dopamine (Sigma), acyldopamine (Sigma), 4-methylcatechol (Sigma), tyrosine (Fluka). Then, one of the non-specific activators of proPO (final concentration 0.02% SDS; cetylpyridium chloride (CPC); CHAPS, all from Sigma) was added to the reaction mixture. The reaction was allowed to proceed at room temperature for 6 h, which is the time period when a maximum L-DOPA oxidation occurs using LPS and β-1-3-glucan to activate the

proPO cascade, and then the $A_{475}$ was measured. Absorbance was calculated as the difference between the values of samples with and without the CF. Control samples were prepared without activator of the proPO or without the substrate for PO.

### Statistical analysis

Three independent experiments were performed with different CF samples. In each experiment, all parameters were measured in duplicate. Data were expressed as mean ± SD of the values obtained in all three experiments. Paired Student's *t* test using GraphPad Prism software was performed to evaluate the significance of the data. Differences were considered significant when $P < 0.05$.

### Electrophoresis

Native and SDS-PAGE was performed on a 10% acrylamide native gel (Laemmli 1970) at 4°C using common buffer system without and with SDS. For native gels, the samples were not denatured before electrophoresis. The CF (200 µl/gel) was applied to the gel and allowed to migrate for 3 h.

### Detection of PO activity in native PAGE and electroelution

Gel was incubated in 50 ml of 50 mM sodium phosphate buffer, pH 6.5 containing 20 mM L-DOPA directly after native PAGE separation. Then, CPC was added (0.05%) to activate the proPO. Bands visible after incubation for 12 h at room temperature were cut from the gel and the material was electroeluted overnight at room temperature in 250 mM Tris, 200 mM glycin buffer, pH 8.5 (Schleicher&Schuell, BIOTRAP electro-separation system). Electroeluted material was concentrated on Vivaspin (Vivascience) concentrator with a cut-off at 15 kDa and subjected to gel filtration chromatography, SDS-PAGE and Edman degradation.

### Protein purification by chromatography

The concentrated electroeluted sample (100 µl) was applied to a preequilibrated (150 mM Tris–HCl, pH 7.5) Superdex S75 column (Pharmacia), run at 100 µl/min and eluted in 25 mM Tris, 20 mM glycin, 0.01% SDS, 0.1 M NaCl, 10 mM β-mercaptoethanol, pH 7.5. Besides the $A_{280}$ (corresponding to protein content), the $A_{475}$ (reflecting the oxidation of L-DOPA to dopachrome) of eluted fractions was directly measured during elution. Collected fractions exhibiting PO activity were pooled, concentrated on Vivaspin (Vivascience) concentrator with a cut-off 15 kDa and subjected to SDS-PAGE and Edman degradation.

### Edman degradation

N-terminal and internal amino acid sequence analysis was performed using an automated protein sequencer LF 3600D (Beckman Instruments, Inc., Fullerton, CA, USA) according to the manufacturer's manual.

## Results and discussion

### Activation of proPO cascade in the CF of E. fetida earthworms

The activation of proPO system in invertebrates can be achieved in vitro using L-DOPA as a substrate for PO and microbial cell wall components like LPS or β-1,3-glucan as activators (Cerenius and Söderhäll 2004). When they were added in vitro to the CF of *E. fetida* earthworms, L-DOPA was oxidized, therefore suggesting the occurrence of PO activity in this species. The level of spontaneous L-DOPA oxidation was marginal, occurring after 8 h of incubation. However, in the presence of an activator, such as LPS or β-1,3-glucan, the L-DOPA oxidation started after 2 h and reached a maximum level between 6 and 10 h of incubation (Fig. 1). This observation confirms our previous results (Bilej et al. 2001) and indicates the presence of proPO system in annelids. Considering that in arthropods, PO activity is detected within minutes after the incubation of body fluid with L-DOPA and microbial cell wall constituents (Cerenius and Söderhäll 2004), our data suggest that level of PO or its activity is lower in earthworms than in arthropods.

To further characterize the proPO cascade in *E. fetida* CF, different substrates (L-DOPA, dopamine, N-acyldopamine, 4-methylcatechol, tyrosine) were used. Moreover, different non-specific activators of proPO like detergents (SDS, CPC, CHAPS) were envisaged (Ashida

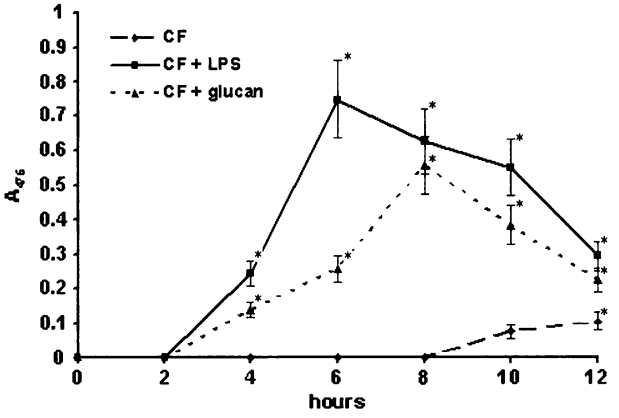



**Fig. 1** Activation of proPO cascade in the coelomic fluid (*CF*) of *E. fetida* earthworms by β-1,3-glucan (laminarin) and *LPS*. *CF* levels of L-DOPA oxidation are expressed as the mean of $A_{475}$ value differences of the sample with and without proteinase inhibitor ± SD calculated from three independent experiments (*significant at $P < 0.05$)

and Yamazaki 1990; Sugumaran and Kanost 1993). As shown in Table 1, the highest activation of the proPO was obtained by combining CPC as an activator and L-DOPA as a substrate. L-DOPA produced the highest values of PO activity in combination with the three activators. Tyrosine used as a substrate and the CF without any substrate/activator of PO/proPO did not reveal significant PO activity. It should be mentioned that the name PO encompasses two similar enzymes, which only differ in their enzymatic properties: tyrosinase (EC 1.14.18.1) and catecholoxidase (EC 1.10.3.1). Tyrosinase catalyzes the hydroxylation of monophenols (i.e., tyrosine, considered as the natural substrate of the enzyme) and the oxidation of diphenols to quinones, whereas catecholoxidase catalyzes only the oxidation of diphenols. The latter enzyme is almost indistinguishable from the different kinds of tyrosinases by sequence and properties other than the enzymatic activity (Sanchez-Ferrer et al. 1995; Decker and Jaenicke 2004). This may be the reason why the term PO is often used for tyrosinases and catecholoxidases of invertebrates in the literature without discrimination between them. A number of POs are reported for arthropods, but only a few have been demonstrated as having tyrosinase activity i.e., when using the monophenol tyrosine as substrate (Fujimoto et al. 1993; Aspan et al. 1995; Chase et al. 2000; Jaenicke and Decker 2003). Since we did not detect PO activity in CF after incubation with any of the activators and tyrosine as a substrate together, we suggest the presence of catecholoxidase rather than tyrosinase in *E. fetida*.

## Identification of proteins having PO activity

To evaluate the observed PO activity, CF content was separated in native SDS-free PAGE and incubated with L-DOPA as a substrate and CPC as an activator. In these conditions only one band showing oxidase activity appeared. Such oxidizing activity was completely abolished when CF was preincubated with irreversible proteinase inhibitor Pefabloc or boiled before separation in native SDS-free PAGE and adding of L-DOPA and CPC, which suggests an enzymatic nature of earthworm material exhibiting PO activity (Fig. 2a). No spontaneous oxidation was visible after incubating gels in the absence of the L-DOPA substrate and/or the CPC activator (data not shown). This could reflect that the *E. fetida* CF contains a minimal amount of PO.

Bands showing detectable oxidation were cut from the native SDS-free PAGE, and electroeluted for further analyses. (1) First, when separated in SDS-PAGE and stained with Coomassie blue, the electroeluted material revealed a high molecular weight band, a strong band of approximately 90 kDa, and a few weak bands of molecular weight lower than 40 kDa (Fig. 2c, lane a). The high molecular weight band is assumed to be formed by precipitated material that did not enter the gel. (2) Second, the electroeluted material was concentrated and separated by gel filtration. The $A_{475}$ detecting the oxidation of L-DOPA to dopachrome (Harisha 2005) and $A_{280}$ as an estimation of protein content were recorded (Fig. 2b) by UV spectrophotometer during elution. Between the two main protein peaks ($A_{280}$) showing PO activity ($A_{475}$) only one (fraction 3–4) contained enough material to be further analyzed in SDS-PAGE, revealing in Coomassie blue a protein band of approximately 90 kDa (Fig. 2c, lane b). (3) Third, the 90 kDa protein band was further subjected to amino acid sequence analysis. Edman degradation was performed three times and revealed only an N-terminal peptide sequence of eight amino acids, due to repeated blocking of N-terminus. We proceeded to sequence internal peptides, hereby obtaining four peptide sequences. Sequences of the identified earthworm peptides as well as those of homologous peptides are shown in Table 2. N-terminal amino acid sequence (peptide no. 1) showed homology with the endogenous inhibitor of PO from housefly *Musca domestica*. In the latter animal, this competitive inhibitor of the PO was found to be a DOPA-containing peptide with a molecular weight of only 4.2 kDa (Daquinag et al. 1995, 1999). It is known that the proPO system also involves inhibitors of proPO activation and PO activity. They can prevent undesired activation of proPO, or prevent over-activation of ppA (Aspan et al. 1990; Liang et al. 1997; De Gregorio et al. 2002). The endogenous inhibitor of PO from *M. domestica* was described as a DOPA-containing peptide, wherein DOPA is a modified tyrosine residue. The

**Table 1** Substrate specificity of *Eisenia fetida* proPO

| Activators | Substrates | | | | | |
|---|---|---|---|---|---|---|
| | L-DOPA | Dopamine | N-acyldopamine | 4-Methylcatechol | Tyrosine | None |
| SDS | 0.159 ± 0.008* | 0.117 ± 0.01* | 0.097 ± 0.005* | 0.125 ± 0.007* | 0.080 ± 0.001 | 0.050 ± 0.001 |
| CPC | 0.237 ± 0.012* | 0.162 ± 0.013* | 0.137 ± 0. 005* | 0.193 ± 0.016* | 0.080 ± 0.001 | 0.050 ± 0.001 |
| CHAPS | 0.122 ± 0.009* | 0.106 ± 0.005* | 0.103 ± 0.008* | 0.117 ± 0.01* | 0.080 ± 0.001 | 0.050 ± 0.001 |
| None | 0.020 ± 0.001 | 0.020 ± 0.001 | 0.020 ± 0.001 | 0.020 ± 0.001 | 0.020 ± 0.001 | 0.020 ± 0.001 |

Detergents that can non-specifically activate proPO and different substrates for PO were tested for their ability to trigger the proPO cascade in the coelomic fluid. Activation of proPO after 6 h of incubation is expressed as the mean of $A_{475}$ value difference of the sample with and without coelomic fluid ± SD calculated from three independent experiments. In control samples, coelomic fluid was incubated without activator of proPO or without the substrate for PO

*Significant at $P < 0.05$

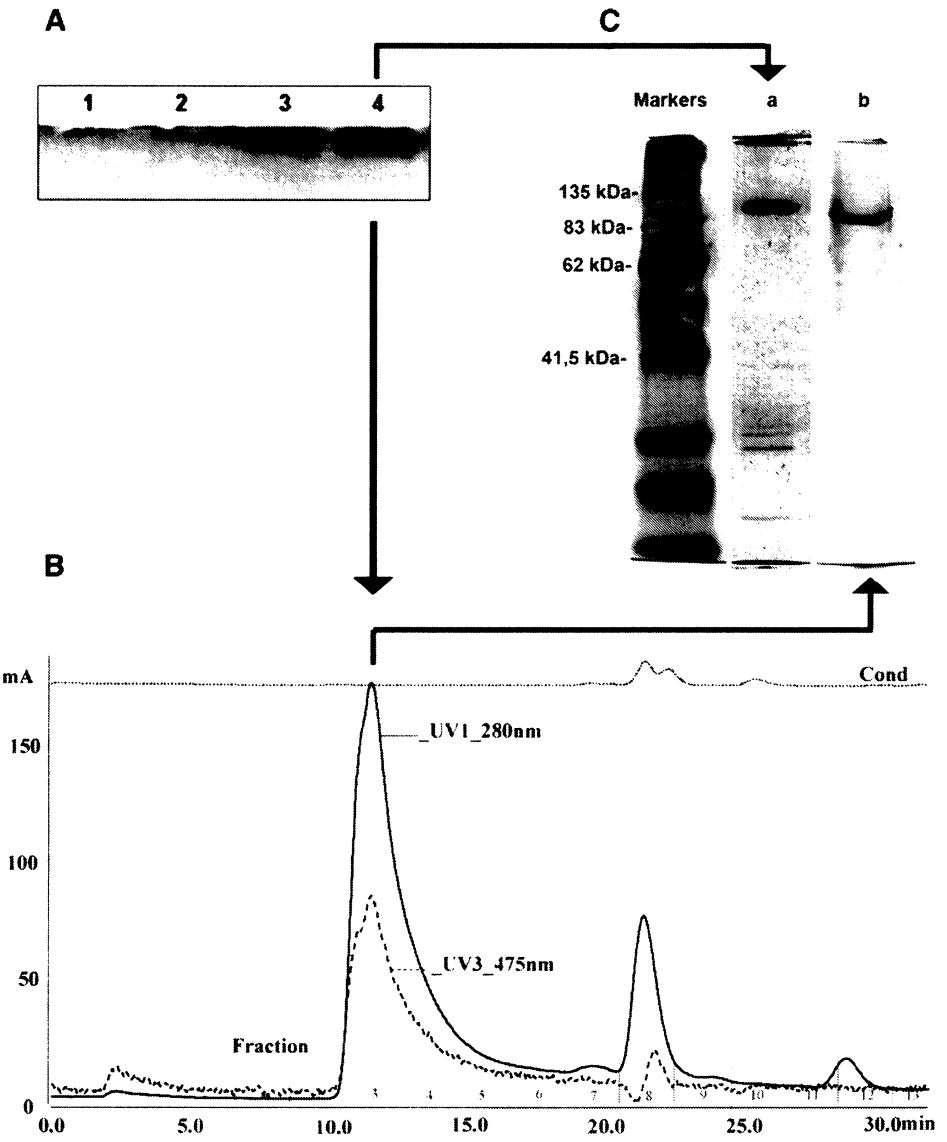



**Fig. 2** **a** Native PAGE of *E. fetida CF*. *CF* run on acrylamid gel was incubated in a mixture of sodium phosphate buffer, L-DOPA and CPC for 12 h at room temperature to detect L-DOPA oxidation. *Lane 1* 20 µl of the *CF* (approx. 200 µg of proteins) boiled before separation. *Lane 2* 20 µl of the *CF* preincubated with proteinase inhibitor Pefabloc. *Lane 3* 2 µl of the *CF* (approx. 20 µg of proteins). *Lane 4* 20 µl of the *CF*. **b** Purification of proteins showing oxidase activity on a Superdex S75 column. Material present in bands detected after native PAGE and incubation with L-DOPA and CPC was electroeluted and separated by gel filtration. The absorbance at 280 nm for estimation of protein content and at 475 nm corresponding to the oxidation of L-DOPA to dopachrome was monitored. The fractions with highest PO activity (fractions 3–4) eluted after gel chromatography were collected. **c** Material exhibiting PO activity obtained in native SDS-free PAGE (*lane a*) and the fractions with highest PO activity (fractions 3–4) eluted after gel chromatography (*lane b*) were electroeluted and analyzed on SDS-PAGE. Proteins were stained with Coomassie blue

presence of such modified tyrosine residue in PO inhibitor that could form a complex with PO in earthworms might explain the correlation of activity and absorbance of 475 nm of fractions obtained during gel filtration of our proteins (Fig. 2b). From observed data we suggest the existence of an inhibitor of PO in earthworms, which might form a complex with PO.

Peptide sequences no. 2 and 3 (15 and 17 amino acid long, respectively), shared partial homologies with the sequences of PO and/or Hc of different invertebrate species. The two remaining earthworm peptide sequences no. 4 and 5 (17 and 8 amino acid long, respectively) did not display homology with known proteins.

Efforts to identify whole proPO/PO sequence including (1) PCR with degenerated primers designed and based on conserved regions in published POs and with degenerated primers designed in accordance with identified peptide sequences, and (2) the screening of *E. fetida* cDNA library with a probe containing a partial sequence of the gene coding for PO in crayfish *P. leniusculus* were so far unsuccessful. One of the plausible reasons for our failure may be the very low level of mRNA for PO in earthworms.

Together, these data indicate that peptides having a partial homology with PO and/or Hc originated from the active fraction from *E. fetida* CF proteins exhibiting PO-oxidizing properties. Therefore, we suggest that PO and related inhibitor exist in earthworms (*E. fetida*), but the level of PO activity is lower than in other invertebrates. Moreover, it is clear that proPO activation is slower as compared to other invertebrate species. We can presume that in contrary to arthropods proPO cascade does not represent the main immunodefense system in earthworms and that earthworms rely on other innate defense mechanisms (Cooper and Roch 2004; Cooper et al. 2006).

**Table 2** Amino acid sequence of peptides derived from a 90 kDa *E. fetida* coelomic fluid protein with oxidase activity obtained by N-terminal and internal sequencing

| | Amino acid sequence | GenBank accession no. |
|---|---|---|
| Peptide no. 1 | **VPQNPANG** | |
| PO inhibitor of *Musca domestica* | **VPQCLANG** | **P81765** |
| Peptide no. 2 | **ALLF-AVSVTTGSPSK** | |
| PO of *Pimpla hydrochondriaca* | **ALLTYAVSVTAMHRDD** | **CAC04150** |
| Hc of *Litopenaeus vannamei* | **DLEFSGVSVTELAVVG** | **CAA57880** |
| Hc of *Litopenaeus vannamei* (var.) | **ELTFAGVSVDSVAIEG** | **CAB85965** |
| Hc sub. 3 of *Palinurus vulgaris* | **DLEFAGVSVDNIAIDG** | **CAC69245** |
| pseudo-Hc of Homarus americanus | **DLLFPGVAVNMIDIDG** | **CAB38043** |
| Hc type 1 of *Haliotis tuberculata* | **ALLNRGSGVAVPYWEW** | **CAB76379** |
| Hc E chain of *Aphonopelma sp.* | **GLFVYAVSVALLHRDD** | **P02242** |
| Hc of *Peneus monodon* | **ELTFAGVSVDKVAIEG** | **ALL27460** |
| Peptide no. 3 | **LGTVEVAD- - - - -QLLAGSSLR** | |
| PO I of *Pimpla hydrochondriaca* | **LGNMIEAS- - -P- -VLSPNFGYY** | **CAC04150** |
| Hc sub. A of *Scutigera coleoptrata* | **LGTVIRAD- - - - -QCPPVPADT** | **CAC69246** |
| proPO sub. 1 of *Anopheles gambiae* | **LGDVVEASSLTPNAQLY-GSLHN** | **AAB94671** |
| proPO of *Pacifastacus leniusculus* | **LGDAFEAD- - - - -AQLSPNYLFY** | **CAA58471** |
| Peptide no. 4 | PPPAELAGGEGHYYLQV | |
| Peptide no. 5 | LRFGFAST | |

Peptides exhibiting sequence homology found by a search in NCBI Blast database are listed below the obtained earthworm peptides (no. 1–5)
Amino acids identical within the different sequences are in bold

**Acknowledgements** This work was supported by the Czech Science Foundation (310/04/0806; B500200613), Institutional Research Concept (AVOZ50200510) and a bilateral international scientific and technological cooperation grant of the Ministry of the Flemish Community (BOF-BWS 03/06) and was performed within the frames of an Interuniversity Attraction Pole Program. Authors are grateful to Prof. Söderhäll, Uppsala, for providing probe containing the gene coding for PO in crayfish *P. leniusculus*. All experiments comply with the current laws of the Czech Republic and Belgium.

## References

Armstrong P, Qiuigley J (1999) Alpha2-macroglobulin: an evolutionarily conserved arm of the innate immune system. Dev Comp Immunol 23:375–390

Ashida M, Yamazaki IH (1990) Biochemistry of the phenol oxidase system in insects: with special reference to its activation. In: Ohnishi E, Ishizaki H (eds) Molting and metamorphosis. Japan Scientific Society Press, Tokyo, pp 239–265

Aspan A, Hall M, Söderhäll K (1990) The effect of endogenous proteinase inhibitors on the prophenoloxidase activating enzyme, a serine proteinase form crayfish haemocytes. Insect Biochem 20:485–492

Aspan A, Huang TS, Cerenius L, Söderhäll K (1995) cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proc Natl Acad Sci USA 92:939–943

Beschin A, Bilej M, Hanssens F, Raymakers J, Van Dyck E, Revets H, Brys L, Gomez J, De Baetselier P, Timmermans M (1998) Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida* earthworm involved in the activation of prophenoloxidase cascade. J Biol Chem 273:24948–24954

Bilej M, De Baetselier P, Van Dijck E, Stijlemans B, Colige A, Beschin A (2001) Distinct carbohydrate recognition domains of an invertebrate defense molecule recognize Gram-negative and Gram-positive bacteria. J Biol Chem 276:45840–45847

Cerenius L, Söderhäll K (2004) The prophenoloxidase-activating system in invertebrates. Immunol Rev 198:116–126

Chase MR, Raina K, Bruno J, Sugumaran M (2000) Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. Insect Biochem Mol Biol 30:953–967

Cooper EL, Roch P (2004) Earthworm immunity: a model of immune competence. Pedobiologia 47:676–688

Cooper EL, Kvell K, Engelmann P, Nemeth P (2006) Still waiting for the toll? Immunol Lett 104:18–28

Dales RP (1983) Observations on granulomata in the polychaetus annelid *Nereis diversicolor*. J Invertebr Pathol 42:288–291

Daquinag AC, Nakamura S, Takao T, Shimonishi Y, Tsukamoto T (1995) Primary structure of a potent endogenous dopa-containing inhibitor of phenol oxidase from *Musca domestica*. Proc Natl Acad Sci USA 92:2964–2968

Daquinag AC, Sato T, Koda H, Takao T, Fukuda M, Shimonishi Y, Tsukamoto T (1999) A novel endogenous inhibitor of phenoloxidase from *Musca domestica* has a cystine motif commonly found in snail and spider toxins. Biochemistry 38:2179–2188

De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata S, Lee BL, Iwanaga S, Lemaitre B, Brey PT (2002) An immune-responsive Serpin regulates the melanization cascade in Drosophila. Dev Cell 3:581–592

Decker H, Jaenicke E (2004) Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. Dev Comp Immunol 28:673–687

Decker H, Rimke T (1998) *Tarantula* hemocyanin shows phenoloxidase activity. J Biol Chem 273:25889–25892

Decker H, Terwilliger N (2000) Cops and robbers: putative evolution of copper oxygen-binding proteins. J Exp Biol 203(12):1777–1782

Decker H, Ryan M, Jaenicke E, Terwilliger N (2001) SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelma californicum*, and *Cancer magister*. J Biol Chem 276:17796–17799

Dishaw LJ, Smith SL, Bigger CH (2005) Characterization of a C3-like cDNA in a coral: phylogenetic implications. Immunogenetics 57:535–548

Dodds AW, Law SKA (1998) The phylogeny and evolution of the thioester bond-containing proteins C3, C4, and alpha2-macroglobulin. Immunol Rev 166:15–26

Field SG, Kurtz J, Cooper EL, Michiels NK (2004) Evaluation of an innate immune reaction to parasites in earthworms. J Invertebr Pathol 86:45–49

Fujimoto K, Masuda K, Asada N, Ohnishi E (1993) Purification and characterization of prophenoloxidases from pupae of Drosophila melanogaster. J Biochem (Tokyo) 113:285–291

Harisha S (2005) Enzymology. In: Harisha S (ed) An introduction to practical biotechnology. Laxmi Publications Ltd, New Delhi, pp 70–72

Jaenicke E, Decker H (2003) Tyrosinases from crustaceans form hexamers. Biochem J 371:515–523

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–665

Lee SY, Lee BL, Söderhäll K (2004) Processing of crayfish hemocyanin subunits into phenoloxidase. Biochem Biophys Res Commun 322:490–496

Levashina EA, Blandin S, Moita LF, Lagueux M, Kafatos FC (2003) Thioester-containing proteins of protostomes. In: Ezekowitz RAB, Hoffmann JA (eds) Innate immunity. Humana Press Inc, Totowa, pp 55–174

Liang Z, Sottrup-Jensen L, Aspan A, Hall M, Söderhäll K (1997) Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. Proc Natl Acad Sci USA 94:6682–6687

Marino R, Kimura Y, De Santis R, Lambris JD, Pinto MR (2002) Complement in urochordates: cloning and characterization of two C3-like genes in the ascidian *Ciona intestinalis*. Immunogenetics 53:1055–1064

Nagai T, Kawabata S (2000) A link between blood coagulation and prophenol oxidase activation in arthropod host defense. J Biol Chem 275:29264–29267

Porchet-Henneré E, Vernet G (1992) Cellular immunity in an annelid (*Nereis diversicolor*, Polychaeta): production of melanin by a sub-population of granulocytes. Cell Tissue Res 269:167–174

Salvato B, Santamaria M, Beltramini M, Alzuet G, Casella L (1998) The enzymatic properties of *Octopus vulgaris* hemocyanin: o-diphenol oxidase activity. Biochemistry 37:14065–14077

Sanchez-Ferrer A, Rodriguez-Lopez JN, Garcia-Canovas F,Garcia-Carmona F (1995) Tyrosinase: a comprehensive review of its mechanism. Biochim Biophys Acta 1247:1–11

Smith VJ, Soderhall K (1991) A comparison of phenoloxidase activity in the blood of marine invertebrates. Dev Comp Immunol 15:251–261

Solomon EI, Baldwin MJ, Lowery MD (1992) Electronic structures of active sites in copper proteins: contributions to reactivity. Chem Rev 92:521–542

Stein E, Cooper EL (1981) The role of opsonins in phagocytosis by coelomocytes of the earthworm, *Lumbricus terrestris*. Dev Comp Immunol 5:415–425

Sugumaran M, Kanost MR (1993) Regulation of insect hemolymph phenoloxidases. In: Beckage E, Thompson SN, Federick BA (eds) Parasites and pathogens of insects. Academic, San Diego, pp 317–342

Söderhäll K, Cerenius L (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 10:23–28

Söderhäll K, Cerenius L, Johansson MW (1994) The prophenoloxidase activating system and its role in invertebrate defence. Ann N Y Acad Sci 712:155–161

Valembois P, Seymour J, Roch P (1991) Evidence and cellular localization of an oxidative activity in the coelomic fluid of the earthworm *Eisenia fetida andrei*. J Invert Pathol 57:177–183

Valembois P, Lassegues M, Roch P (1992) Formation of brown bodies in the coelomic cavity of the earthworm *Eisenia fetida andrei* and attendant changes in shape and adhesive capacity of constitutive cells. Dev Comp Immunol 16:95–101

Zhu Y, Thangamani S, Ho B, Ding JL (2005) The ancient origin of the complement system. Embo J 24:382–394

Zlateva T, Di Muro P, Salvato B, Beltramini M (1996) The o-diphenol oxidase activity of arthropod hemocyanin. FEBS Lett 384:251–254