CHARLES UNIVERSITY IN PRAGUE

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Molecular characterization of selected defense factors in *Lumbricidae*

Molekulárna charakterizácia vybraných obranných faktorov v čeľadi *Lumbricidae*

DIPLOMA THESIS

Prague 2011 **Veronika Mančíková**

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Molecular characterization of selected defense factors in *Lumbricidae*

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I hereby declare that I have written this diploma thesis by myself, using only cited sources. I agree with lending and distribution of the thesis.

Prague, april 2011 Veronika Mančíková_____

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Abstract

Earthworms belonging to oligochaete annelids have been a model for comparative immunology for over 40 years. They possess various defense mechanisms efficiently recognizing and responding to non-self substances. Among these there are molecules with many biological activities including cytolytic, antimicrobial and proteolytic.

This work is aimed to compare the immunological features of two closely related earthworm species *Eisenia andrei* and *Eisenia fetida.* Due to many morphological and life cycle similarities they have been, until recently, regarded as members of subspecies. Interestingly, their natural habitat varies considerably, and it was of particular interest to investigate how these environmental differences affect the features of innate immunity of both species.

Key words: annelids, innate immunity, *Eisenia andrei, Eisenia fetida,* CCF, fetidin, lysenin, lysozyme

Abstrakt

Dážďovky patriace medzi máloštetinavce sú modelom porovnávacej imunológie už viac ako 40 rokov. Sú schopné efektívne rozpoznať a reagovať na nevlastné látky vďaka rôznym obranným mechanizmom, medzi ktoré sa zahrňujú aj molekuly s mnohými biologickými aktivitami zahrňujúcimi cytolytické, antimikróbne a proteolytické.

Táto práca je zameraná na porovnanie imunologických vlastností dvoch blízkych druhov dážďoviek *Eisenia andrei* a *Eisenia fetida.* Vďaka mnohým podobnostiam medzi ich morfológiou a životnými cyklami boli až donedávna považované za členov poddruhu. Zaujímavým však je, že sa ich prirodzené prostredia života od seba veľmi líšia, a preto bolo veľmi zaujímavé skúmať aký vplyv majú rozdiely v prostredí na vlastnosti prirodzenej imunity oboch druhov.

Kľúčové slová: dážďovky, prirodzená imunita, *Eisenia andrei, Eisenia fetida,* CCF, fetidín, lysenín, lyzozým

Contents

Abbreviations

12C9	monoclonal antibody against CCF
AP	adapter primer
APS	ammonium persulfate
AUAP	abridged universal amplification primer
BSA	bovine serum albumin
CCF	coelomic cytolytic factor
cDNA	complementary DNA
CF	coelomic fluid
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
GSP	gene specific primer
Ig	immunoglobulin
IPTG	isopropyl β-D-1-thiogalactopyranoside
LPS	lipopolysaccharide
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PO	phenoloxidase
PP-1	a lumbricin I analog
ppA	prophenoloxidase-activating enzyme
proPO	prophenoloxidase
Q-PCR	real-time quantitative PCR
RACE	rapid amplification of cDNA ends
ROIs	reactive oxygen intermediates
RT	reverse transcription
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
TdT	terminal deoxynucleotidyl transferase
TEMED	tetramethylethylenediamine

TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
X-Gal	bromo-chloro-indolyl-galactopyranoside

1 Introduction

The success of invertebrate taxa in evolution buttresses arguments about the potential value of gaining deep insights in their immunology. In this respect, observation of the less complex invertebrate defense strategies has already led to understanding of some of the most basic immunological processes. One of these – phagocytosis – was discovered in the late 1800s by Elie Metschnikoff, who was the first to observe cells digesting bacteria while studying starfish larvae. As it turned out, it is an important and evolutionarily conserved defense mechanism of innate immunity. Since Metschnikoff's pioneering work, invertebrate immunology has become a proper topic of study.

Yet another example of such a discovery are Toll-like receptors that were originally described as factors involved in dorsoventral determination in *Drosophila melanogaster* (Hashimoto et al. 1988) but were also found to activate antimicrobial mechanisms (Lemaitre et al. 1996). Nowadays, these molecules are known to play an important part in the innate immunity system acting as pattern-recognition receptors that are widely distributed in both plant and animal kingdoms.

Invertebrates have evolved for hundreds of millions of years, often surviving in very hostile environment. Their successful survival strategies are likely based on short life span combined with numerous offspring. Despite the absence of an adaptive immune system based on antigen-specific T and B cells and antibodies observed in vertebrates only, invertebrates still possess a variety of defense mechanisms efficiently recognizing and responding to non-self substances. The common defense mechanisms used by most invertebrates to protect themselves against infectious agents are the synthesis and secretion of antibacterial and antifungal proteins, agglutination and nodule formation, encapsulation of foreign objects, pattern-recognition receptors, and phagocytosis.

The annelids (*Annelida*) constitute an important invertebrate phylum of the animal kingdom comprising of more than 9000 species including three classes: marine worms (*Polychaeta*), earthworms

(*Oligochaeta*), and leeches (*Hirudinea*). There are more than 3000 earthworm species adapted to a wide range of soil habitats as well as freshwater lakes and streams. They can also be found in leaf litter, manure, under stones and logs as well as some arid areas, but most species prefer wetter, more heavily vegetated regions.

The earthworms are regarded as a model of comparative immunology since early sixties when transplantation experiments proving the existence of self/nonself recognition were performed (Bailey et al. 1971; Cooper 1968). These results initiated extensive studies of the earthworm immune mechanisms. In the last four decades important cellular and humoral pathways were described and numerous biologically active compounds characterized.

This study focuses on comparison of two closely related earthworm species *Eisenia andrei* (Bouché 1972) and *Eisenia fetida* (Savigny 1826) from the immunological point of view. The two species were first described as different morphotypes of *Eisenia fetida* according to differences in body pigmentation (André 1963), and subsequently they were given subspecific status (Bouché 1972) and named *Eisenia fetida fetida* and *Eisenia fetida andrei.* Nowadays, despite their morphological and life cycle similarities and possibility of cross – hybridization, they are accepted as two different species.

Eisenia fetida corresponds to the striped or banded morph, with the area around the intersegmental groove having no pigmentation and appearing pale or yellow, whereas *Eisenia andrei*, the common „red“ worm, corresponds to the uniformly reddish morph (Reinecke et al. 1991).

These two earthworm species share many similarities, but their natural environment varies greatly. Whereas *Eisenia andrei* naturally lives in compost and manure rich in possible pathogens, populations of *Eisenia fetida* earthworm can be found exclusively in litter layer of South Bohemian forests that are considerably poorer in number of pathogenic organisms (Pižl 2002). Therefore, it was of interest to inquire how the natural environment affects various defense mechanisms involved in innate immunity of the earthworms.

2 Anatomical structure

The earthworms are protostomian animals possessing true coelomic cavity of mesenchymal origin. The coelomic cavity is filled with the coelomic fluid (CF) containing free coelomocytes originating in the mesenchymal lining of the cavity. The coelomic cavity is metameric and the segments are separated by transversal septa. Transport of the coelomic fluid and coelomocytes between the segments is regulated by channels. Each segment of the coelomic cavity is opened to the outer environment by a pair of nephridia and by a dorsal pore (Fig. 1).

The earthworms possess a simple closed circulatory system. They have two main blood vessels extending through the length of the body: a ventral blood vessel pumping the blood by a series of "hearts" (aortic arches) to the posterior end, and a contractile dorsal blood vessel carrying the blood anteriorly. From the ventral vessel the blood is being distributed via capillaries to the entire body.

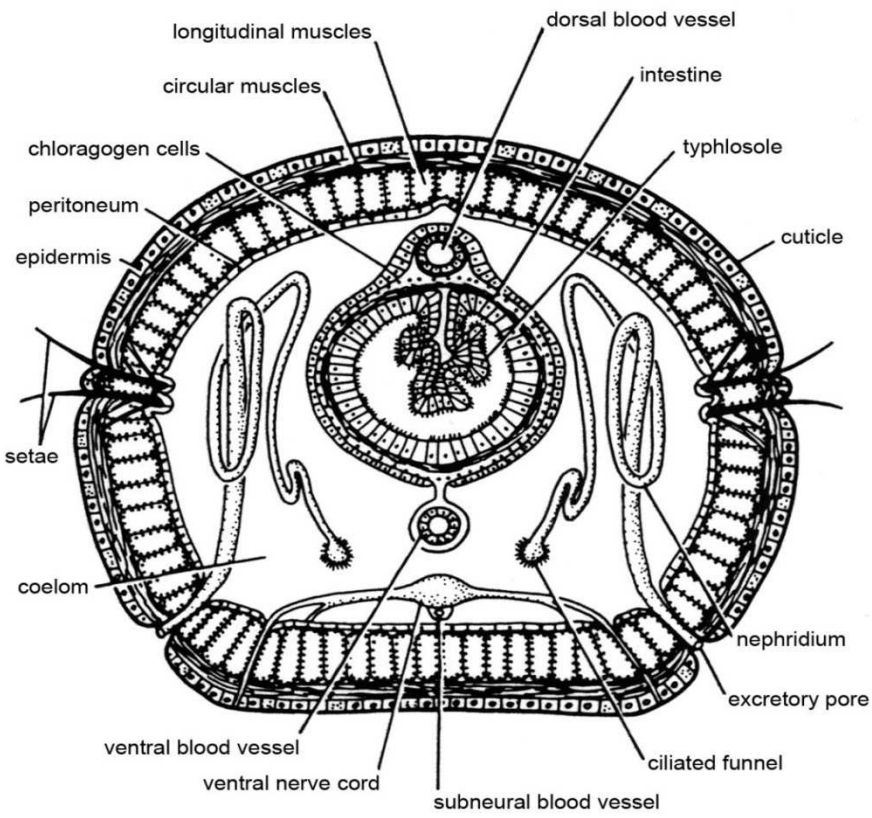

Fig. 1. Earthworm cross section (adopted from Biology of the Invertebrates, Pechenik, 1996).

3 Defense of the earthworms

The first natural defense mechanism is represented by anatomical and chemical protective barriers helping to prevent damage of the underlying tissues, body fluid losses, and microbial infections of the body cavity. The body wall of the earthworms serves as the first protective barrier between the outside environment and the internal organ systems, as a structure holding the body parts together, and it provides the physical external structures of the sensory receptors.

The first layer of the body wall is the skin, which consists of the epidermis and a thin cuticle. The epidermis is formed by a single-layer epithelium of supporting cells, basal cells, and secretory cells. Basal cells play an important role in wound healing and graft rejection, often exerting phagocytotic activity (Chapron 1970; Valembois 1971). Secretory cells of different types form epidermal glands secreting mucus containing the so-called mucopolysaccharide-lipid-protein complex (Dall Pai et al. 1981). The mucus funcions in diverse ways, such as defense against predators, resistance to desiccation, structural support, locomotion, and aid in blood plasma clotting (Valembois et al. 1988). Mucus due to its gel–like structure can entrap microorganisms and effectively removes entrapped particles. Antibacterial factors secreted into the mucus kill the entrapped microorganisms.

A well–developed basement membrane separates the epithelium from longitudinal and circular muscular layers. These layers are separated by a simple squamous epithelium from the fluid–filled coelomic cavity. Muscular layers and epithelium well protect the coelomic cavity from mechanical injury and are little permeable for microorganisms.

Microorganisms passing through the body wall barrier enter the coelomic cavity mainly through dorsal pores. Consequently, the coelomic cavity is not aseptic and always contains bacteria, protozoans and fungi from the outer environment. Nevertheless, there are efficient mechanisms that keep the growth of microorganisms under control (Dales et al. 1992). It was reported that coelomic fluid contains 6×10^5/ml naturally occurring

bacteria while the number of potentially phagocytic cells is more than ten times higher. These abundant phagocytes combined with the presence of various humoral factors can easily prevent the microorganisms from outgrowth.

The immune defense mechanisms of annelids consist of cellular mechanisms (Chap. 3.1) such as phagocytosis, nodule formation and encapsulation, blood coagulation and wound healing, and humoral mechanisms (Chap. 3.2). These antibacterial mechanisms of the coelomic fluid are associated with components such as lysozyme-like proteins, proteases, cytolytic proteins, antimicrobial peptides and components of prophenoloxidase activation cascade; humoral defense also includes pattern recognition and lectin-like molecules that are designed to recognize few highly conserved structures present in many different microorganisms.

3.1 Cellular defense mechanisms

Coelomic fluid of the earthworms contains various types of coelomocytes. The nomenclature of coelomocytes is based mainly on morphological and cytochemical criteria (Stein et al. 1977). In general, they include eleocytes, hyaline amoebocytes, and granular amoebocytes. Eleocytes (free chloragogen cells type I and type II) have mainly accessory and nutritive function. The hyaline amoebocytes contain basophils and neutrophils, granular amoebocytes comprise acidophils type I and type II. Hyaline and granular amoebocytes represent effectory immunocytes, both possessing the ability of phagocytosis, although their activity differs.

The nonspecific cellular defense responses, basic for the clearance of body cavity from intruders, include phagocytosis (Bilej et al. 1990b; Dales et al. 1992), encapsulation and nodule formation (Ratcliffe et al. 1985), and wound healing (Burke 1974a).

3.1.1 Phagocytosis

Phagocytosis is regarded as the primary cellular response in all invertebrates. It is a multistep process that involves recognition, chemotaxis,

attachment, ingestion, and intercellular elimination by several mechanisms – either by proteolytic and lysozyme-like enzymes or by an oxidative burst, which involves the production of highly reactive oxygen radicals.

Hyaline and granular amoebocytes, as mentioned above, have phagocytic properties. In contrast to granular amoebocytes, the cytoplasm of hyaline amoebocytes is occasionally full of engulfed material (Bilej et al. 2006; Stein et al. 1977). It should be mentioned that amoebocytes engulf all kinds of material including inert particles, microbial cell wall components as well as foreign cells. However, the phagocytosis of eukaryotic cells depends on the source of the cells. Unlike allologous cells, xenologous cells (both from different earthworm species and from non-invertebrate species) are rapidly phagocytosed (Cameron 1932; Stein et al. 1981).

Phagocytosis by coelomocytes, similarly to that of vertebrates, can be modulated by humoral components, opsonins that coat the engulfed particle and thus promote its phagocytosis. It was proven that preincubation of both yeast and synthetic particles with the coelomic fluid significantly increased their phagocytosis (Bilej et al. 1991; Bilej et al. 1990a).

It is noteworthy that also mammalian opsonins, IgG immunoglobulin and C3b complement fragment, were described to enhance coelomocyte phagocytic activity, in contrast to IgM and C3d fragment, which did not affect phagocytosis (Laulan et al. 1988).

3.1.2 Encapsulation

Large foreign bodies, such as agglutinated bacteria, gregarines, incompatible graft fragments, and altered self structures such as setae or necrotic muscle cells are eliminated by encapsulation resulting in the formation of so called brown bodies due to the content of brown pigment – melanin (Valembois et al. 1992; Valembois et al. 1994).

Amoebocytes and eleocytes participate both in encapsulation and formation of brown bodies (Cooper 1996). Encapsulation begins like phagocytosis with recognition of nonself, but the engulfment cannot accur because of the size of the particle. Within 24 hours the coelomocytes surround the foreign particle and form multicellular sheets called capsules.

The initial capsule rapidly increases in volume by aggregation of new coelomocytes and various waste particles. Later on melanization occurs, starting from disintegrated cells of the inner layer of capsule and discharged material near the foreign surface. A dense capsule composed of flattened cells is formed during several days. The encapsulating cells are closely packed, producing thin strands of fibrous tissue and finally, a fibrous capsule is produced. When the capsule reaches 1-2 mm in diameter, its external cells lose their adhesiveness. The capsule starts to migrate to posterior segments of the coelomic cavity where it can be eliminated by autotomy (Keilin 1925).

Parasites are killed within the capsule owing to starvation or asphyxiation. Lytic enzymes secreted by coelomocytes forming the capsule may facilitate destruction of the encapsuled parasite. The by-products released during the melanization in brown bodies may also destroy the encapsuled parasites.

3.1.3 Wound healing

Blood coagulation and wound healing effectively prevent excessive loss of fluids and the intrusion of possible infectious microorganisms into the tissues of the earthworms. The reaction at the site of injury involving vasoconstriction, coelomocyte accumulation and formation of a wound plug, represents an effective mechanism to prevent infections. Hemostatic responses are accompanied by muscle constriction that reduces the size of the wound, which might serve as a portal of entry for infecting agents. Wound healing is initiated by the migration of amoebocytes to the site of injury, where they aggregate to form a wound plug. There is no evidence that wounding in annelids is accompanied by “leukopoiesis” as it is generally accepted in vertebrates, since increased mitotic activity of free coelomocytes was not observed (Burke 1974a; Burke 1974b; Burke 1974c).

3.2 Humoral defense mechanisms

The development of molecules designed to prevent infection by potential pathogens and parasites is essential for the survival of the earthworms in microbially polluted soils. Subsequently, the coelomic fluid exerts numerous biological activities. It was documented that it contains various antimicrobial factors, such as lysozyme (Cotuk et al. 1984) or antimicrobial peptides (Cho et al. 1998; Liu et al. 2004; Ovchinnikova et al. 2008; Ovchinnikova et al. 2007; Wang et al. 2003). Nevertheless, particular attention has been devoted to cytolytic components secreted by coelomocytes into the coelomic cavity. Firstly, this activity was demonstrated on vertebrate erythrocytes. The majority of proteins with hemolytic properties have hemagglutination activity as well as, and more interestingly, a spectrum of antibacterial and bacteriostatic activities against pathogenic soil bacteria (Roch 1979; Roch et al. 1991a; Valembois et al. 1982). Later, it was observed that coelomic fluid lyses eukaryotic cells other than erythrocytes, namely chicken fibroblasts, guinea-pig polymorphonuclear leukocytes, and insect hemocytes (Kauschke et al. 1987). Furthermore, compounds with antitumor activities were described (Bilej et al. 1995; Cooper et al. 1995).

3.2.1 Proteases

Proteases are enzymes that conduct the proteolysis by hydrolyzing the peptide bond linking amino acids together in the polypeptide chain. Proteases present in invertebrates are usually found in coelomic cavity, where they play a role in immunodefense by activating the prophenoloxidase cascade (Ashida et al. 1988) or attending in the coagulation of plasma (Fortes-Dias et al. 1993). In earthworms, proteolytic enzymes have been found in the coelomic fluid (Mohrig et al. 1989). Moreover, a significant increase of proteolytic activity of coelomic fluid of *L. terrestris* within 24 hours after antigenic challenge was observed indicating the involvement of proteases in the innate immunity (Kauschke et al. 1997).

3.2.2 Prophenoloxidase cascade

The prophenoloxidase cascade represents one of the most important defense mechanisms in many invertebrates. Mechanical injuries or presence of foreign objects such as parasites and microorganisms result in melanin deposition around the damaged tissue or intruding object. Melanin, which happens to be the final product of the prophenoloxidase cascade, deposited on or near the pathogen serves to physically shield invertebrates from the pathogen, and therefore prevents or retards its growth. Perhaps even more importantly during the melanin formation, highly reactive and toxic quinone intermediates are produced (Cerenius et al. 2004; Soderhall et al. 1998).

The prophenoloxidase-activating system is a sensitive non-self-recognizing cascade triggered by the presence of minute amounts of microbial compounds known as pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, and β-1,3-glucan. Following the recognition of microbial saccharides by pattern recognition molecules, proteinases cleave inactive prophenoloxidase to its active form phenoloxidase (PO). This conversion is achieved by a cascade of serine proteinases, 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 to melanin (Fig. 2) (Soderhall et al. 1994). In addition, the quinone intermediates generated during the synthesis of melanin can enter enzymatic and non-enzymatic redox cycling with their corresponding semiquinone radicals and as a result generate reactive oxygen intermediates (ROIs).

Prophenoloxidase as well as phenoloxidase were characterized in numerous arthropod groups, namely crustaceans and insects. Indeed, PO activity was recorded in mollusks (Smith et al. 1991) and more recently in earthworms (Field et al. 2004; Prochazkova et al. 2006b).

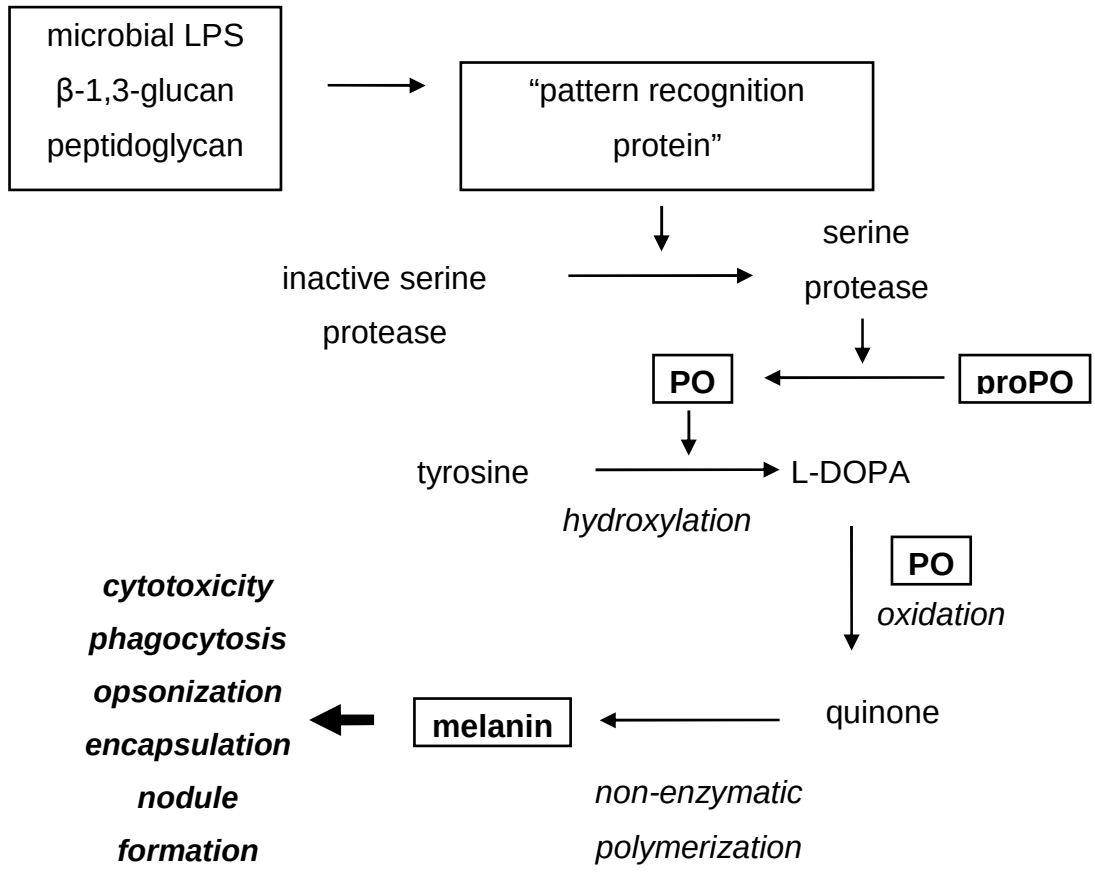

Fig. 2. Prophenoloxidase activating cascade. The recognition of pathogen associated molecular patterns leads to the cleavage of inactive prophenoloxidase to its active state phenoloxidase. Active enzyme catalyzes both hydroxylation and oxidation of phenols to quinones, which are subsequently polymerized to melanin. Melanin exerts various biological activities important in defense reactions.

Molecular weight of proPO and PO differs among species, and varies usually between 70 and 90 kDa. The primary structure of known proPOs reveals the presence of two functional copper-binding sites, as well as another conserved peptide motif GCGEQNM (Armstrong et al. 1999; Dodds et al. 1998), which is present in α2-macroglobulins both in vertebrates and invertebrates. This motif is present also in the vertebrate thioester-containing complement proteins C3, C4 and in the complement-related proteins of some invertebrates (Marino et al. 2002).

The finding of the PO activity in the coelomic fluid of *Eisenia fetida* suggested the presence of proPO activating cascade in annelids (Beschin et al. 1998). It was documented that proPO cascade of *Eisenia fetida* is directly activated by Gram-negative bacteria and yeasts, while Gram-positive bacteria have to be treated with lysozyme to activate the cascade (Bilej et al. 2001). Moreover, a 90-kDa fraction of the coelomic fluid of *Eisenia fetida* was identified to possess detectable phenoloxidase activity. This fraction was further subjected to amino acid sequence analysis, which revealed a partial homology with PO originated from other invertebrates. Moreover, it was observed that proPO activation is slower as compared to other invertebrate species. Therefore, it was suggested that in contrary to arthropods proPO cascade, does not represent the main immunodefense system in the earthworms (Prochazkova et al. 2006b).

3.2.3 Coelomic cytolytic factor CCF

As it was already mentioned, coelomic fluid of the earthworms exerts a large variety of biological effects. Among these, the cytotoxic effect has always driven a particular attention. The toxic effect of *Eisenia fetida* coelomic fluid on different cell types, such as chicken fibloblasts and insect hemocytes, was observed, while the viability of coelomocytes of other lumbricids as well as the cells of some mollusks, nematodes, and protozoans was not affected (Kauschke et al. 1987). Moreover, compounds with antitumor activities have been isolated from body homogenates of lumbricids *Eisenia fetida, Lumbricus rubelus* and *Lumbricus terrestris* (Hrzenjak et al. 1992).

A significant lytic activity of *Eisenia fetida* coelomic fluid on the tumor necrosis factor (TNF)-sensitive cell line L929 was observed. Interestingly, coelomic fluid exerts cytolytic activity on the TNF-sensitive cell line and the TNF-resistant subclone indicating that the lytic activity is mediated by TNF-unrelated mechanisms. Subsequent isolation of lytic proteins resulted in the identification of 42-kDa protein named coelomic cytolytic factor – CCF (Bilej et al. 1995). This protein,

apart from its involvement in cytotoxicity, also participates in opsonization and hemolysis.

Analysis of the cellular expression revealed the CCF expression on the surface of free coelomocytes and in the cells of mesenchymal lining adjacent to the gut wall (Bilej et al. 1998).

Further research revealed more information about the sequence of CCF. The CCF cDNA encodes for a 384-amino acid protein, synthesized with a 17-amino acid signal peptide. Consequently, the mature CCF is a 367-amino acid protein of 42 kDa with a predicted pI of 4.2. Calculated molecular mass is similar to that of native CCF suggesting the absence of post-transational modifications. This is corroborated by the fact that the CCF cDNA contains no potential *N*-glycosylation site (Beschin et al. 1998).

Once revealed, the amino acid sequence of CCF allowed a comparison with other proteins to be carried out. The analysis showed close matches with highly conserved amino acid residues commonly shared by bacterial and animal β-1,3-glucanases (Bachman et al. 1996; Kozhemyako et al. 2004; Yamamoto et al. 1993). Unlike these though, CCF does not exhibit glucanase activity. Furthermore, CCF also shows homology with the α subunit of the β-1,3-glucan-sensitive coagulation factor G from horseshoe crab *Tachypleus tridentatus* (Seki et al. 1994), with the Gram-negative bacteria-binding proteins of various insects (Kim et al. 2000; Lee et al. 1996; Shin et al. 1998) and β-1,3-glucan recognition protein of arthropods (Ma et al. 2000; Ochiai et al. 2000). These invertebrate homologs have been suggested to play a role in invertebrate innate immunity by acting as pattern recognition molecules.

In summary, CCF acts in earthworm defense as a pattern-recognition molecule. Upon binding microbial pathogen-associated molecular patterns, namely O-antigen of LPS of Gram-negative bacteria, muramyl dipeptide and muramic acid of peptidoglycan from the cell walls of Gram-positive bacteria and β-1,3-glucans and *N*, *N'*-diacetylchitobiose of yeast, CCF triggers the activation of the prophenoloxidase cascade, which results in the formation of cytotoxic and antimicrobial compounds and thus represents an important invertebrate defense mechanism (Chap. 3.2.2). The broad specificity of CCF for pathogen-associated molecular patterns

results from the presence of two spatially distinct pattern recognition lectin-like domains. Domain localized in the central part of the molecule shows homology with the polysaccharide-binding and glucanase motifs of β-1,3-glucanases and other invertebrate defense molecules. This domain interacts with LPS and β-1,3-glucans. The C-terminal tryptophan-rich domain mediates interactions with *N*, *N'*-diacetylchitobiose, muramyl dipeptide and muramic acid (Fig. 3) (Beschin et al. 1998; Bilej et al. 2001).

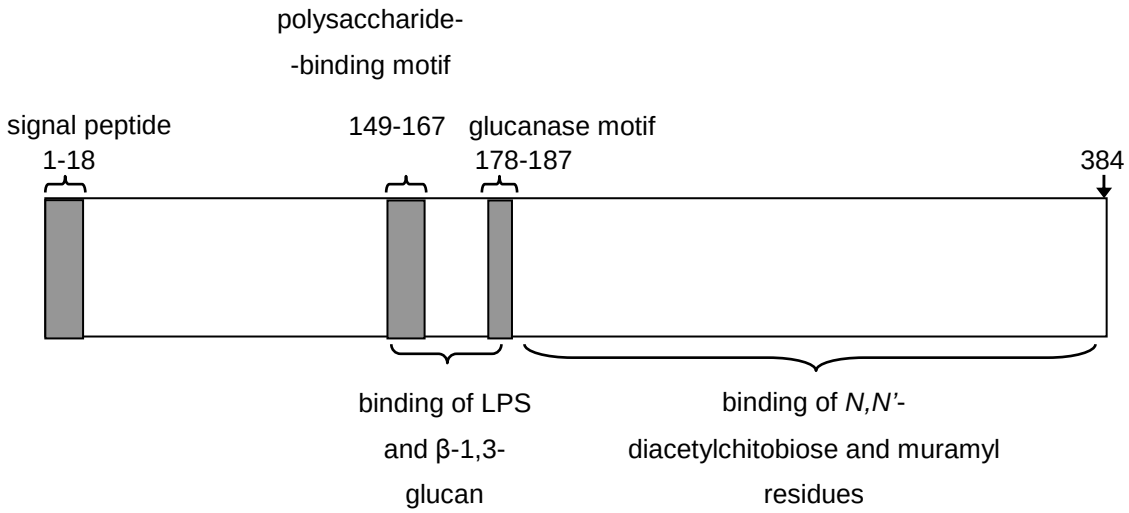

Fig. 3. Structure of *E. fetida* CCF.

Another study aimed to characterize CCF-based biological properties of CF of different lumbricid species, namely *Aporrectodea caliginosa, Aporrectodea icterica, Aporrectodea longa, Aporrectodea rosea, Dendrobaena veneta, Lumbricus rubellus* and *Lumbricus terrestis* revealed that all species express CCF-like molecules. These molecules share a high level of homology, particularly in polysaccharide-binding and glucanase motifs. However, the pattern recognition specifity of CCF of *E. fetida* is broader, namely, it is the only one possessing the lectin-like specifity for *N,N'*-diacetylchitobiose. Therefore, the CF of *E. fetida* is capable to lyse TNF-sensitive cell line L929 as well as to trigger the prophenoloxidase pathway upon binding *N,N'*-diacetylchitobiose. It was suggested that the broad saccharide recognition repertoire of *E. fetida* CCF reflects a particular microbial environment this species lives in (Silerova et al. 2006).

3.2.3.1 Coelomic cytolytic factor as a TNF analogue

CCF was originally identified as the protein responsible for the lysis of TNF-sensitive tumor L929 cell line in a proteinase-independent way. The cytolytic effect of CCF is not inhibited by anti-TNF neutralizing monoclonal antibodies suggesting that the mechanisms of TNF- and CCF-mediated lysis differ (Bilej et al. 1995). Moreover, CCF expression is up-regulated in macrophage-like coelomocytes upon lipopolysaccharide stimulation (Bilej et al. 1998) while TNF is produced by macrophages (Aggarwal et al. 1985). CCF is involved in the opsonizing properties of the earthworm CF (Bilej et al. 1995). Similarly, TNF was reported to possess opsonin-like properties that mediate the attachment of bacteria to macrophages (Luo et al. 1993). CCF and TNF bind β-1,3-glucans and *N*, *N'*-diacetylchitobiose *via* a lectin-like interaction (Beschin et al. 1998; Olson et al. 1996). In addition, monoclonal antibodies elicited against the lectin-like domain of TNF cross-react with CCF and, conversely, monoclonal antibody against CCF reacts with TNF without impairing the interaction of TNF with its specific receptor (Lucas et al. 1994; Magez et al. 1997).

Since the lectin-like domain of TNF was shown to be involved in the killing of African and American trypanosomes (Lucas et al. 1994; Magez et al. 1997), the possible trypanolytic activity of CCF was investigated (Beschin et al. 1999). Indeed, the coelomic fluid of *E. fetida* as well as purified CCF shows a potent trypanolytic activity. This effect can be inhibited not only by anti-CCF monoclonal antibodies but also by *N,N'*-diacetylchitobiose and anti-TIP TNF antibodies. Moreover, anti-CCF antibodies neutralize TNF-mediated trypanolysis.

Finally, it was documented that TNF increases the membrane conductance in endothelial cells and peritoneal macrophages, interacting with ion-channels or ion-channel-coupled molecules through a lectin-like domain (Hribar et al. 1999). Similarly, it was documented that CCF activates an amiloride-sensitive cationic channel in murine endothelial cells and macrophages *via* its *N,N'*-diacetylchitobiose lectin-like domain (Bilej et al. 2006; Bloc et al. 2002).

Surprisingly, the functional analogies of CCF and TNF are not based on gene or amino acid sequence homology, indicating a lack of common evolutionary origin (Beschin et al. 1999).

3.2.4 Hemolytic molecules

The coelomic fluid of *E. fetida* earthworms was described to exhibit strong hemolytic activity. A heterogeneous group of proteins is responsible for these hemolytic properties of CF but their relationship is still not clear.

The first described hemolytic proteins were named **EFAF** (*Eisenia fetida andrei* factors) and characterized as two glycoproteins that constitute the major CF peptide component (20% of total CF proteins) and are secreted by chloragocytes and eleocytes (Du Pasquier et al. 1968; Roch 1979; Roch 1984; Roch et al. 1981). The 45-kDa protein is encoded by a single nonpolymorphic gene and has a pI of 6.0, while the 40-kDa protein is encoded by a gene having several alleles, each representing one of isoforms with pI of 6.3, 6.2, 5.95 and 5.9. Each individual earthworm possesses the 45 kDa protein and either two or three isoforms of the 40 kDa protein. In addition to EFAFs hemolytic activity, these proteins were found to agglutinate red blood cells (Valembois et al. 1984) and to participate in the cytotoxic activity of the coelomic fluid (Kauschke et al. 1987). Moreover, they were found to participate in the antibacterial activity of the CF against both Gram-positive and Gram-negative bacteria (Hirigoyenberry et al. 1991), particularly against strains that are pathogenic for earthworms and posses antigen(s) common with red blood cells (Roch et al. 1991a; Roch et al. 1987). In addition to their antibacterial activity, they may also participate in the process of opsonization (Sinkora et al. 1993) and clotting of the coelomic fluid (Valembois et al. 1988). The coagulation cascade is likely triggered by a serine proteinase and EFAFs polymerize to give rise to a network of globular units of about 4 nm. It was documented, that upon binding to sphingomyelin, a major lipid constituent of plasma membranes of most mammalian cells, these proteins polymerize and form 10-nm channels through the lipid bilayer (Roch et al. 1981).

Later, when EFAFs were characterized on the molecular level, they were named, they were named fetidins (Lassegues et al. 1997; Milochau et al. 1997). By screening a cDNA library with a polyclonal anti-fetidin serum, a cDNA that encodes the 40-kDa fetidin was cloned. It encondes a peptide sequence of 300 amino acid residues that is rich in aromatic residues (11 %). The molecular mass of the putative 300-residue peptide was calculated to be 34 kDa. At position 250-252 an asparagine residue in a consensus sequence Asn-Xaa-Ser was identified to be able to accept an oligosaccharide. Both fetidins are *N*-glycoproteins with no O-linked sugar moieties and the molecular mass of the deglycosylated fetidins corresponds to that of the predicted peptide. Therefore, the difference of the molecular mass between these two proteins is mainly due to the size of the saccharide components. Moreover, in residues 52-62 a peroxidase signature similar to the proximal ligand of several heme-binding enzymes, especially plant peroxidase, was identified. Accordingly, both fetidins display peroxidase activity.

Independently, a 41-kDa hemolysin was discovered and named **lysenin**. This protein, isolated from the coelomic fluid of *E. fetida*, was characterized due to its ability to cause contraction of rat vascular smooth muscle (Sekizawa et al. 1996). Simultaneously, two 42-kDa lysenin-related proteins with weak contraction activity were identified (Sekizawa et al. 1997). More recently, a new member of this lysenin-like multi-gene family was cloned and was provisionally called lysenin-related protein 3 (Bruhn et al. 2006).

Upon screening a cDNA library of *E. fetida*, a clone containing the cDNA of lysenin was obtained and the protein was characterized on the molecular level (Sekizawa et al. 1997). The open reading frame of lysenin encoded a putative protein of 297 amino acid residues. The predicted sequence exhibited no significant homology to the sequences of previously characterized vasoactive compounds, but lysenin has a high amino acid homology with fetidin (89 % identity, 95 % positivity), with lysenin-related protein 1 (76 % identity, 89 % positivity) and lysenin-related protein 3 (81 % identity, 90 % positivity). Moreover, amino acid sequence of lysenin-related protein 2 corresponds to that of fetidin (99 % homology

at the nucleotide level, 100 % identity at the amino acid level). All the data suggest a close relationship between these lytic molecules.

Interestingly, the molecular mass based on the predicted sequence of lysenin was calculated to be 33 kDa, while M_r of the native protein is 41 kDa. One potential site of Asn-linked glykosylation was found at amino acid residue 248. However, deglycolysation of the native protein did not result in a significant reduction of the molecular mass, suggesting that the discrepancy in the molecular mass might be due to the high level of acidic amino acid residues or due to some unique features in the secondary or tertiary structure of lysenin.

Nothern blot analysis was used to investigate the tissue of *E. fetida* where the lysenin mRNA is expressed. A positive cDNA of approximately 1,6 kb was detected in coelomocytes, but not in the intestine, body wall and other tissues (Sekizawa et al. 1997) .

Due to its homology with fetidins, the hemolytic properties of lysenin were tested. Indeed, a strong hemolytic activity of this protein was observed. The sensitivity of erythrocytes to lysenin varies among animal species and it is closely correlated with the relative level of sphingomyelin in the membranes (Yamaji et al. 1998). Sheep erythrocytes contain sphingomyelin as a major phospholipid and thus are the most sensitive to the lysenin treatment. Therefore, it was postulated that the hemolytic activity of lysenin is dependent on the presence of sphingolipids in the membrane. Moreover, the presence of cholesterol in the membrane facilitates hemolysis. Upon binding to the spingomyelin, lysenin forms oligomers and subsequently pores 3 nm in diameter in the target membranes (Yamaji-Hasegawa et al. 2003; Yamaji et al. 1998). In this process various molecules of the protein polymerize together and hide the N-terminal parts of the protein inside of the created complex. This process is dependent both on incubation temperature and lysenin concentration.

As sphingomyelin is crucial for the lytic activity of lysenin, it has been proposed to use lysenin as a valuable probe for sphingomyelin detection in sphingomyelin storage diseases, particularly in the cells of Niemann-Pick A patients (Yamaji et al. 1998), although the multiplicity

of hemolysins in the natural source and the cytolytic activity appeared to be a major obstacle.

At the same time, a 38-kDa protein named **eiseniapore**, also capable to lyse red blood cells, was isolated from the coelomic fluid of *E. fetida* and described (Lange et al. 1999; Lange et al. 1997). Since receptors for eiseniapore on target cells were not known, lipid vesicles of various compositions were used to determine whether specific lipids may serve as receptors. This hemolysin binds to and disturbs the lipid bilayer only when distinct sphingolipids consisting of a hydrophilic head group as phosphorylcholine or galactosyl as well as the ceramide backbone, *e. g.* sphingomyelin, are present. The presence of cholesterol in the membrane was again shown to affect the lysis caused by this protein by enhancing the lytic effect towards sphingomyelin-containing vesicles probably due to interaction with sphingomyelin. Leakage of vesicles was most efficient when the lipid composition resembled that of the outer leaflet of human erythrocytes. Moreover, no membrane (glyco-)proteins as receptors for eiseniapore are required as long as specific sphingolipids are present and the lytic activity does not require any other component from the coelomic fluid. Presumably, an oligomeric protein pore formed by six monomers is responsible for disruption of sphingomyelin-containing vesicles. The pore has an outer diameter of 10 nm and an inner diameter of 3 nm.

Furthermore, three proteins **H_1**, **H_2**, **H_3** were isolated from the coelomic fluid of *E. fetida* by preparative polyacrylamide gel electrophoresis and characterized. Their molecular weight is 46, 43 and 40 kDa, respectively. H_1 and H_2 were shown to be stable unter SDS treatment, while H_3 splits into two fragments with molecular weight of 18 and 21 kDa. Moreover, isoelectrical focusing revealed that each protein consists of various isoforms with pI between 5.1 and 6.2. Despite the cross-reactivity of the monospecific antisera, hemolysins functionally differ. H_3 protein is a bifunctional – besides hemolytic properties which are common to all hemolysins it has also agglutinating activity (Eue et al. 1998). Later on, Koenig *et al* isolated and analyzed two hemolytic proteins from the cell lysate (**CL_{39}** and **CL_{41}**). Nevertheless, more detailed analyses of these molecules revealed close relathionships with lysenin, fetidin and lysenin-related

proteins. Moreover, they showed that H_{1-3} proteins share sequence components with fetidin but they seem to be glycosylated (Koenig et al. 2003).

To conclude, the primary function of the hemolytic system is presumably to destroy membranes of foreign cells, a mechanism that causes cell death by cytosol release, and is attributed to coelomocytes which secrete humoral effectors into the coelomic fluid. Moreover, numerous hemolytic factors exert agglutination and antibacterial activities against pathogenic soil bacteria.

3.2.5 Lysozyme

Lysozyme is a ubiquitous enzyme that is widely distributed within the animal and plant kingdoms. Based on the differences in their structure, catalytic character and original source, lysozymes are classified into six groups, the invertebrate-type lysozyme being one of these groups (Prager et al. 1996). Lysozyme is a bacteriolytic enzyme that possesses the hydrolytic activity to specifically cleave β-1,4-glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan present in bacterial cell walls and thus efficiently protects against infections caused by Gram-positive bacteria.

Already in 1960 a lysozyme-like activity was reported in the coelomic fluid of the earthworm *Nephtys* (Jolles et al. 1960). Subsequently, lysozyme-like effect was detected in the coelomic fluid and the extract of coelomocytes of *E. fetida.* This activity was reported to be low under normal conditions, however it was shown that the enzyme was released from the eleomocytes following microbial infection (Cotuk et al. 1984).

Later, the active protein was isolated and the N-terminal sequence up to the 33rd residue was determinated. The purified lysozyme has a molecular weight of 13 kDa and shows a considerable homology with other three lysozymes purified from other invertebrate species (a marine bivalve, a marine conch and a starfish), while the homology with other known types

of lysozyme was negligable. Thus, a novel class of invertebrate lysozyme family was discovered in invertebrates (Ito et al. 1999).

Recently, a full-length cDNA coding for this novel invertebrate-type lysozyme was assembled from *Eisenia andrei* earthworm. The whole coding sequence with an open reading frame of 480 bp encoding for 160 amino acids was obtained. Subsequent analysis of the deduced amino acid molecule revealed a high content of cysteine residues, which was observed not only in the invertebrate-type of lysozyme, but also within the other types. These residues are vital for proper protein folding and, more importantly, for the stability of the molecule. Besides the conserved cysteine residues, the molecule contains also three amino acid residues potentially important for the lysozyme activity – Glu14, Asp26 and Ser29 (Joskova et al. 2009).

3.2.6 Antimicrobial peptides

Antimicrobial peptides are an abundant and diverse group of molecules that are produced by many cell types in invertebrates, vertebrates and plants. Depending on their tissue distribution, they ensure either a systemic or a local protection of the organism against pathogens. These peptides exert their antimicrobial activity through either the lipid bilayer of the cell membrane by the formation of multimeric pores (Hancock 1997) or the interaction with DNA or RNA after penetrating into the cell membranes (Boman et al. 1993; Cabiaux et al. 1994; Park et al. 1998; Zasloff 2002).

Most of antimicrobial peptides of invertebrates were found in arthropods. To date, only a limited number of bioactive peptides have been described in annelids. An antimicrobial peptide named Lumbricin I was identified in *Lumbricus rubellus* (Cho et al. 1998). Lumbricin I is a proline-rich antimicrobial peptide which is constitutively expressed in adult animals and is not induced by bacterial infection. A Lumbricin I analog named PP-1 was found in the Asian earthworm *Pheretima tschiliensis* (Wang et al. 2003). PP-1 is synthesized in the body wall only and its localization in the mucus of the epidermis suggests its role in the mucosal defense. Furthermore, an antimicrobial short peptide OEP3121 of only 5 amino acids was found in *Eisenia fetida* earthworms (Liu et al. 2004).

3.3 Modulation of the defense molecules during in vivo immune response

As indicated in the sections 3.1 and 3.2, the earthworms are able to protect themselves against invading pathogens due to efficient innate defense mechanisms. Apart from the effectory immunocytes present in the coelomic fluid, they possess various types of antimicrobial factors including lysozyme-like molecules, factors with hemolytic activity, as well as a pattern recognition protein (CCF). Clearly, it was of interest to address the modulation of the earthworm defense system during in vivo immune response.

The earthworms respond to body injury, antigenic stimulation or stress conditions by non-specific changes in the protein concentration of the coelomic fluid. When the body cavity of the earthworms is injected with bacteria or β-1,4-glucan, a significant increase in protein concentration is observed within 48 hours after the injection (Tuckova et al. 1988). It was already reported that the lysozyme-like activity in the CF of *E. fetida* is low under normal conditions, yet the enzyme is released from coelomocytes after bacterial infection. Indeed, an increase of lysozyme-like activity was observed within 2 days after microbial stimulation (Cotuk et al. 1984).

Suprisingly, when the effect of experimental infection on the expression of mRNA coding for CCF and fetidin was investigated, it was shown that the biosynthesis of CCF, but not fetidin, is up-regulated upon microbial stimulation. The decreased hemolytic activity of the CF reflects the increase of the whole protein content in the absence of synthesis of hemolytic proteins (Kohlerova et al. 2004).

Interestingly, CCF was described to be released into the coelomic fluid within the first 24 hours following microbial challenge (Bilej et al. 1998), i.e. earlier than lysozyme-like activity, which increases 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 the earthworms.

4 Main aims and scheme of experiments

The aim of this work was to characterize selected defense factors and their expression in two closely related earthworm species *Eisenia andrei* and *Eisenia fetida.* It was of interest to address the differences between immunological features of these two species, because although they share many similarities, their natural environment varies greatly. Whereas *Eisenia andrei* naturally lives in compost and manure rich in possible pathogens, population of *Eisenia fetida* earthworm can be found exclusively in litter layer of moist forests that is considerably poorer in heterogeneity and quantity of microorganisms. Therefore, it was expected to discover some discrepancies between the immunological features of the two species. The differences were analyzed at three levels that are included in the experimental part of this work.

First, differences at the gene sequence level were analyzed. Some of the genes, coding for immunologically active proteins, were sequenced and compared.

Second, discrepancies between the gene expression of selected compounds were assesed using quantitative RT-PCR.

Third, using various bioassays, proper biological activities of coelomic fluids isolated either from *E. andrei* or *E. fetida* earthworms were evaluated and subsequently compared.

4.1 Scheme of experiments

● Sequence comparison of selected immunologically active compounds

-RNA isolation from coelomocytes of *E. andrei* and *E. fetida*

-cDNA synthesis using reverse transcriptase (RT-PCR)

-PCR of the middle gene parts

-synthesis of the terminal gene parts using 3´RACE and 5´RACE

-cloning of amplification products and bacterial transformation

-plasmid isolation and DNA sequencing

● Comparison of mRNA levels coding for selected immunological proteins

-RNA isolation from coelomocytes of *E. andrei* and *E. fetida*

- cDNA synthesis using reverse transcription PCR (RT-PCR)

-quantitative PCR (Q-PCR)

● Comparison of immunological activities of coelomic fluid

-isolation of coelomic fluids of *E. andrei* and *E. fetida*

-determination of coelomic fluid protein concentration

-SDS-PAGE

-cytolytic assay

-hemolytic assay

-lysoplate assay

-protease assay

-native PAGE and substrate gel electrophoresis

5 Material and methods

5.1 Isolation of coelomic fluid and coelomocytes

Adult individuals of *E. andrei* and *E. fetida* were used as the experimental model of this thesis. *E. andrei* earthworms were collected in compost kept at the Department of Ecology, Institute of Microbiology of the Academy of sciences of the Czech republic in Prague, and *E. fetida* earthworms were collected in the moist forests of South Bohemia (near Hluboké, E 14.46813 °, N 49. 08531 °).

Coelomic fluid was obtained by puncturing post-clitellum segments of the coelomic cavity with Pasteur micropipette and kept on ice. Coelomocytes were isolated by subsequent centrifugation (10 min, 500×g, 4 ℃). The supernatant was collected, re-centrifuge d (10 min, 1000×g, 4 ℃), frozen and stored at -20 ℃.

5.2 RNA isolation and cDNA synthesis

5.2.1 RNA isolation

Total RNA was isolated from coelomocytes performing a modified method of Chomczynski and Sacchi (1987) using TRIzol® reagent (Invitrogen), which is suited for isolation of total RNA from cells and tissues. The reagent is a monophasic solution of phenol and guanidine isothiocyanate that maintains the integrity of RNA, while disrupting cells and dissolving cells components. All the laboratory equipment (plastic) was RNAse-free, eppendorf tubes were treated with DEPC H_2O (0.01 % DEPC) and autoclaved.

Approximately 10^7 coelomocytes were lysed by adding 1 mL of TRIzol® reagent and passing the cell solution several times through a pipette. The homogenized samples were incubated at room temperature for 10 min and then 0.2 mL of chloroform was added. The tubes were shaken vigorously, incubated at room temperature for 3 min and centrifuged subsequently (15 min, 12000×g, 4 ℃). Following cen trifugation, the mixture

separated into three phases: a lower chloroform phase, an interphase, and an upper aqueous phase containing RNA. The aqueous phase was transferred to a new tube, and the RNA was precipitated by adding 0.5 mL of isopropanol. The mixture was incubated at room temperature for 10 min and centrifuged (10 min, 12000×g, 4 ℃). The RNA pr ecipitate formed a gel-like pellet on the bottom of the tube. The supernatant was removed and the pellet was washed with 75 % ethanol and centrifuged (5 min, 7500×g, 4 ℃). Ethanol was removed and the pellet w as dried at room temperature for 10 min. Finally, RNA pellet was dissolved in 20 µL of DEPC H_2O and stored at -80 ℃.

In order to provide an indication of the degree of contamination of the isolated RNA as well as to determine its concentration, a spectrophotomeric analysis was performed. RNA was diluted in DEPC H_2O (1:50) and its absorbance at 260 and 280 nm was measured. The RNA purity was determined as the 260 nm/280 nm ratio.

5.2.2 DNase treatment

When cDNA is supposed to be used for real-time PCR, a total elimination of any possible contaminating genomic DNA is advised. Therefore, in this case, DNase treatment is a preparation step of cDNA synthesis. One µg of total RNA from each sample was used and DEPC H_2O was added to a final volume of 7 µL. Afterwards, 2 µL of DNase I (Fermentas; 1 U/µL) and 1 µL of DNase I buffer was added to the mixture and incubated for 20 min at 37 ℃. To inactivate th e enzyme, 1 µL of 25 mM EDTA (Fermentas) was added and the solution was incubated for 10 min at 65 ℃. Afterwards, cDNA was prepared using the p rotocol given in the Chapter 5.2.3.

5.2.3 cDNA synthesis

For synthesis of the first-strand cDNA a commercial kit SuperScript II® First-Strand Synthesis System for RT-PCR (Invitrogen) was used according to manufacturer´s protocol. One µg of total RNA from each

sample was used and DEPC H_2O was added to a final volume of 10 µL. Then, 1 µL of primer (Oligo(dT)$_{12-18}$ Primer; 0.5 µg/µL) was added and the solution was incubated for 10 min at 70 ℃. Afterwards, 4µL of reaction buffer (5× First strand buffer), 2 µL of 0,1M DDT, 1 µL of 10 mM dNTP (RNAse free), 1 µL of RNAse inhibitor (RNAseOUT™; 40 U/µL) and 1 µL of reverse transcriptase (SuperScript® II Rnase H^- Reverse Transcriptase; 200 U/µL) were added to each reaction tube and incubated for 50 min at 42 ℃. Afterwards, the enzyme was ina ctivated by incubation at 70 ℃ for 15 min. The mixture was then stored at -20 ℃.

5.3 PCR

The polymerase chain reaction (PCR) is a technique that generates thousands to millions of copies of a particular DNA from a single or a few copies of a DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification (Mullis et al. 1986).

Unless stated otherwise, in all following PCR experiments the basic composition of the reaction mixture (25 µL/reaction) was as follows:

12.5 µL of 2× PPP Master mix
1 µL of 10 mM forward primer
1 µL of 10 mM reverse primer
1 µL of cDNA
9.5 µL of H_2O,

where 2× PPP Master mix (Top-Bio) is a ready-to-use solution designed for rapid preparation of reaction mixture containing all the necessary components, including a heat-stable polymerase (Taq-Purple DNA polymerase; 100 U/mL).

For amplification of desired cDNA fragments the following PCR program was used:

1) initialization step – 94 ℃/3 min
2) denaturation step – 94 ℃/30 sec
3) annealing step – 58 ℃/40 sec
4) extension step – 72 ℃/90 sec
5) final elongation – 72 ℃/10 min
6) final hold – 4 ℃,

where steps 2-4 were repeated 35x.

Products of PCR reactions were analyzed in 1.1 % agarose gel prepared in 1× TAE buffer, which was stained with SYBR® Safe DNA gel stain (10000× concentrate in DMSO; Invitrogen). GeneRuler™ Express DNA Ladder (Fermentas) was used as marker.

In this study, two genes coding for immunologically active proteins were subjected to sequencing and compared: CCF and lysozyme. The first step was to sequence middle parts of these genes using simple PCR technique with a set of primer specific for each gene. The primers were designed on the bases of sequences already published in gene database NCBI (CCF accession no. AF030028.1; lysozyme accession no. DQ339138.1) and their sequences are given in the Table 1. The product molecules were subsequently cloned in pCR2.1-TOPO (Chapter 5.5) and sequenced.

5.3.1 Real-time PCR

To measure the levels of CCF, fetidin, lysenin and lysozyme mRNA in coelomocytes from *E. fetida* and *E. andrei* earthworms, a quantitative real-time PCR assay was performed using the iCycler^{TM} iQ5^{TM} real-time PCR detection system (Bio-Rad).

The cDNA samples of the two species synthesized as described above (see chapter 5.2.3) were used for iQ^{TM} SYBR® green (Biorad) real-

time PCR. The composition of reaction mixture in real-time PCR experiment was as follows:

12.5 µL of iQ^{TM} SYBR® green supermix (Biorad)
1 µL of 10 mM forward primer
1 µL of 10 mM reverse primer
4 µL of 20xdiluted cDNA
6.5 µL of H_2O

'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:

1) initialization step – 95 ℃/5 min
2) denaturation step – 95 ℃/60 sec
3) annealing step – 58 ℃/45 sec
4) extension step – 72 ℃/70 sec
5) final elongation – 72 ℃/7 min
6) final hold – 4 ℃,

where steps 2-4 were repeated 40x.

Quantitative measurements were normalized using mRNA levels of *E. andrei* ribosomal protein L17 (RPL 17) as a housekeeping gene (GenBank accession no. BP524444). Gene-specific primers used in this experiment are listed in the Table 2.

The differences in the threshold cycle values (C_T) of CCF, fetidin, lysenin or lysozyme and the internal control ribosomal protein L17 were calculated. The SYBR® green assay was done in duplicates and repeated in three independent experiments.

Primer	Gene	Sequence (5´→3´)	Direction	Gene occurrence
A1	CCF	ATCAATATCACATCGTCTGGCAG	sense	55-78
110	CCF	TCAGTTGCGCTTGTAGACTCG	antisense	1134-1155
RT-Ly1	lysozyme	GGACGAGTGACGGAGAGAAG	sense	97-117
RT-Ly2	lysozyme	CAGTAGGGTTCCTTGATTTGG	antisense	370-390

Table 1. PCR primers used in PCR experiments from Chapter 5.3.

Primer	Gene	Sequence (5´→3´)	Direction	Gene occurrence	Product size
RPL17 for	RPL17	GCAGAATTCAAGGGACTGGA	sense	83-103	159
RPL17 rev	RPL17	CTCCTTCTCGGACAGGATGA	antisense	241-261	
RT-CCF 1	CCF	CTTCACCGACTGGGATCAAT	sense	50-70	296
RT-CCF 2	CCF	CGTTGTTGTCCGTATTCGTG	antisense	326-346	
Lys2up	lysenin/fetidin	AAGCATGCGGACAGGAAGGAGTAT	sense	lysenin 648-671 fetidin 604-627	397
VAV2	lysenin	CCCCATTTCAGGGGTTAACCAACCA	antisense	1021-1045	
VAV3	lysenin	GCTATTACAATCTACACCGC	antisense	1133-1154	Lys2up+ VAV3 506
Lys3up	lysenin/fetidin	CAGCGCTGGGCAATCAATAAGTCA	sense	lysenin 864-887 fetidin 820-843	Lys3up+VAV4 490
VAV4	fetidine	TGGTTGTACACTTGGTAAAG	antisense	1291-1310	Lys2up+ VAV4 706
NELA3	lysozyme	GCCATTCCAAATCAAGGAAC	sense	147-167	129
NELA4	lysozyme	TAGGTACCGTAGCGCTTCAT	antisense	256-276	

Table 2. Primers used in real-time PCR experiment (Charter 5.3.1).

5.4 RACE

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a mRNA template between a defined internal site and either the 3´ or the 5´ end of the mRNA. This methodology of amplification with single-sided specificity has been described as “one-sided” PCR (Ohara et al. 1989) or “anchored” PCR (Loh et al. 1989). For synthesis of the 3´ and the 5´ end of the mRNA commercial kits (3´ RACE System for Rapid Amplification of cDNA Ends, 5´ RACE System for Rapid Amplification of cDNA Ends; Invitrogen) were used according to manufacturer´s protocol.

5.4.1 3´RACE

The 3´ RACE protocol takes advantage of the natural poly(A) tail found in mRNA as a generic priming site for PCR. In this procedure, mRNAs are converted into cDNA using reverse transcriptase and an oligo(dT) adapter primer. Specific cDNA is then amplified by PCR using a gene-specific primer (GSP) that anneals to a region of known exon sequences and an adapter primer that targets the poly(A) tail region. This permits the capture of unknown 3´-mRNA sequences that lie between the exon and the poly(A) tail.

Three µg of total RNA from each sample were combined with DEPC H_2O to a final volume of 11 µL. Then, 1 µL of primer (Adapter primer AP, Invitrogen; 10 mM) was added and the solution was incubated for 10 min at 70 ℃. Afterwards, 2 µL of reaction buffer (10× PCR buffer), 2 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTP, 2 µL of 0.1 M DTT and 1 µL of reverse transcriptase (SuperScript™ II RT; 200 U/µL) were added and the solution was incubated for 50 min at 42 ℃. The reaction was terminated by incubating the mixture for 15 min at 70 ℃. The RNA template was removed from the cDNA:RNA hybrid molecule by adding 1 µL of RNAse H (2 U/ µL) and incubation for 20 min at 37 ℃.

Two µL of the mixture were then used in the specific amplification of the target cDNA. Ten mM gene specific primers used in PCR reaction

are listed in the Table 3. As antisense primer in each reaction Abridged Universal Amplification Primer (AUAP, Invitrogen; 10 mM) was used. The end products were cloned in pCR2.1-TOPO and sequenced (Chapter 5.5).

5.4.2 5´RACE

The 5' RACE protocol, or “anchored” PCR, is a technique that facilitates the isolation and characterization of 5' ends. First strand cDNA synthesis is primed using a gene-specific antisense oligonucleotide (GSP1). This permits cDNA conversion of specific mRNA, or related families of mRNAs, and maximizes the potential for complete extension of the 5' -end of the message. Following cDNA synthesis, the first strand product is purified from unincorporated dNTPs and GSP1. TdT (Terminal deoxynucleotidyl transferase) is used to add homopolymeric tails to the 3' ends of the cDNA. Tailed cDNA is then amplified by PCR using a mixture of two primers: a gene-specific primer 2 (GSP2), which anneals 3' to GSP1; and a complementary homopolymer-containing primer which permits amplification from the homopolymeric tail. This allows amplification of unknown sequences between the GSP2 and the 5'-end of the mRNA.

Three µg of total RNA, 1 µL of 10 mM GSP1 (all primers used in 5' RACE reactions are listed in the Table 3) and DEPC H_2O were combined to a final volume of 15.5 µL. The mixture was incubated for 10 min at 70 ℃. Afterwards, 2.5 µL of reaction buffer (10 × PCR buffer), 2.5 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTP, 2.5 µL of 0.1 M DTT and 1 µL of reverse transcriptase (SuperScript^{TM} II RT; 200 U/ µL) were added and the solution was incubated for 50 min at 42 ℃. The reaction was terminated by incubating the mixture for 15 min at 70 ℃. After cDNA synthesis, RNase mix, a mixture of RNase H and RNase T_1, was used to degrade the RNA. One µL of the RNAse mix was added to the reaction tube and the solution was incubated for 30 min at 37 ℃.

For the following purification of the first strand products a commercial kit QIAquick PCR purification kit (QIAGEN) was used according to manufacturer´s protocol. Firstly, 5 volumes of binding buffer

(Buffer PB) were mixed with one volume of the PCR sample, transferred on a QIAquick column and centrifuged (1 min, 13000×g, 4 ℃). The column was washed with 0.75 mL of washing buffer (buffer PE) and centrifuged (1 min, 13000×g, 4 ℃). The column was then transferred to a clean microcentrifuge tube and DNA was eluted by addition of 20 µL of H_2O and centrifugation (2 min, 13000×g, 4 ℃).

Ten µL of eluted DNA were used in the cDNA tailing. DNA was combined with 5 µL of reaction buffer (5× Tailing buffer), 2.5 µL of 2 mM dCTP and 6.5 µL of DEPC H_2O and incubated for 3 min at 94 ℃. Afterwards, 1 µL of terminal deoxynucleotidyl transferase (TdT) was added to the reaction tube and the mixture was incubated for 10 min at 37 ℃. The reaction was terminated by incubation at 65 ℃ for 10 min.

Tailed cDNA (2.5 µL) obtained from the preceding protocol was amplified directly by PCR using 10 mM GSP2 (listed in the table 3). As sense primer in each rection Abridged Anchor Primer (AAP, Invitrogen; 10 mM) was used. The final products were subsequently ligated into pCR2.1-TOPO cloning vector and sequenced (Chapter 5.5).

5.5 Cloning and bacterial transformation

To clone the final products of 3´RACE, 5´RACE and PCR a highly efficient cloning system TOPO TA Cloning® (Invitrogen) was used according to manufacturer´s protocol. This system provides a one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR product into a plasmid vector (Shuman 1994). The plasmid vector (pCR®2.1-TOPO®) is supplied linearized with topoisomerase I covalently bound to the vector and single 3´-thymidine overhangs. *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine to the 3´ ends of PCR products. This allows PCR inserts to ligate efficiently with the vector.

Once ligated, the vector was transformed into chemically competent cells (One Shot® TOP10F´ Chemically Competent *E. coli*, Invitrogen).

Primer	Reaction	Gene	Sequence (5´→3´)	Direction	Gene occurrence
A1	3´RACE-GSP	CCF	ATCAATATCACATCGTCTGGCAG	sense	55-78
RT-Ly1	3´RACE-GSP	lysozyme	GGACGAGTGACGGAGAGAAG	sense	97-117
AP	3´RACE		GGCCACGCGTCGACTAGTAC$(T)_{16}$	antisense	
AUAP	3´RACE		GGGCACGCGTCGACTAGTAC	antisense	
110	5'RACE-GSP1	CCF	TCAGTTGCGCTTGTAGACTCG	antisense	1134-1155
A4	5'RACE-GSP2	CCF	TTTCGTATTGAGGAAGCCAG	antisense	852-872
RT-Ly2	5'RACE-GSP1	lysozyme	CAGTAGGGTTCCTTGATTTGG	antisense	370-390
NELA4	5'RACE-GSP2	lysozyme	TAGGTACCGTAGCGCTTCAT	antisense	256-276
AAP	5' RACE		GGCCAGGCGTCGACTAGTACGGGIIGGGIIGGGIGG	sense	

Table 3. Primers used in RACE procedure (Chapter 5.4).

This strain overexpresses the Lac repressor (*lacZ*α gene). For blue/white screening, addition of IPTG to the plates is needed to obtain expression from the *lac* promoter. This strain contains the F episome and can be used for single-strand rescue of plasmid DNA containing an f1 origin.

In order to detect a successful ligation of insert into vector blue/white screening was used. Its molecular mechanism is based on a genetic engineering of the *lac* operon in TOP10F´ serving as a host cell combined with a subunit complementation achieved with the cloning vector pCR®2.1-TOPO®. The vector encodes the α subunit of LacZ protein with an internal multiple cloning site, while the chromosome of the host strain encodes the remaining Ω subunit to form a functional β-galactosidase enzyme. The foreign DNA can be inserted within the *lacZ*α gene, thus disrupting the production of functional β-galactosidase. When incubated with X-gal, a colourless modified galactose sugar that is metabolized by β-galactosidase, the functional enzyme hydrolyzes the sugar causing the characteristic blue colour in the colonies; it shows that the colonies contain vector without insert. White colonies indicate insertion of foreign DNA and loss of the cells' ability to hydrolyse the marker.

5.5.1 TOPO TA cloning

The purification of PCR products using QIAquick columns (Chapter 5.4.2) was a preliminary step for cloning. Once purified, 1 µL of cDNA was combined with 1 µL of TOPO® vector, 1 µL of salt solution and 3 µL of water and incubated at room temperature for 15 min.

5.5.2 Transformation of TOP10F´ competent cells

Two µL of vector solution were transferred directly to a vial with competent TOP10F´ *E. coli* cells and the vial was incubated for 30 min on ice. Then, the cells were heat-shocked for 30 sec at 42 ℃ and immediately transferred on ice. 250 µL of S.O.C. medium were added. The vial was placed in a shaking incubator (200 rpm) at 37 ℃ for 1 hour. 50 µL of each transformation were spread on selective LB plates containing

ampicilin (50 µg/mL) as well as 40 µL of X-Gal (40 mg/mL) and 40 µL of IPTG (23.8 mg/mL) spread on the surface of each plate for subsequent blue/white screening of transformed clones. The plates were incubated overnight at 37 ℃.

If the ligation was successful, the bacterial colony was white, if not, the colony was blue. Approximately 10 white colonies from each transformation were picked for further analysis. Each colony was cultured overnight in 96-well plates at 37 ℃ in 100 µL LB m edium containing 50 µg/ml ampicilin. Even though blue/white screening was used to determine whether inserts were present, colony PCR determined whether the insert size was of expected length.

The composition of reaction mixture in colony PCR was as follows:

12.5 µL of 2× PPP Master mix
1 µL of forward primer (10 mM M13 sense)
1 µL of reverse primer (10 mM M13 antisense)
9.5 µL of H_2O
1 µL of bacterial suspension overnight cultured

For amplification of desired inserts the following PCR program was used:

1) initialization step – 94 ℃/10 min
2) denaturation step – 94 ℃/45 sec
3) annealing step – 58 ℃/45 sec
4) extension step – 72 ℃/90 sec
5) final elongation – 72 ℃/8 min
6) final hold – 4 ℃,

where steps 2-4 were repeated 35x.

Products of PCR reactions were analyzed in 1.1 % agarose gel. The colonies with inserts of desired size were picked, their plasmids were purified and sequenced.

5.5.3 Plasmid purification and DNA sequencing

For plasmid purification a commercial kit FastPlasmid Mini Kit (Eppendorf) was used according to manufacturer´s protocol. 3 mL of bacterial culture were centrifuged (1 min, 13000×g, 4 ℃). The supernatant was eliminated by decanting and 500 µL of ice-cold lysis buffer (Complete Lysis buffer) were added to the pellet. The tube was vortexed until the pellet became completely resuspended and the tube was incubated at room temperature for 3 min. The lysate was transferred on a spin column and centrifuged (1 min, 13000×g, 4 ℃). The column was then washed by adding of 400 µL of washing buffer (Wash buffer) and centrifuged (1 min, 13000×g, 4 ℃). The spin column was transferred to a collection tube and DNA was eluted.

Isolated and purified plasmid DNA was sequenced with 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 according to manufacturer's protocol. Finally, sequences were determined using ABI PRISM 3100 DNA sequencer (Applied Biosystems).

The full length cDNA sequence was obtained by combination of aquired sequences of PCR and RACE products. The resulting sequences were analyzed using BLAST.

5.6 Determination of coelomic fluid protein concentration

Protein concentration of the coelomic fluid was determined by Lowry assay using commercial kit DC Protein Assay (Bio-Rad).

The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps leading to color development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins effect a reduction of the Folin reagent

by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 405 nm (Lowry et al. 1951).

To determine protein concentration a microplate 96-well plates assay was used according to manufacturer´s protocol. 5 µL of coelomic fluid diluted in PBS 1:10 was combined with 25 µL of solution A containing alkaline copper tartrate and 200 µL of solution B, which is the diluted Folin reagent. The samples were incubated at room temperature for 15 min and subsequently absorbance at 680 nm was measured with Microplate reader EL800 (BioTek).

Calibration curve was prepared from solutions of Bovine Serum Albumin (BSA, Sigma) in concentration range 0–2 mg/mL. Protein concentration of each sample was determined using linear regression. The assay was done in duplicates.

5.7 Cytolytic assay

In this assay L929 fibrosarcoma TNF-sensitive murine fibroblast cell line was used. Cells were cultivated in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 2 mM glutamine, 1 × 10^6 U/l of penicillin, 100 mg/l of streptomycin and 250 µg/l of amphotericin B in a cell incubator at 37 ℃ and 5 % CO_2 atmosphere. After a continuous layer of cells was established in the culture vessel (Nunc SoLo Flasks Nunclon ™Δ; Thermo) the supernatant was eliminated and 10 mL of 0.25 % Trypsin-EDTA (Sigma) solution was added. The vessel was incubated at 37 ℃ for 10 min and 20 mL of RPMI-1640 was added. Cell solution was transferred to a centrifuge tube and centrifuged (10 min, 250×g). The pellet was resuspended in 1 mL of medium and cells were counted in Bürker counting chamber. Afterwards, cells were either used in the experiment or cultured in a new culture vessel.

Hundred µL of 10^6 cells/mL cell suspension was adhered in 96-well flat-bottomed culture plates (Microwell 96F Nunclon D) for 1 hour at 37 ℃. Then 100 µL of serially diluted (protein concentration range

0-1.5 mg/mL) coelomic fluid were added into each well and incubated for 16 hours at 37 ℃. Cells were than fixed and stained using 100 µL of a 0.5 % solution of crystal violet dissolved in 22 % ethanol and 8 % formaldehyde for 10 min at room temperature. The plates were rinsed in water, 100 µL of 30 % acetic acid were added and dye uptake was measured at 620 nm using Microplate reader EL800 (BioTek). The lysis percentage was subsequently determined using linear regression. The cytolytic assay was done in duplicates and repeated in three independent experiments.

5.8 Hemolytic assay

Hemolytic activity of coelomic fluid was tested in 96-well round-bottomed microtiter plates (type U). Samples of *E. andrei* or *E. fetida* coelomic fluid with or without inhibitor of serine proteases Complete™ (Roche) were diluted in 145 mM NaCl (pH 7.4). Starting dilution of *E.fetida* CF was 1:100 while *E. andrei* CF dilution started at 1:1000. The samples were further diluted serially with dilution index 2. Hundred µL of each diluted sample were combined in 96-well plates with 100 µL of 3 % sheep erythrocyte suspension and incubated for 2 hours at room temperature. The plates were centrifuged (10 min, 100×g, 4 ℃), 100 µL of supernatants were transferred to a micro-titer flat-bottomed plate and absorbance at 405 nm was measured using Microplate reader EL800 (BioTek). The percentage of hemolysis was subsequently determined using linear regression. The hemolytic assay was done in duplicates and repeated in three independent experiments.

5.9 Lysoplate assay

To evaluate the lysozyme activity a lysoplate assay was performed according to a modified protocol by Lie (Lie et al. 1986). A solution of 1 % agarose in 50 mM phosphate buffer (monosodium phosphate monohydrate and disodium phosphate heptahydrate, pH 6.0) containing 1 mg/mL lyophilized *Micrococcus lysodeicticus* (Sigma) was prepared. Samples

of *E. andrei* or *E. fetida* coelomic fluid were serially diluted with dilution index 2 in PBS (final protein concentrations of samples were 10, 5, 2.5 and 1.25 mg/mL). Five µL of each sample as well as 5 µL of standard (5 mg/ml hen egg white lysozyme; Roche) were placed on Petri dish and incubated at 37 ℃. The diameter of lysed zone (mm) was measured after 24 hours. The assay was done in duplicates and repeated in three independent experiments.

5.10 Protease assay

To evaluate the protease activity of coelomic fluid a commercial kit QuantiCleave™ protease assay kit (Pierce) was used according to manufacturer´s protocol.

The assay method uses succinylated casein and trinitrobenzenesulfonic acid (TNBSA). Succinylated casein is native casein that has been treated with succinic anhydride to block primary amines on the surface of the protein. In the presence of protease, the succinylated casein is cleaved at peptide bonds, thereby exposing primary amines (predominantly α-amines). TNBSA reacts with these exposed primary amines to produce an orange-yellow product which intensity is measured at 450 nm. The assay method requires use of a control blank for each unknown and standard sample. Each blank contains buffer and protease sample (unknown or standard) but no Succinylated Casein Solution. The increase in color relative to sample without succinylated casein is a measure of protease activity in the sample.

Hundred µL of succinylated casein solution was added to one set of microplate wells, while 100 µL of assay buffer serving as blank were added to another set of microplate wells. Samples of coelomic fluid were diluted in PBS 1:1000, 1:10000 and 1:100000 and 50 µL were added to both succinylated casein wells and corresponding blank wells. The plate was incubated at 37 ℃ for 20 min. Afterwards, 50 µL of TNBSA Working solution were added to each well and the plate was incubated for 20 min at room temperature. Absorbance at 450 nm was measured using Microplate reader EL800 (BioTek) and the change of absorbance ΔA_{450} for each sample was

calculated. ΔA_{450} is the absorbance generated by the proteolytic activity of the protease.

Calibration curve was prepared from solutions of trypsin in concentration range 0-0.5 mg/mL. Standard curve was plotted using logarithmic scale and used to assess relative protease activity of the samples. The protease assay was done in duplicates and repeated in three independent experiments.

5.11 Polyacrylamide gel electrophoresis

5.11.1 SDS-PAGE

Electrophoresis in 12 % polyacrylamid gel containing anionic detergent SDS was run vertically according to a modification of the Laemmli method (1970).

12 % running gel solution was prepared according to the following protocol:

H_2O	3.3 mL
Rotiphorese® Gel 30	4 mL
1.5 M Tris (pH 8.8)	2.5 mL
10 % SDS	0.1 mL
10 % APS	0.1 mL
TEMED	0.004 mL

Stacking gel solution:

H_2O	2.7 mL
Rotiphorese® Gel 30	0.67 mL
1.5 M Tris (pH 6.8)	0.5 mL
10 % SDS	0.04 mL
10 % APS	0.04 mL
TEMED	0.004 mL

Individual samples of non diluted coelomic fluid (3 µL, 5 µL, 10 µL combined with H_20 to a final volume of 10 µL) were mixed with 10 µL of reducing (0.5 M Tris, 10 mM 2-mercaptoethanol, 10 % SDS, 20 % glycerol, 0.01 % bromophenol blue) or non-reducing (0.5 M Tris, 10 % SDS, 20 % glycerol, 0.01 % bromophenol blue) sample buffer and boiled for 5 min. Five µL of PageRuler™ Plus Prestained Protein Ladder (Fermentas) with a range of 10-250 kDa was used as molecular weight standard.

The proteins migrated in running buffer (0.25 M Tris, 0.2 M glycin, 0.1 % SDS) under constant electric current of 35 mA for about two hours until the blue dye front reached the bottom.

Afterherwards, the gels were stained with Coomassie® Brilliant Blue R-250 solution (50 % methanol, 10 % acetic acid, 0.2 % Coomassie® Brilliant blue R-250). After 2 hours of incubation with the staining solution, the gels were washed various times in destaining solution (40 % methanol, 10 % acetic acid).

5.11.2 Native and substrate gel electrophoresis

Analysis of proteases by substrate gel electrophoresis was performed by the method of Heussen and Dowdle (1980) with modifications.

Electrophoresis was performed in native conditions (without the presence of SDS) according to the protocol described above. However, BSA (Sigma) was added to one of 12 % running gels at 0.5 %. Samples were prepared by combining 2 µL or 5 µL of non diluted coelomic fluid with 10 µL of sample buffer (0.5 M Tris, 10 % SDS, 20 % glycerol, 0.01 % bromophenol blue) and H_2O to a final volume of 20 µL. In parallel, corresponding control samples containing serine protease inhibitor Complete^{TM} (Roche) was prepared. Five µL of PageRuler™ Prestained Protein Ladder (Fermentas) with a range of 10-170 kDa was used as molecular weight standard. The proteins migrated in running buffer at 0 ℃ for about two hours until the blue dye front reached the bottom.

After separation, the gel containing BSA was incubated in an ice-cold solution containing 0.2 M boric acid, 0.1 M sodium chloride and 1 % Triton X-100 for 13 min. Afterwards, it was briefly washed in a 0.2 M solution

of boric acid containing 0.1 M sodium chloride for 3 min and incubated again for 20 min at 37 ℃ in the first solution. Then, to gether with the gel that did not contain BSA, it was stained using Coomassie® Brilliant Blue R-250 solution. Afterwards, only the native gel was destained using destaining solution (10 % methanol, 10 % acetic acid).

5.12 Chemicals used in experiments

- 2-mercaptoethanol (Fluka)
- 50× TAE buffer (5 PRIME)
- Acetic acid (LACHEMA)
- Agar (Difco)
- Agarose I (Amresco)
- Ammonium persulfate (APS; SERVA)
- Ampicilin (Sigma)
- Boric acid (LACHEMA)
- Bovine serum albumin (BSA; Sigma)
- Bromo-chloro-indolyl-galactopyranoside (X-Gal; Sigma)
- Bromphenol blue (SERVA)
- Chloroform (Sigma)
- Complete protease inhibitor coctail tablets (Roche)
- Coomassie® Brilliant Blue R-250 (SERVA)
- Crystal violet (SERVA)
- Diethyl pyrocarbonate (DEPC; Sigma)
- Disodium phosphate heptahydrate (LACHEMA)
- Ethanol (MERCK)
- Ethylenediaminetetraacetic acid (EDTA, Fermentas)
- Fetal bovine serum (FBS; Sigma)
- Formaldehyde (Sigma)
- Glycerol (LACHEMA)
- Hydrochloric acid (LACHEMA)
- Isopropanol (Sigma)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma)
- LB Broth (Sigma)

- L-glutamine (Sigma)
- Lysozyme (Roche)
- Methanol (Sigma)
- *Micrococcus lysodeicticus* (lyophilized; Sigma)
- Monosodium phosphate monohydrate (LACHEMA)
- Penicillin-Streptomycin solution (Sigma)
- Phosphate buffered saline (PBS; Sigma)
- Rotiphorese® Gel 30 (37,5:1) (Roth)
- RPMI-1640 Medium (Sigma)
- Sodium chloride (LACHEMA)
- Sodium dodecyl sulfate (SDS; SERVA)
- Sodium hydroxide (LACHEMA)
- SYBR® Safe DNA gel stain (10000× concentrate in DMSO; Invitrogen)
- Tetramethylethylenediamine (TEMED; SERVA)
- Tris-(hydroxymethyl)-aminomethane (Tris; TRIZMA® Base; Sigma)
- Triton X-100 (Sigma)
- Trizol (TRIZOL® Reagent, Invitrogen)
- Trypan Blue Solution (0,4%; Sigma)
- Trypsin-EDTA (Sigma)

5.13 Laboratory equipment

- Analytical balance Adventurer Pro (Ohaus)
- Biometra personal cycler 48 (Biometra)
- Centrifuge Jouan MR22i (Jouan S.A.)
- CO_2 incubator MCO-18AIC(UV) (Sanyo)
- iCycler^{TM} iQ5^{TM} real-time PCR detection system (Bio-Rad)
- Mastercycler® Pro (Eppendorf)
- Microplate reader EL800 (BioTek)
- Mini protean II gel kit (Bio-Rad)
- Minicentrifuge Eppendorf Minispin Plus (Eppendorf)
- Nanodrop 2000C (Thermo)
- pH meter 3305 (Jenway)
- Power supply PS-300B (Hoefer)

- Shaking incubator NB-205 (N-NIOTEK.INC.)
- Thermoblock TB2 Biometra (Biometra)
- Watherbath nb5 (Nuve)

5.14 Enzymes and commercial kits

- 2× PPP Master mix (Top-Bio)
- 3´ RACE System for Rapid Amplification of cDNA Ends (Invitrogen)
- 5´ RACE System for Rapid Amplification of cDNA Ends (Invitrogen)
- DC Protein assay (Bio-Rad)
- DNAse I (Fermentas; 1U/ µL)
- FastPlasmid Mini Kit (Eppendorf)
- iQ™ SYBR® green supermix (Biorad)
- QIAquick PCR purification kit (QIAGEN)
- QuantiCleave™ protease assay kit (Pierce)
- SuperScript II® First-Strand Synthesis System for RT-PCR (Invitrogen)
- TOPO TA Cloning® (Invitrogen)

6 Results

6.1 Sequence comparison of selected immunologically active compounds

The full-length cDNA sequences of two immunologically active proteins, CCF and lysozyme of *E. andrei* and *E. fetida* were assembled and subsequently aligned.

Total RNAs from coelomocytes of either *E. andrei* or *E. fetida* were isolated and reverse-transcribed. Using specific primers designed on the basis of known nucleotide sequences of CCF and lysozyme, fragments of approximate length of 1100 bp and 300 bp, respectively, were amplified. In order to assemble the full-length cDNAs sequence, RACE amplifications of 5´ and 3´ ends were performed. Resulting PCR products were cloned into pCR®2.1-TOPO® vector and subsequently TOP10F´ bacterial cells were transformed. Using blue/white screening and colony PCR, colonies with successfully ligated inserts of desired lengths were picked, plasmids were purified and fragments sequenced. Finally, the full-length cDNA sequences and deduced amino acid sequences of both molecules were aligned using the BLAST program.

6.1.1 CCF

The cDNA of *E. andrei* CCF with an open reading frame of 1155 bp encodes for 384 amino acids, while *E. fetida* CCF has an open reading frame of 1158 bp encoding for 385 amino acids. The alignment of nucleotide and deduced amino acid sequences is shown in Fig. 4a and 4c, respectively. Both cDNA and amino acid sequences of CCF from the two earthworm species display high homology (94 % and 95 %, respectively).

6.1.2 Lysozyme

The full-length lyzozyme cDNA sequences of the two earthworm species are aligned in the Figure 4b. They display 90 % sequence identity. *E. andrei* nucleotide sequence with an open reading frame of 480 bp encodes 160 amino acids. *E. fetida* lysozyme´s ORF has 492 bp and encodes 164 amino acids. Compared to *E. andrei* lysozyme, there are four extra amino acids in N-terminal part of *E. fetida* peptide. The alignment of lysozyme amino acid sequences is shown in the Figure 4d.

```
E. andrei   1     atgaggtggactttggtggtcttgtgtctgctgttcggtgaagggt///tcgccttcaccgactgggatcaatatcacatcgtctggcaggacgaattcg
E. fetida   1     ---------g--gca----------c--ct--c-------g--a-----------------------------------------------g----

E. andrei   98    attactttgatggcgcgaagtggcaacatgaggtcacagcaactggcggagggaacagcgaattccaactgtacacacaggatggggccaacagcttcgt
E. fetida   101   ----------c----------------g------------t-----------------------------------g-----c----------------

E. andrei   198   tcgagatggaaagcttttcattaagccgacgttgctggctgacaacatcaacccacagacgggtgcgccatttggaaccgatttcatgtacaatggagtt
E. fetida   201   -----------------a-------------------------------------g--------c-----g-----------------------------

E. andrei   298   ctagatgtctgggctatgtacgggacctgcacgaatacggacaacaacggatgctacaggacgggagccgctggcgacattccaccggccatgtcggcac
E. fetida   301   ----------------------tg---------------------------------t-------------------------g----t---------g-

E. andrei   398   gagttcgaaccttccagaaatacagcttcacccacggacgcgttgtcgttcacgccaagatgcccgtcggagactggctctggccagccatttggatgtt
E. fetida   401   ----g------------g--------------------a--------cag-----------------------------------------------

E. andrei   498   gccggaggattgggtctatggcggatggcctcgatcgggcgagatcgacatcattgaaacaatcggcaaccgagatttcaagaacactggtggagagttc
E. fetida   501   -----------------c-----------------------------------------g-------t--------------------c-----------

E. andrei   598   cttggaattcagaagatgggatcaacgatgcactggggtccaggatgggacgacaaccgatactggctgaccagccttccgaaacactcagacgactgga
E. fetida   601   ------------------------------------------------------------------t---------------------------------

E. andrei   698   actacggtgacaacttccacacgttctggttcgactggagtcccaacggactgaggttcttcgtagatgacgagaaccaggctctgctcgatgttccata
E. fetida   701   -------a--------------------------------c--------------------a----c--------------------t--------t--

E. andrei   798   tcctctgattgatgcgaatccatggtgggtggatttctgggagtggggaaagccctggcttcctcaatacgaaaatgacaatccatgggctggaggaacg
E. fetida   801   ---------------------------------c--------------g-----------------------------t---------------------

E. andrei   898   aacctggctcccttcgaccaaaatttccatttcattctgaacgtggccgttggaggaacgaacggcttcatcccggacggttgcatcaatcgcggcggaa
E. fetida   901   ---t---------------------------------------t-----c-----------t-----------a--t------g--------------g

E. andrei   998   acccggccctgcagaagccgtggagcaatggtgactggtacaacgatgcaatgaggaaattcttcgacgccagagggaactggaagtggacgtgggatga
E. fetida   1001  -----------------a-------------------------------------------------t-------------------------------

E. andrei   1098  cgagggagacaacaatgccatgcaggtcgattacatccgagtctacaagcacaactga
E. fetida   1101  -----------------------------c---c----------------------
```

Fig. 4a. Sequence alignment of nucleotide sequences of CCF molecules of *E. andrei* and *E. fetida*. Nucleotides that match are indicated by a dash, gaps are indicated by a slash.

```
E. andrei   1    atgtttatctactttgcgctgtcatgtatcttggccaccgcggcagctcaaatctcggaaaactgccttaattgcatctg
E. fetida   1    ----------t----------------c----------a--------c-----------------tt-g----------

E. andrei   81   ccagatcgaaggatgtgagagccagatcggaaagtgtcgcatggatgtcggatctctgagctgcgggccattccaaatca
E. fetida   81   t---g-g---------c----------------------------c--t-----a--------t---------------

E. andrei   161  aggaaccctactggattgactgcggcagacctggaggagactggaagtcatgtacaactcagatggactgctcgcggaca
E. fetida   161  -------a----------------------c-----------------g----------------c---------c---

E. andrei   241  tgtgtcaggagctacatgaagcgctacggtacctattgcactggcggccgagctcccacctgccaggattacgcccgcat
E. fetida   241  -------------------c--------------------c--g----------t--------a--c-----t-----

E. andrei   321  ccacaacggaggtcccaaaggctgccaacatgcgagtaccgtcggttactggaacaaggtgaagcagtgctgctcatcga
E. fetida   321  -----------------g----------ag-t----------------------g------c-a---------g-----t

E. andrei   401  aacccggcggctgtggtttagaccacgaggttctgaggttcgaaggagtagacatcgaagaagacaccgtctatcgtcag
E. fetida   401  c----------------------------g-------a--gg-cg-------------g-----------------

E. andrei   481  taa
E. fetida   481  gcagaagacgtgtag
```

Fig. 4b. Sequence alignment of lysozyme molecules of *E. andrei* and *E. fetida*. Nucleotides that match are indicated by a dash.

```
E. andrei      1    MRWTLVVLCLLFGEG/FAFTDWDQYHIVWQDEFDYFDGAKWQHEVTATGG
E. fetida      1    ---AA------L---C------------------------R-------

E. andrei      50   GNSEFQLYTQDGANSFVRDGKLFIKPTLLADNINPQTGAPFGTDFMYNGV
E. fetida      51   ----------------------Y-------------------------

E. andrei      100  LDVWAMYGTCTNTDNNGCYRTGAAGDIPPAMSARVRTFQKYSFTHGRVVV
E. fetida      101  --------A-------------------V---------R----------

E. andrei      150  HAKMPVGDWLWPAIWMLPEDWVYGGWPRSGEIDIIETIGNRDFKNTGGEF
E. fetida      151  S-----------------------------------A------------

E. andrei      200  LGIQKMGSTMHWGPGWDDNRYWLTSLPKHSDDWNYGDNFHTFWFDWSPNG
E. fetida      201  --------------------------------------------------

E. andrei      250  LRFFVDDENQALLDVPYPLIDANPWWVDFWEWGKPWLPQYENDNPWAGGT
E. fetida      251  ----I---------------------------------------------

E. andrei      300  NLAPFDQNFHFILNVAVGGTNGFIPDGCINRGGNPALQKPWSNGDWYNDA
E. fetida      301  ----------------------------V----D----------------

E. andrei      350  MRKFFDARGNWKWTWDDEGDNNAMQVDYIRVYKHN  384
E. fetida      351  ----------------------------L------  385
```

Fig. 4c. Alignment of amino acid sequences of *E. andrei* and *E. fetida* CCF molecules. The polysaccharide-binding motif (underlined), the glucanase motif (double-underlined) and the signal peptide (dotted line underlined) are indicated.

```
E. andrei      1    MFIYFALSCILATAAAQISENCLNCICQIEGCESQIGKCRMDVGSLSCGP
E. fetida      1    ---F-----L-----------------V---Q----------------

E. andrei      51   FQIKEPYWIDCGRPGGDWKSCTTQMDCSRTCVRSYMKRYGTYCTGGRAPT
E. fetida      51   -------------------------A----------T-------------

E. andrei      101  CQDYARIHNGGPKGCQHASTVGYWNKVKQCCSSKPGGCGLDHEVLRFEGV
E. fetida      101  ------------R---SS------K--Q---A-S------------YGA-

E. andrei      151  DIEEDTVYRQ      160
E. fetida      151  ----------AEDV  164
```

Fig. 4d. Alignment of amino acid sequences of *E. andrei* and *E. fetida* lysozyme. The conserved cysteine residues of i-type lysozyme are highlighted with rectangles and the signal peptide is dotted line underlined.

6.2 Comparison of mRNA levels coding for selected immunological proteins

Levels of mRNAs coding for defense factors CCF, fetidin, lysenin and lysozyme were compared between *E. andrei* and *E. fetida*. Real-time PCR analysis using iQ5^{TM} real-Time PCR Detection System was used.

Quantitative measurements were normalized using RPL17 mRNA levels as housekeeping gene. The differences in the mean C_T values of lysozyme (CCF) and the internal control were calculated. Relative changes in the expression of lysozyme (CCF) were shown as the normalized lysozyme expression in *E. fetida* compared to that of *E. andrei*.

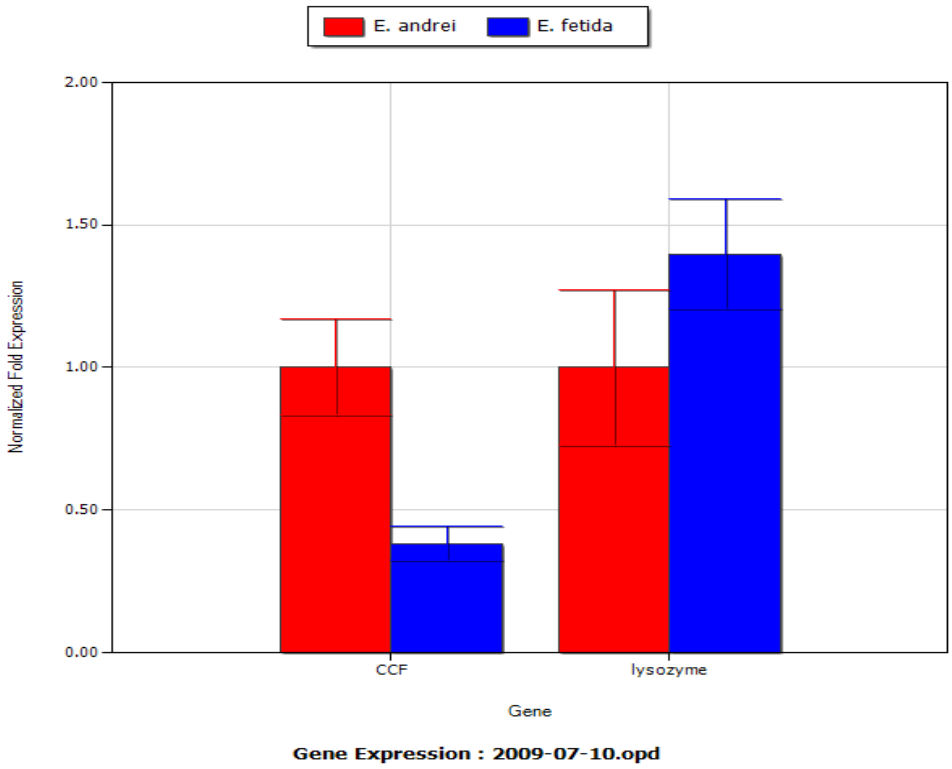

Fig. 5. Gene expression levels of CCF and lysozyme of *E. andrei* and *E. fetida* earthworms.

Expression levels of fetidin and lysenin are not included in the Figure 5, since none of these genes was expressed in *E. fetida* earthworm (Fig. 6). The level of CCF mRNA was slightly elevated in *E. andrei* earthworms, while there was more lysozyme mRNA expressed in *E. fetida* earthworms.

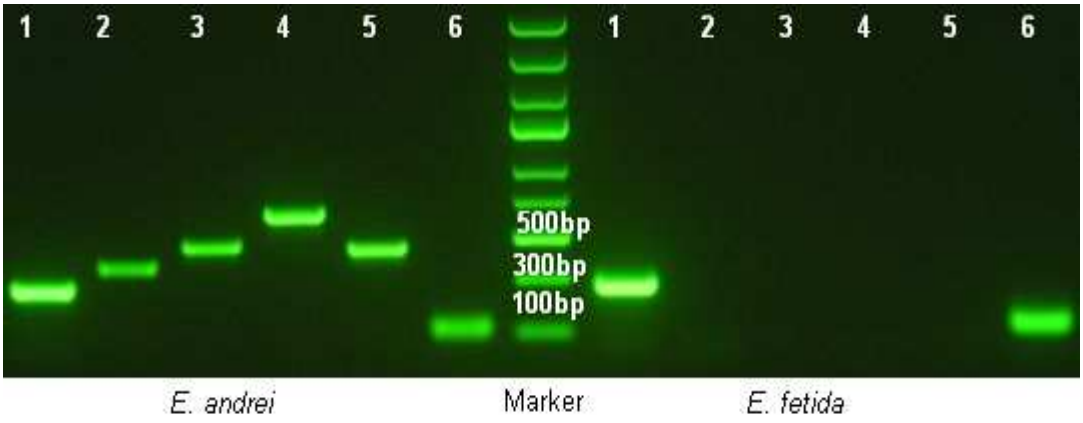

Fig. 6. Electrophoretic analysis of real-time PCR experiment. Numbers above lines indicate genes and the corresponding set of primers: 1) CCF (RT-CCF1 and RT-CCF2), 2) lysenin1 (Lys2up and VAV2), 3) lysenin2 (Lys2up and VAV3), 4) fetidin1 (Lys2up and VAV4), 5) fetidin2 (Lys3up and VAV4) and 6) lysozyme (NELA3 and NELA4).

6.3 Comparison of immunological activities of coelomic fluid

In order to compare the immunological features of coelomic fluid of *E. andrei* and *E. fetida* various bioassays were performed.

Using the Lowry method protein concentration of coelomic fluid was determined to be 12 ± 3 mg/mL. Electrophoretic analysis proved the presence of a large amount of proteins in both *E. andrei* and *E. fetida* coelomic fluid. Three, 5 and 10 µL of coelomic fluid were applied on a gel under both reducing and non-reducing conditions. After the electrophoretic separation gels were stained using Coomassie® Brilliant Blue R-250 solution (Fig. 7 and Fig. 8).

Electrophoretic analysis under non-reducing condition revealed many differences between coelomic fluid of *E. andrei* and *E. fetida*. Interestingly, these differences were not observed as significantly under reducing conditions suggesting that the quaternary protein structure of coelomic fluid proteins may differ between the compared species.

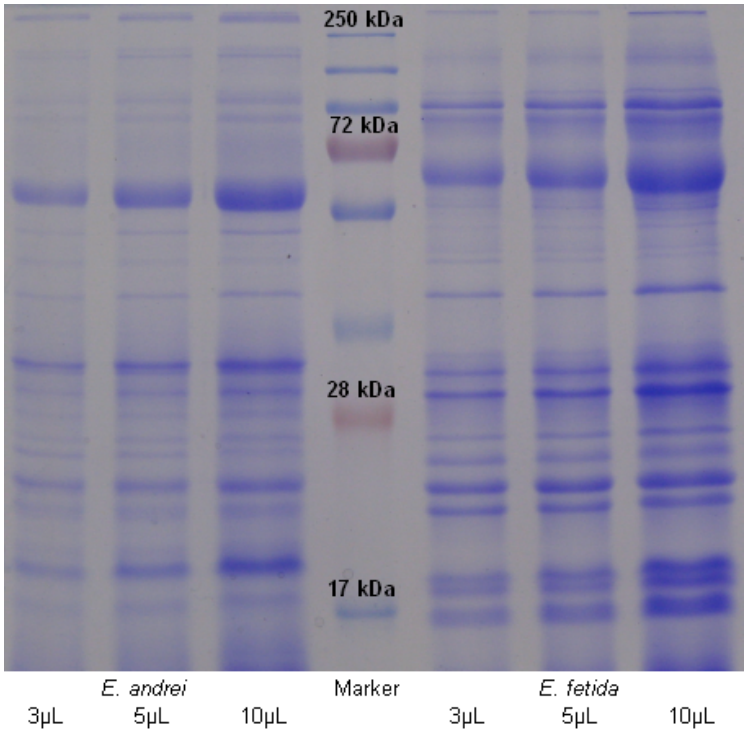

Fig. 7. Electrophoretic analysis of coelomic fluid of *E. andrei* and *E. fetida* under non-reducing conditions.

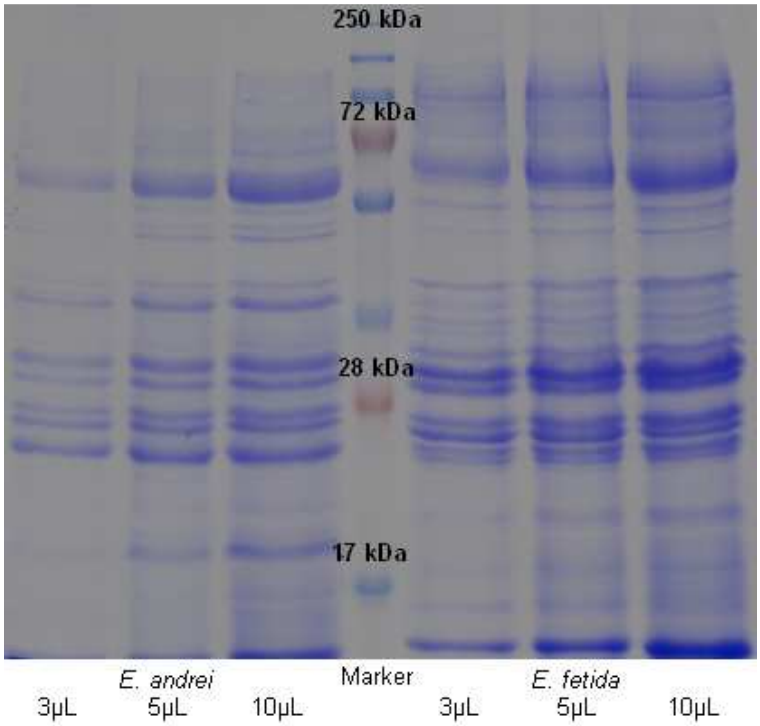

Fig. 8. Electrophoretic analysis of coelomic fluid of *E. andrei* and *E. fetida* under reducing conditions.

6.3.1 Cytolytic activity

Cytolytic activity was determined using the cytolytic assay. Coelomic fluid in concentration range 0-1.5 mg/mL was added to 10^5 adhered fibrobasts L929. After overnight incubation the cells were fixed, the viable cells were stained with crystal violet and the dye was solubilized in 30% acetic acid. Cytolytic activity was evaluated as a percentage of lysis depending on the concentration of coelomic fluid (Fig. 9). Coelomic fluid of both earthworm species showed a strong cytolytic activity. Almost 100 % of lysis was observed when cells were treated with coelomic fluid with concentration 600 µg/mL and higher. The cytolytic activity of *E. fetida* was significantly higher in the concentration range 200-600 µg/mL than the cytolytic activity of *E. andrei*, however the protein concentration necessary for 50 % of lysis was very similar in both species.

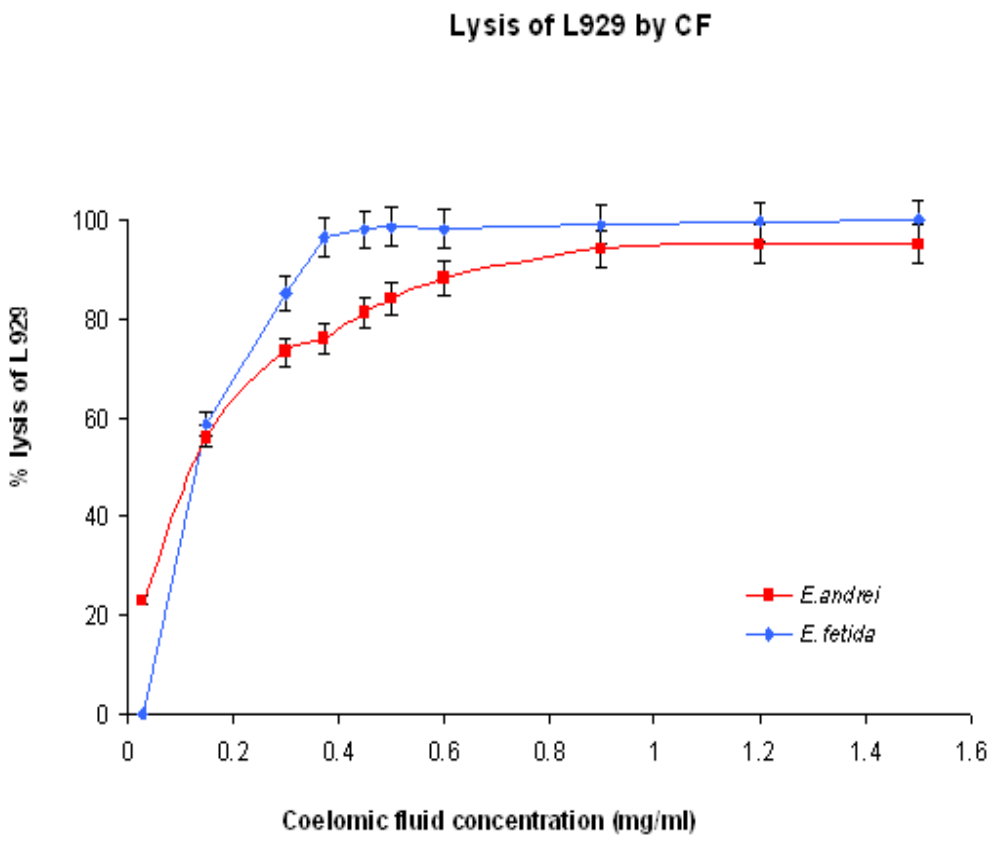

Fig. 9. Cytolytic activity. Cytolytic activity of coelomic fluid was measured as lysis of L929 fibroblasts at different CF concentrations (representative data of one of three independent experiments).

6.3.2 Hemolytic activity

In order to evaluate hemolytic activity of coelomic fluid, hemolytic assay using sheep erythrocytes was performed. 3% erythrocyte suspension was combined with samples of CF of *E. andrei* or *E. fetida* in concentration range 0-5 µg/mL.

Coelomic fluid of *E. andrei* exhibited higher hemolytic activity than coelomic fluid of *E. fetida*. *E. andrei* caused 50 % hemolysis when diluted to 0.07 µg/ml whereas *E. fetida* had to be diluted up to 3.00 µg/ml to cause the same amount of hemolysis (Fig. 10).

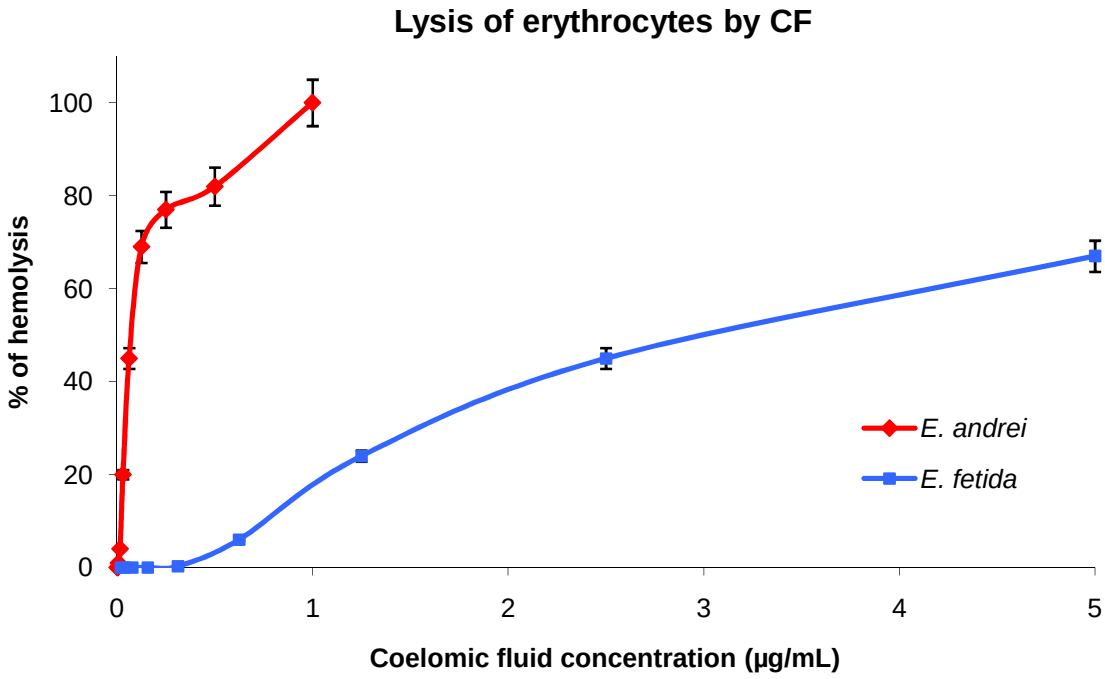

Fig. 10. Hemolytic activity. Hemolytic activity was evaluated as a percentage of hemolysis depending on the concentration of coelomic fluid (representative data of one of three independent experiments).

Coelomic fluid also exhibits strong proteolytic activity, which unspecifically helps to lyse erythrocytes. Thus, previous incubation of CF of *E.andrei* and *E.fetida* with inhibitor of serine proteases Complete™ (Roche), which eliminates this unspecific effect, provided a clearer view of the activity of hemolytic proteins.

After incubation with inhibitor of serine proteases, the ability to lyse erythrocytes was reduced considerably in coelomic fluid of both earthworm

species. In coelomic fluid of *E. andrei* protein concentration required for 50% lysis was increased by more than 25 % (to 0.09 µg/ml) and in coelomic fluid of *E. fetida* it increased by more than 35 % (to 4.12 µg/ml).

6.3.3 Lysozyme activity

The lysozyme activity in coelomic fluid of *E. andrei* and *E. fetida* was evaluated qualitatively using a lysoplate assay. For this assay a Petri dish with 1 % agar solution in 50 mM phosphate buffer (pH 6.0) containing 1 mg/mL lyophilized *Micrococcus lysodeicticus* was prepared. Samples of *E. andrei* or *E. fetida* coelomic fluid and standard were applied on the surface of the Petri dish and after incubation the diameter of lysed zone (mm) was measured (Fig. 11).

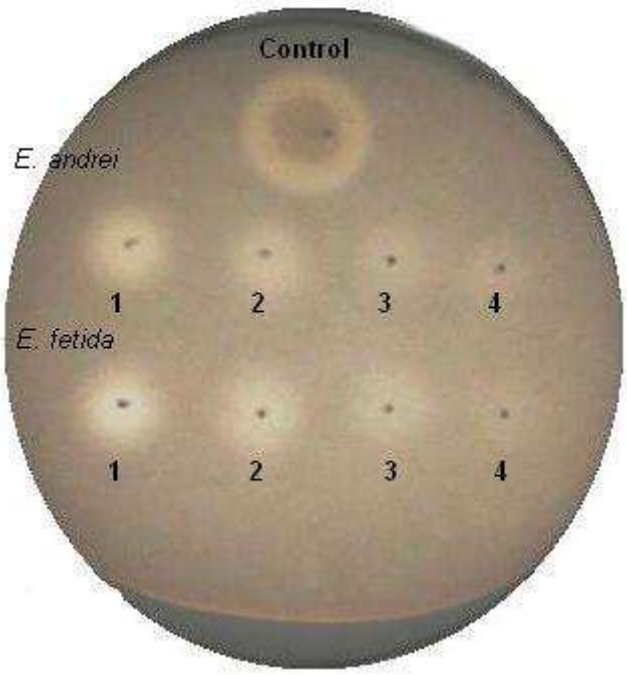

Fig. 11. Lysozyme activity of coelomic fluid isolated from *E. andrei* or *E. fetida* was evaluated by measurement of the diameters of the cleared zones (representative data of one of three independent experiments). As positive control hen egg white lysozyme (25 µg) was used. Lysozyme activity of coelomic fluid with total protein amount of (1) 50 µg, (2) 25 µg, (3) 12.5 µg and (4) 6.25 µg was measured.

The diameter of control lysed zone was 18 mm. The diameters of zones cleared by *E. andrei* coelomic fluid were (1) 9 mm, (2) 7 mm and (3) 5 mm, whereas zones lysed by coelomic fluid isolated from *E. fetida* had diameters of (1) 10 mm, (2) 8 mm and (3) 5 mm, respectively. There was no measurable cleared zone when 6.25 µg of total coelomic fluid proteins were used.

6.3.4 Protease activity and proteolytic pattern

To measure the protease activity, firstly a commercial kit QuantiCleave™ protease assay kit was used. The kit uses succinylated casein as substrate for proteases of the sample. After the proteolytic cleavage, trinitrobenzenesulfonic acid is added to each sample to produce a colored product (Fig.12). To assess relative protease activity of the samples, a standard curve is plotted using logarithmic scale from solutions of trypsin used as standard. Relative protease activity of *E. andrei* and *E. fetida* coelomic fluid was calculated from the samples of coelomic fluid diluted 1/10000 in H_2O. Using the standard logarithmic curve, the protease concentration in coelomic fluid of *E. andrei* was determined to be 2.0 mg/mL while the protease concentration in coelomic fluid of *E. fetida* was calculated to be 1.7 mg/mL.

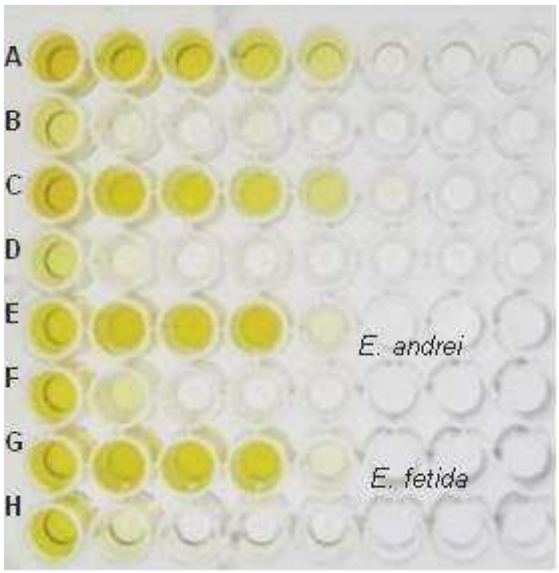

Fig. 12. An example of arrangement of the protease assay. Lines A and C contain samples of standard (trypsin) with substrate, while lines B and D do not contain substrate for proteolytic cleavage. Lines E and G contain samples of coelomic fluid of different protein concentration with substrate for proteolytic cleavage, whereas lines F and H do not contain this substrate.

Furthermore, to compare the proteolytic patterns of coelomic fluid of *E. andrei* and *E. fetida* a substrate gel electrophoresis along with native polyacrylamide electrophoresis was performed. Two and 5 µL of coelomic fluid with or without inhibitor of serine protease were applied on a gel. After the electrophoretic separation gels were stained using Coomassie® Brilliant Blue R-250 solution. Patterns of proteolytic proteins of coelomic fluid were observed as transparent bands in the blue background. Using this technique, it was determined that the proteolytic patterns of *E. andrei* and *E. fetida* coelomic fluid differ one from the other (Fig. 13). Both proteolytic patterns are formed by a wide variety of proteins, but there is one clear proteolytic band present in the pattern of *E. fetida* that is missing in the other pattern.

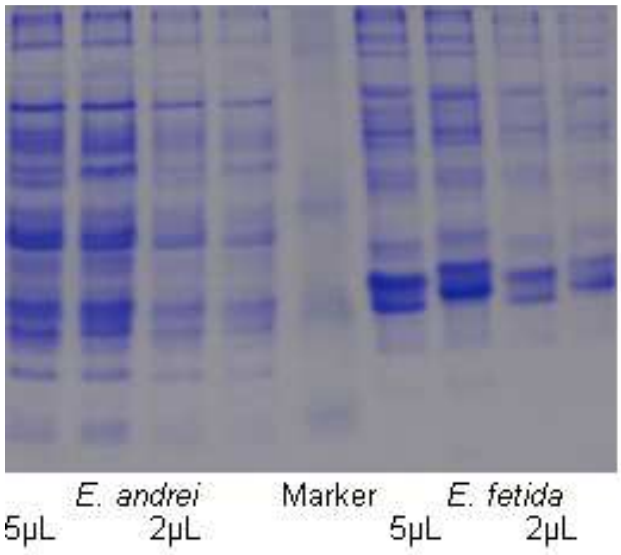

Fig. 13a. Native polyacrylamid electrophoresis. Two and 5 µL of samples were applied on the gel with or without inhibitor of serine protease.

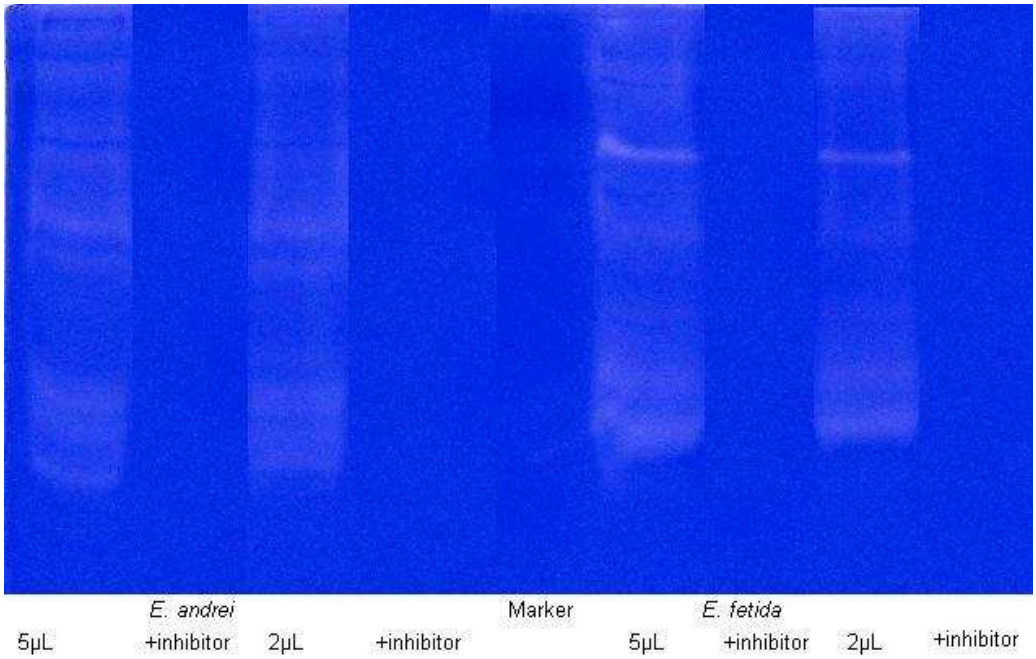

Fig. 13b. Substrate gel electrophoresis. Proteolytic patterns of coelomic fluid were observed as transparent bands in the blue background. For each sample there was a control included containing inhibitor of serine protease.

7 Discussion

The aim of this work was to compare two closely related earthworm species *Eisenia andrei* and *Eisenia fetida* from the immunological point of view. The two species were long believed to be members of sub-species. More recently, they have been accepted as two different species due to the differences in mitochondrial and nuclear DNA sequences (Pérez-Losada et al. 2005). This is supported by the reproductive isolation as well (Domínguez et al. 2005). Furthermore, their natural habitats vary considerably. *Eisenia andrei* naturally lives in compost rich in possible pathogens such as protozoan, fungi, viruses and mainly bacteria, whereas *Eisenia fetida* inhabits the litter layers of moist forests, an environment with lower antigenic pressure. In this study, the effect of the natural environment on various defense mechanisms involved in the innate immunity of the earthworms was investigated.

First we focused on the sequence comparison and functional differences between the pattern recognition molecule CCF of *E. andrei* and *E. fetida*.

CCF was originally isolated from coelomic fluid of *E. fetida* due to its ability to lyse the tumor necrosis factor (TNF)-sensitive cell line L929 (Bilej et al. 1995). Subsequently, CCF was characterized as a pattern recognition molecule recognizing cell wall components of different microorganisms (so called microbe associated molecular patterns) by its two lectin-like domains. The central domain shows a strong homology with other invertebrate pattern recognition molecules and binds to O-antigen of lipopolysaccharides of Gram-negative bacteria and β-1,3-glucans of yeast (Beschin et al. 1998). The C-terminal tryptophan-rich domain mediates interactions with yeast *N*, *N'*-diacetylchitobiose and also muramyl dipeptide and muramic acid from the cell walls of Gram-positive bacteria (Beschin et al. 1999; Bilej et al. 2001). Upon binding microbial pathogen-associated molecular patterns, CCF triggers the activation of the prophenoloxidase cascade, which results in the formation of melanin with cytotoxic and antimicrobial properties.

In the present study we assembled and aligned cDNA sequence of CCF molecules of *E. andrei* and *E. fetida.* Both nucleotide and deduced amino acid sequence display approximately 95 % homology (Fig. 4a and Fig. 4c). The amino acid sequences are nearly identical in the polysaccharide-binding motif with an exception in the position 150, where histidine residue is substituted by serine in *E. fetida,* and glucanase motif with another substitution in the position 186, where threonine residue is replaced by alanine in *E. fetida*. Furthermore, there is one extra amino acid inserted in the signal peptide of *E. fetida* protein. Equal substitutions in these motifs were already described in previous work that was aimed to characterize CCF-like molecules of other lumbricid species (Silerova et al. 2006). Both molecules contain a high number of tryptophan residues (7 %) located mainly in the C-terminal part of the molecules. Moreover, this part of both molecules is the less variable. These data suggest that the two described molecules share both lectin-like domains allowing them to act as pattern recognition molecule and efficiently recognize antigens specific for yeast, Gram-positive and Gram-negative bacteria.

Furthermore, we analyzed the mRNA expression levels of CCF molecules of *E. andrei* and *E. fetida.* We determined that the expression of these genes is very similar in both species (Fig. 5), even though the expression of *E. andrei* CCF mRNA was slightly, but not significantly, elevated as compared to *E. fetida*.

The cytolytic effect of *E. fetida* coelomic fluid on different cell types including various mammalian tumor cell lines was long known (Hrzenjak et al. 1992; Nagasawa et al. 1991). Among these lines, the TNF-sensitive L929 was widely used to investigate the cytolytic properties of coelomic fluid. It was found that CCF is responsible for approximately 50-60 % of the cytolytic effect of the coelomic fluid on this cell line (Bilej et al. 1995). In this study, a cytolytic assay using L929 cell line was used to compare cytolytic activities of coelomic fluid of *E. fetida* and *E. andrei*.

Considering high sequence homology of CCF isolated from both species and comparable expression level of CCF mRNA in both species, similar cytolytic activity of the compared coelomic fluids could have been expected. Indeed, as shown in the Figure 9, both earthworm species exerted

a comparably strong cytolytic activity. Interestingly, even though the cytolytic activity of *E. fetida* seemed to be slightly stronger compared to the cytolytic activity of *E. andrei*, the protein concentration necessary for 50 % of lysis was very similar in both species (128.2 µg/mL in *E. andrei* and 128.1 µg/mL in *E. fetida*). Therefore, it could be suggested that the compared earthworm species possess similar ability to lyse the TNF-sensitive L929 cell line.

Lysozyme was the second molecule subjected to sequencing and subsequent comparison. Lysozyme is one of the important molecules involved in the innate immunity and is widely distributed throughout the animal and plant kingdoms. The lysozyme activity of the coelomic fluid of *Eisenia fetida* was reported more than 20 years ago (Cotuk et al. 1984). Afterwards, the active protein was isolated and the N-terminal sequence was determinated. It showed a considerable homology with lysozymes purified from other invertebrate species (Ito et al. 1999). More recently, the complete sequence of *E. andrei* lysozyme has been determined. The amino acid sequence analysis revealed a high content of cysteine residues characteristic not only for the invertebrate type of lysozyme, but also for the other types. These residues are essential for proper folding and for the stability of the protein. Besides the conserved cysteine residues, the *E. andrei* lysozyme was also reported to contain three amino acid residues potentially important for the lysozyme activity – glutamic acid at the position 14, aspartic acid at the position 26 and serine at the position 29 (Joskova et al. 2009).

Both nucleotide and deduced amino acid sequences display 90 % identity (Fig. 4b and Fig. 4d) with twelve conserved cysteine residues, as well as three amino acid residues potentially important for the lysozyme activity – Glu14, Asp26 and Ser29. In addition, the N-terminal 16-amino acid residues long signal peptide was predicted. To sum up, both lysozyme molecules contain the amino acid residues believed to be crucial for the lysozyme activity of the protein.

We also investigated the levels of mRNA expression in the coelomocytes of *E. andrei* and *E. fetida* and determined very similar expression of these molecules (Fig. 5). In this case, *E. fetida* expressed lysozyme mRNA slightly more. Again, the difference between the threshold

cycles was very low (one and a half cycle), thus, biologically, these expression levels can be considered very similar.

Lysozyme activity was previously described to be very low under normal conditions, however it was shown that the enzyme was released from coelomocytes in response to microbial infection as a part of defense mechanism (Cotuk et al. 1984).

In this study, the lysozyme activity in coelomic fluid of *E. andrei* and *E. fetida* was evaluated qualitatively using a lysoplate assay. As shown in the Figure 11, lysozyme activity of the coelomic fluids was very similar, which is in accordance with the previous results of the current study (high degree of sequence homology, similar expression level). Interestingly, protein concentrations applied in this assay in order to observe the lysozyme activity were considerably higher (10-10000×) as compared to all other used bioassays. This observation is in accordance with the previous data stating that lysozyme activity is under normal conditions weaker as compared to other biological activities exhibited by the coleomic fluid.

The coelomic fluid of earthworms exhibits numerous biological activities. Among these activities, particular attention has always been devoted to hemolytic components secreted by coelomocytes into the coelomic fluid. Various proteins were described to be responsible for the hemolytic properties of coelomic fluid and their relationship remains unclear. The majority of these proteins, apart from hemolytic properties, possess also a spectrum of antibacterial and/or bacteriostatic activities against pathogenic soil bacteria (Roch 1979; Roch et al. 1991a; Valembois et al. 1982). Fetidin and lysenin belong to the most extensively studied protein of *Eisenia andrei* coelomic fluid (Prochazkova et al. 2006a). Therefore, we focused on the comparison of these two molecules. Surprisingly, we did not prove the expression of fetidin and lysenin in *E. fetida* earthworm, despite the presence of both genes in *E. fetida* genome (data not shown). On the other hand, these two molecules are the most abundantly expressed proteins in *E. andrei* coelomic fluid. The absence of the expression of fetidin and lysenin in *E. fetida* species might reflect the natural environment of *E. fetida*, which is considerably poorer in the number of microorganisms compared to *E. andrei* natural

habitat. We suggest that there is some early termination signal included in the genomic sequences preventing them to be expressed in *E. fetida* earthworms.

Moreover, hemolytic activities of *E. fetida* and *E. andrei* were assessed using a hemolytic assay with sheep erythrocytes with or without inhibitor of serine proteases added to the coelomic fluid. As shown in Figure 10, *E. andrei* coelomic fluid exerted hemolytic activity 40 times stronger compared to *E. fetida*. The protein concentration needed to lyse 50 % of erythrocytes was in the case of *E. andrei* 0.07 µg/ml, whereas *E. fetida* had to be diluted up to 3.00 µg/ml. This observation is in accordance with the lack of expression of lysenin and fetidin in E. fetida species. Moreover, when the coelomic fluid was incubated with inhibitor of serine proteases, the ability to lyse erythrocytes was reduced more considerably in E. fetida coelomic fluid, suggesting that erythrocyte lysis depends more on nonspecific killing of erythrocytes based on the proteolytic activity.

Therefore, proteolytic activity of coelomic fluid of *E. andrei* and *E. fetida* was compared. The presence of proteases in coelomic fluid of *E. fetida* was shown already 20 years ago (Roch et al. 1991b). More recently, the active involvement of proteases in earthworm resistance and immunodefense has been confirmed (Kauschke et al. 1997). In this study, a protease assay and a substrate gel electrophoresis were carried out in order to evaluate the proteolytic activities of compared species. According to the results of the protease assay, the protease concentration in coelomic fluid of *E. andrei* was 2.0 mg/mL while the protease concentration in coelomic fluid of *E. fetida* was calculated to be 1.7 mg/mL. These values are considerably high taking into account the measured protein concentration of coelomic fluid (12 ± 3 mg/mL) suggesting that proteases constitute up to 16 % of total proteins in the coelomic fluid. Moreover, the comparison of the proteolytic patterns of both coelomic fluids showed some differences (Fig. 13b). These data suggest that even though the protease concentration in both coelomic fluids was comparable, the specific protease activity in *E. andrei* and *E. fetida* coelomic fluid differ.

To sum up, immunological properties of the coelomic fluid of *E. andrei* and *E. fetida* are different. The main difference was observed

in the expression of antibacterial/hemolytic proteins fetidin and lysenin that can reflect a different microbial environment. *E. andrei* living in decaying organic matter, in compost and mold appears to be better equipped to resist microbial activity than *E. fetida* living in litter layer of moist forests. The proteolytic pattern differed even though the protease concentration of the two species was measured to be very similar. Finally, it was confirmed that lysozyme of both species is under normal conditions present in the coelomic fluid in low concentration compared to the other immunologically active compounds.

8 Conclusion

Present study has focused on the immunological comparison of two closely related earthworm species *Eisenia andrei* and *Eisenia fetida* with the following results:

● high degree of homology was observed at the gene sequence level of selected immunological compounds – lysozyme and pattern recognition molecule CCF;

● expression of CCF and lysozyme molecules was similar in both earthworm species;

● genes encoding for lysenin and fetidin are present in the genome of both *Eisenia andrei* and *Eisenia fetida* earthworms, however in *E. fetida* are not expressed; accordingly, hemolytic activity exhibited by the coelomic fluid of *Eisenia andrei* was 40 times stronger compared to *Eisenia fetida*, most likely due to the nonspecific effect of proeases;

● a difference in proteolytic patterns of the compared coelomic fluid was observed;

● described changes in the innate immunity can reflect different microbial environment.

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