Innate Immunity in Earthworms

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„It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly, organized creatures."

(Charles Darwin, "The Formation of Vegetable Mould through the Action of Worms", 1881)
I should like to express my gratitude to many people who contributed in various ways to successful finishing of my studies. First and foremost, I would like to thank my supervisor, Dr. Martin Bilej, for the opportunity to work in his laboratory, for the guidance and encouragement he gave me during my studies, and especially for his kind and calm way of dealing even in stressful situations.

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Finally, I wish to thank my family for their moral support and, last but not least, I would like to express my sincere gratitude to my husband Luboš for his understanding, support and especially patience over all these years.

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Petra Procházková
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<th>Description</th>
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<tbody>
<tr>
<td>CF</td>
<td>coelomic fluid</td>
</tr>
<tr>
<td>CCF</td>
<td>Coelomic cytolytic factor</td>
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<tr>
<td>rCCF</td>
<td>recombinant CCF</td>
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<tr>
<td>HEMA</td>
<td>2-hydroxyethylmetacrylate</td>
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<tr>
<td>ABP</td>
<td>antigen-binding protein</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>ARS-HSA</td>
<td>arsanylated human serum albumin</td>
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<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>LBSS</td>
<td><em>Lumbricus</em> balanced salt solution</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>proPO</td>
<td>prophenoloxidase</td>
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<tr>
<td>PO</td>
<td>phenoloxidase</td>
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<tr>
<td>L-DOPA</td>
<td>L-β-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>ppA</td>
<td>prophenoloxidase activating enzyme</td>
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<tr>
<td>PAMPs</td>
<td>pathogen associated molecular patterns</td>
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<tr>
<td>PRPs</td>
<td>pattern recognition proteins</td>
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<tr>
<td>CPC</td>
<td>cetylpyridinium chloride</td>
</tr>
<tr>
<td>12C9</td>
<td>monoclonal antibody against CCF</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>VSG</td>
<td>variant specific glycoprotein</td>
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<td>UTR</td>
<td>untranslated region</td>
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1 Introduction

From the total number of extant animal species 95% are included in the invertebrate taxa. Invertebrates represent a heterogeneous group of organisms ranging from unicellular protozoans to the complex protochordates. Successful survival strategy of a particular invertebrate species is generally based on a short life-span and numerous offspring. However, this rule is not universal - individuals of some species survive a number of years (for example, earthworm can reach the age of 10-12 years, oyster even up to 60 years).

![Simplified phylogenetic tree of metazoans.](image)

Earthworms belong to a large phylum, Annelida, to the class Oligochaeta. This name means “few bristles” and refers to the few bristles or setae on each body segment, compared with the many setae of marine annelids in the class Polychaeta. There are over 3 000 species of earthworms around the world. Earthworms are mainly free-living terrestrial, but also freshwater worms. They are found in soil, leaf litter and under stones and logs in most habitats, including arid areas, but most species are found in wetter, more heavily vegetated regions.
Earthworms range in size from several millimeters to two or three metres in length. They are beneficial in many different ways. They are used as an ecological monitor of the maintenance of soil fertility, they may reflect environmental changes that are not readily recognized with chemical measures and they may provide an early warning of soil degradation and contamination. The Organization for Economic Cooperation and Development (OECD) and the American Environmental Protection Agency therefore accepted an official protocol to follow immunological parameters in earthworms to monitor environmental pollution - OECD Guidelines for Testing of Chemicals 1984 (Green et al. 1989; Goven and Kennedy 1996). Beside that they might be considered as a source of biologically active compounds with potential industrial or medical use (see below, chapter 2.2.6). Earthworm powder has been used in traditional medicine as a drug for treatment of various diseases in the Far East since several thousand years ago. Further, they provide bait for fishing and are a source of protein for food. Moreover, they represent a model for comparative immunologists since the early 1960s, when results of transplantation experiments were reported (see below, chapter 2.1.2 (Cooper and Roch 1994)). These experiments proved the existence of self and non-self recognition in earthworms and their ability to reject foreign grafts. Certain advantages of this model of comparative immunology represent inexpensive, non-controversial, and socially acceptable usage for experimentation.
1.1 Earthworm body plan

Earthworms are protostomian animals having a true coelom of mesenchymal origin. The coelomic cavity is metameric and the segments are separated by transversal septa. Each segment contains a complete set of digestive and excretory organs and a nerve center. The coelomic cavity is filled with coelomic fluid containing free coelomocytes. Annelids generally have a closed circulatory system, consisting of a dorsal vessel (carrying blood anteriorly), a ventral vessel (carrying blood posteriorly), and capillaries connecting the two (Pechenik 1996).

Earthworms are hermaphroditic (having both male and female reproductive systems), but two worms are required to mate and reproduce. The reproductive organs are in the clitellum (the enlarged segments in the 1st half of earthworm body), where are later formed cocoons that protect the developing eggs.

Generalized anatomy of an earthworm. The earthworm shows the well-developed segmentation that is characteristic of all animals in the phylum Annelida. Although the major nervous, circulatory, and digestive organs are located near the head, more posterior segments contain peripheral structures for all of these systems. These posterior segments are virtually identical to each other. Earthworms are hermaphroditic, possessing both male and female internal reproductive organs.
(http://encarta.msn.com/media_461517341/Generalized_Anatomy_of_an_Earthworm.html)
Skin of earthworms consists of the epidermis and cuticle. Earthworms have no lungs, and breathe through the skin. For gas exchange, the outermost layers of an earthworm are thin and must be kept moist. To keep the skin moist, mucus is excreted onto the skin. Mucus moreover contains several antibacterial factors and thus represents the first protective barrier against invaders (Valembois et al. 1984, 1986).

The coelomic cavity is filled with the coelomic fluid serving as transporting medium for various things and assuring hydromechanic functions such as a hydrostatic skeleton. Moreover, it ensures the defense reaction of earthworms. The coelomic fluid is not aseptic and always contains bacteria, protozoa and fungi from the outer environment; due to some efficient mechanisms the growth of invaders is kept under control (Porchet-Hennere and M’Berri 1987; Valembois et al. 1988).
The coelomic fluid exerts proteolytic, hemolytic, agglutinating, antibacterial, and cytolytic properties that ensure an effective defense. During the defense reactions, humoral factors collaborate with cellular compartments represented mainly by free coelomocytes phagocytizing bacteria and most of harmful particles. Dales and Kalac (Dales and Kalac 1992) reported that the coelomic fluid contains approximately $6 \times 10^9$/ml of naturally occurring bacteria, while the number of potentially phagocytic cells is more than ten times higher. The excess of phagocytes combined with the humoral factors can easily prevent the coelomic bacteria from multiplying. Exhausted phagocytes can be then excluded through dorsal pores that represent another way of transport of soluble metabolites and corpuscular material from the body in each segment (Cameron 1932). Large foreign bodies or agglutinated bacteria can be eliminated by a process known as encapsulation (Ratcliffe et al. 1985). The cellular and fibrous capsules are often called “brown body” because of its melanin content, which results from the activation of the prophenoloxidase cascade, an important defense system of invertebrates. The process of
encapsulation in *Eisenia fetida* earthworms was detailed by Valemois *et al.*, who showed that most brown bodies contain tissue wastes such as setae or necrotic muscle cells, agglutinated bacteria, gregarines, and nematodes. Encapsulation begins like phagocytosis with recognition of nonself; foreign body is surrounded by free coelomocytes and after several days a massive capsule with melanin content is created (Valemois *et al.* 1992). The capsule then migrates to posterior segments of the coelomic cavity, where it can be eliminated by autotomy (Keilin 1925). Autotomy of caudal segments followed by wound healing and a rapid and efficient regeneration of the damaged area is under the neurohormonal control and is well developed in earthworms.

*Eisenia fetida* earthworms. 
(photo Procházková, Šlerová, 2004)

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*Note: The spelling „fetida“ was changed a few years ago by taxonomists to „foetida“ then subsequently changed back. The different spellings do not denote different species. Information on this species can be found under both spellings, though the correct spelling is „fetida“. Therefore now we used the name „fetida“, but the name „foetida“ was used in previous works. To make the story more complicated, two subspecies of *Eisenia fetida* originating from different region are distinguished - *Eisenia fetida fetida* (the name *Eisenia fetida* is currently used) and *Eisenia fetida andrei* (the name *Eisenia andrei* is currently used).*
2 Defense of the earthworms

2.1 Cellular defense mechanisms

2.1.1 Mechanisms of elimination of heterologous material

The coelomic fluid of earthworms contains different types of cells named generally coelomocytes and classified according to morphological and cytochemical criteria (Stein et al. 1977; Sima 1994). Free chloragon cells (eleocytes) have mainly accessory and nutritive functions. Hyaline or granular amoebocytes represent effector immunocytes and they possess strong phagocytic activity.

Recently, Engelmann et al. (Engelmann et al. 2004) studied earthworm coelomocyte populations and they described a strongly immunoreactive coelomocyte population containing effector hyaline and granular coelomocytes but not chloragocytes. They suggest that these cells can combat the pathogens by releasing the contents of their granules (lysosomes) into the coelomic cavity as an extracellular lysis. Further, they developed a panel of monoclonal antibodies against coelomocytes using hybridoma technology and identified four distinct annelid coelomocyte markers (Engelmann et al. 2005). Their anti-coelomocyte clones recognized common antigens present on all earthworm cells and tissues, they bound to certain patterns in snails, but they did not react with Drosophila melanogaster hemocytes or with mammalian cells or tissues suggesting a closer relationship between cellular defense systems of earthworms and snails than those of insects or vertebrates. Nevertheles, these data remain to be elucidated in the context of function.

Phagocytosis represents a key defense mechanism in earthworms. Though the phagocytic properties of coelomocytes were already mentioned by Metchnikoff in 1887, the first detailed description of this process was published by Cameron (Cameron 1932), when he observed phagocytosis of numerous inert particles, cells and bacteria in vitro and in vivo. He observed phagocytosis in all types of coelomocytes with the exception of chloragogen cells, but their activity differs in the amount of engulfed particles and in the type of ingested material. While inert particles were engulfed mainly by granulocytes, bacteria and foreign cells were phagocytized by basophilic and neutrophilic amoebocytes. Cameron’s results were confirmed later in reports describing an uptake of bacteria (Dales and Kalac 1992), yeast (Stein and
Phagocytosis begins by the recognition of non-self, which is followed by the engulfment and destruction of the particles. Phagocytosis by coelomocytes, similarly to vertebrates, can be affected by humoral components of the coelomic fluid - opsonins that coat the engulfed particles. The opsonins are then recognized by receptors on phagocytic cells thus facilitating the uptake. Important role of opsonins in the coelomic fluid was proven both in the experiment of phagocytosis of preincubated yeasts (Stein and Cooper 1981), and in an experiment with the phagocytosis of synthetic 2-hydroxyethylmethacrylate copolymer (HEMA) particles (Bilej et al. 1990b, 1991a), when phagocytic activity was considerably higher in the case of opsonized particles. Experiments carried by Laulan et al. were based on the fact that some components of the coelomic fluid interact with particular components of the complement. Supernatant of coelomocyte culture is able to degrade a C3 fragment of complement similarly as C3 convertase (Laulan et al. 1983). Later they followed the effect of two main mammalian opsonins, immunoglobulins and complement C3 fragments on the phagocytic activity of coelomocytes (Laulan et al. 1988). They found that phagocytosis was enhanced by vertebrate IgG and C3b complement fragments but not by IgM and C3d fragments. These data suggest the presence of receptors on the surface of coelomocytes with structure and function similar to some complement fragments of vertebrates.
Exhausted cells with phagocytized material can be eliminated by several mechanisms mentioned above (expulsion via dorsal pores, excretion by nephridia, encapsulation). Engulfed material is also intracellularly killed and degraded by proteolytic enzymes, lysozyme-like substances or via oxidative burst. As an oxidative burst are generally called processes initiated by activation of NADPH-oxidase that are connected with a high consumption of oxygen. The oxidative burst involves the production of highly reactive oxygen radicals, which disturb the structure of microorganism biopolymers, destroy the activity of their enzymes and harm their DNA. The production of reactive oxygen radicals was detected both in the coelomic fluid and in chloragosomes of chloragogen cells (Valembois et al. 1991). However, the production of highly reactive oxygen radicals does not occur in the case of the phagocytosis of inert, synthetic particles (Bilej et al. 1990b, 1991a) suggesting the possible discrimination of coelomocytes between antigenic and non-antigenic materials (Tuckova and Bilej 1996).
General armamentarium of innate defense of the earthworm. The first protective barrier of earthworms is represented by skin together with the secretion of mucus containing agglutinins. Invading microorganisms can be excreted by nephridia, expelled via dorsal pores or phagocytized by coelomocytes. Furthermore, humoral factors are involved in their elimination: antimicrobial substances and cytolytic molecules protect earthworms against bacteria, coelomic cytolytic factor (CCF) triggers the activation of the phenoloxidase cascade, agglutinated bacteria can be encapsulated and eliminated by autotomy.
(modified according to Tučková and Bilej 1996)

2.1.2 Transplantation immunity

The capability of recognition and response to grafting was observed in some annelids, in the case of xenografts as well as allografts. The beginning of the process resembles the reaction to injury and wound healing. After the earthworm body wall was injured either by wounding or grafting, there is an increase in the number of coelomocytes in association with the graft. Initial changes that occur within 24 hours after the transplantation result in an increased amount of free coelomocytes surrounding the graft. Later on, infiltration of coelomocytes into the graft section occurs regardless of whether it is xenograft or allograft. The infiltration of coelomocytes in the case of xenograft is so large that it ends with a complete rejection of the graft. The amount of surrounding and invasive coelomocytes is markedly lower in the case of autograft (Cooper 1970; Hostetter and Cooper 1973). In some cases, coelomocytes of the acceptor surround the graft and isolate it from the inner environment by a process similar to encapsulation (Parry 1978).
Comparison of the changes of the number of coelomocytes during injury and transplantation showed that coelomocyte responses rise most rapidly within 24 hours, and return to normal by 72 hours (Hostetter and Cooper 1973). Autografts elicit a weaker coelomocyte response. In contrast, cellular response to xenografts is slower, reaches its peak in 3 to 4 days and requires 7 days for coelomocytes to return to normal level. The destruction of xenografts was complete in a mean time of 17 days. If the second-set grafts are transplanted at this time, rejection occurred in an accelerated mean time of 6 to 7 days. Moreover, also the number of coelomocytes is 20 - 30 % higher in comparison with first grafts (Hostetter and Cooper 1973). These data suggest the existence of the memory, which is based solely on cells, because the transfer of the coelomic fluid nor of other substances does not induce the accelerated rejection. Experiments demonstrating the adoptive transfer of memory (Bailey et al. 1971; Valembois 1971) support a role of coelomocytes in graft rejection. Host *L. terrestris* were first xenografted with *E. fetida*, then coelomocytes were harvested at 5 day posttransplantation and injected into control *L. terrestris*. These specimens were used as second acceptors of grafts from the original donor *E. fetida*. *L. terrestris* earthworm responded to first allograft and to injected isolated coelomocytes only slightly. Animals with injected primed coelomocytes showed accelerated rejection of allograft (Cooper and Rubiotta 1969).

Probable reason of the increase of the number of coelomocytes during the response to transplantation or injury is the proliferating activity of mesenchymal lining of the coelom and septa (Hostetter and Cooper 1974).
2.2 Humoral defense mechanisms

2.2.1 Protein-antigen recognition

As it has been mentioned above, invertebrates have evolved a variety of active defense pathways efficiently recognizing and responding to non-self substances despite the absence of an adaptive immune system based on antibodies and lymphocytes. Invertebrates rely on innate defense strategies, which are based mainly on pattern recognition receptors recognizing surface determinants common to potential pathogens, and on antimicrobial factors. However, in some invertebrates some inducible factors with a certain degree of specificity were described. Various inducible antimicrobial peptides were described in numerous insect species (for review see: (Kanost and Zhao 1996; Karp 1996)). They possess a selective activity based on the presence of different saccharides on the surface of cells recognized by pattern recognition proteins (PRP). In earthworms, inducible agglutinins recognizing non-self saccharide moieties with a broad specificity were described (Stein et al. 1982; Wojdani et al. 1982). Moreover, the existence of a short-term immunological memory after xeno- and allografting ((Bailey et al. 1971; Valembois 1971) chapter 2.1.2) and humoral defense factors synthesized in response to foreign substances support the idea of existence of adaptive defense mechanisms.

The first attempt at a detailed analysis of adaptively synthesized substances was done by Laulan et al. (Laulan et al. 1985) who parenterally injected L. terrestis earthworms with synthetic haptens coupled to a protein carrier. They found that earthworms formed specific molecules that bound the hapten-carrier complex used for immunization. The response was dependent on the amount of introduced antigen and on the type of the carrier protein. A maximum amount of the anti-hapten substances was observed between the 5th and 8th day after the immunization. The second administration of the same antigen on day 24 after the first stimulation resulted in a faster and more intense response. Moreover, they showed a certain specificity of formed substances by testing their capability to bind different haptens used for stimulation. The highest level of binding was detected when the same hapten molecule was used both for stimulation and detection.

Similar results were published by Tučková et al. (Tuckova et al. 1988, 1991b). They used arsanilic acid as a hapten coupled to human serum albumin (ARS-HSA) although they
were never able to show such a high degree of specificity of the hapten-binding protein as did the Laulan’s group. The injection of ARS-HSA into the coelomic cavity of *L. terrestris* and *E. fetida* earthworms led to the formation of a protein, which bound the stimulating antigen. This protein was isolated from the coelomic fluid and was called antigen-binding protein (ABP). The molecular weight of the native ABP in the coelomic fluid is 56 kDa and it is formed by two disulfide-linked polypeptide chains (31 and 33 kDa in *L. terrestris*, 30-kDa homodimer in *E. fetida*) forming the antigen-binding site (Tuckova *et al.* 1991a, 1991b). The highest response after the primary stimulation occurred between the 4th and 8th day and the secondary response was found to be faster and more pronounced. Antigen binding capacity of neutrophilic coelomocytes and the presence of ABP on their surface suggest the requirement of cellular compartment in the induction of ABP with the antigen (Bilej *et al.* 1990a, 1991b).

Earthworms possess strong proteolytic activity and their proteolytic system exerts considerably high heterogeneity (Tuckova *et al.* 1986; Roch *et al.* 1991b; Leipner *et al.* 1993). Protein antigen administered into the coelomic cavity is rapidly degraded by proteolytic enzymes and is bound by cells of the mesenchymal lining (Rejnek *et al.* 1993). Proteolytic activity was observed both in the coelomic fluid and in free coelomocytes (Bilej *et al.* 1993; Rejnek *et al.* 1993). It was found that up to 80% of the injected antigen is degraded proteolytically within the first 48 h. This fact is in accordance with the finding that the proteolytic degradation of administered antigen is required to trigger the ABP synthesis and its release (Bilej *et al.* 1994; Tuckova and Bilej 1994), but the rapid digestion of protein antigen contrasts with a relatively delayed antigen-binding protein response, which reaches maximum values between the day 4 and 8 (Tuckova *et al.* 1991b). It was found that proteolytic degradation in earthworms is limited, resulting in short peptides of molecular weights in the range 700-1100 Da (Hanusova *et al.* 1999). Moreover, the ABP response after the injection of such peptides to the earthworms is significantly faster as compared with the response to the intact antigen. Further, administration of protein antigen together with proteinase inhibitor does not trigger the ABP formation, but the ABP response to already degraded peptides is not influenced by proteinase inhibitor (Bilej *et al.* 1994; Tuckova and Bilej 1994; Hanusova *et al.* 1999).

Taken together, the administered antigen is at first rapidly proteolytically degraded, digested fragments are bound by precursor cells in mesenchymal lining and induce their
proliferation, differentiation and release to the coelomic cavity (Bilej et al. 1992; Rejnek et al. 1993; Hanusova et al. 1999).

2.2.2 Lysozyme

Lysozyme is another molecule representing humoral antibacterial defense of invertebrates. Lysozyme provides an efficient protection of animals against Gram-positive bacteria infections by hydrolyzing 1,4-β-D-linked glycosidic bonds of peptidoglycan in bacterial cell walls. In Annelids including Eisenia fetida, molecules exerting lysozyme-like activity were detected in body fluids and cell lysates (Schubert and Messner 1971; Dales and Dixon 1980; Cotuc and Dales 1984). Inoculation of earthworms with a bacterial suspension resulted in increased levels of lysozyme-like activity (Lassalle et al. 1988; Hirigoyenberry et al. 1990). Recently, a lysozyme-like active protein from E. fetida earthworm was partially sequenced and characterized (Ito et al. 1999). Based on the N-terminal sequence, it was suggested to form a novel class of lysozyme shared by mollusks, echinoderms and the nematode Caenorhabditis elegans.

2.2.3 Hemolytic molecules

The coelomic fluid of E. fetida earthworms exhibits numerous biological activities including hemolytic activity. The majority of the proteins with hemolytic activity identified so far show bactericidal and/or bacteriostatic activities against pathogenic soil bacteria (Roch 1979, 1991a; Valembois et al. 1982, 1986) and they represent a heterogeneous set of proteins described by different groups in the last 35 years.

Hemolytic E. fetida proteins were described for the first time in 1968 by Du Pasquier and Duprat (Du Pasquier and Duprat 1968). Later on, they were named EFAF (Eisenia fetida andrei factors), and characterized as two glycoproteins of 40 and 45 kDa with different extent of glycosylation, which are secreted by chloragocytes and eleocytes (Roch 1979, 1981, 1984). The 45-kDa protein was characterized as having a pl of 6.0 and, possibly, being encoded by one nonpolymorphic gene, while the 40-kDa protein was defined as being encoded by a gene
having four alleles encoding four isoforms with pI of 6.3, 6.2, 5.95 and 5.9. All animals possess either 2 or 3 isoforms of the 40-kDa molecule, with one isoform, with pI of 6.0, invariably present. As a result of the polymorphism, six different hemolytic patterns were found in *E. fetida* individuals (Roch 1979, 1987). Besides the hemolytic activity they cause the agglutination of erythrocytes (Valembois et al. 1984; Kauschke and Mohrig 1987b) and they can participate in cytotoxic activity of the coelomic fluid (Kauschke and Mohrig 1987a). Moreover, they display antibacterial activity against both Gram-negative and Gram-positive bacteria (Lassegues et al. 1989; Hirigoyenberry et al. 1990, 1992), particularly on strains that are pathogenic for earthworms (Valembois et al. 1986; Roch et al. 1987, 1991a; Roch and Cooper 1991). Moreover, hemolytic factors bind to sphingomyelin, which is a major lipid constituent of plasma membranes of most mammalian cells. Then they polymerize and form channels of 10 nm through the lipid bilayer (Roch et al. 1981, 1989). In addition to their bacteriolytic/bacteriostatic activity, hemolytic factors may also mediate opsonization (Sinkora et al. 1993) and participate in the clotting of the coelomic fluid (Valembois et al. 1988).

Later, in 1997, EFAF were characterized at the molecular level and were named fetidins. Gene coding for the 40-kDa was cloned (Lassegues et al. 1997; Milochau et al. 1997). Fetidin amino acid sequence comprises an N-glycosylation site (position 250-252) and a peroxidase motif (position 52-62) and both fetidins display peroxidase activity.

Independently, a 41-kDa hemolytic protein produced by coelomocytes and causing the contraction on rat vascular smooth muscle, was cloned and described as lysenin by Sekizawa et al. (Sekizawa et al. 1996, 1997). Simultaneously, two 42-kDa lysenin-related proteins with weak contraction activity were identified. Lysenin displays a high amino acid sequence homology (89 % identity, 95 % positivity) with fetidin and with lysenin-related protein-1 (76 % identity, 89 % positivity). Moreover, lysenin-related protein-2 was found to correspond at the amino acid level to fetidin. Lysenin specifically recognizes sphingomyelin and forms oligomers, which leads to the formation of pores in target membranes with a diameter of ~3 nm (Yamaji et al. 1998; Yamaji-Hasegawa et al. 2003). Experiments with erythrocytes of various animal species showed that sheep erythrocytes are very sensitive to lysenin due to the high sphingomyelin content in the membranes. Sphingomyelin is the predominant phospholipid (51 % of total phospholipids) in the sheep erythrocytes, whereas human and rat erythrocyte
membranes contain much less sphingomyelin (25 and 13 % of total phospholipids in human and rat erythrocyte membranes, respectively). Also the treatment of the erythrocyte membrane with sphingomyelinase leads to inhibition of the hemolysis (Yamaji et al. 1998). Lysenin protein is expressed in large coelomocytes and in free large chloragocytes present in the lumen of the typhlosole (Ohta et al. 2000).

At the same time, another 38 kDa cytolytic protein was identified in the coelomic fluid of *E. fetida* earthworms and named eiseniapore (Lange et al. 1997). This protein requires sphingomyelin or galactosylceramide, but not ceramide or galactosylsphingosine, to bind to red blood cell membranes or phospholipid vesicles, inducing lysis. Lange et al. showed that eiseniapore forms stable oligomers and induces pores in sphingomyelin-containing membranes with a central channel with outer and inner diameters of 10 and 3 nm, respectively (Lange et al. 1997, 1999). They found that eiseniapore lysis is activated by thiol groups but inhibited by metal ions. Binding of eiseniapore to target membranes can be inhibited among others by vitronectin, which resembles the action of the C9 complement fragment, because vitronectin is known to inhibit the hemolysis of erythrocytes by perforin and complement (Tschopp et al. 1988). Nevertheless, the suppression of the binding of eiseniapore to erythrocytes does not affect its pore-forming lytic activity (Lange et al. 1999).

Eue et al. (Eue et al. 1998) described 3 hemolytic proteins from the coelomic fluid of *E. fetida*, H₁, H₂, and H₃, with molecular weight of 46, 43, and 40 kDa. They consist of several isoforms (with pl of 6.2, 6.0, 5.8 and 5.4 for H₁, 6.0 and 5.2 for H₂, and 6.1 and 5.1 for H₃). While H₃ exerts lytic and hemagglutinating activity, H₁ and H₂ show only lytic activity. Moreover, H₃ splits into two fragments of 18 and 21 kDa and is not influenced by heat treatment up to 65 °C.

In 2003, Koenig et al. (Koenig et al. 2003) isolated native hemolytic proteins both from coelomocyte lysate (CL₃₀ and CL₄₁) and from the coelomic fluid (H₁, H₂, H₃) of *E. fetida* earthworms. Using mass spectrometric analyses, they have demonstrated the identity of CL₃₀ with fetidin and CL₄₁ with lysenin, and the probable identity of H₁, H₃ hemolysins with fetidin. Standing nomenclature and identity of *E. fetida* hemolytic proteins are confusing and controversial and this paper is the first that shed light on the nomenclature of hemolytic proteins described in *E. fetida* earthworms.
2.2.4 Coelomic cytolytic factor

The coelomic fluid of *E. fetida* lyses a number of various cell types like chicken fibroblasts, guinea-pig polymorphonuclear leukocytes and insect hemocytes (Kauschke and Mohrig 1987a). On the contrary, it does not cause the lysis of the coelomocytes of other earthworm species nor of hemocytes of mollusks, nematodes and protozoans.

The cytolytic effect of the coelomic fluid was also observed in experiments with TNF-sensitive tumor cell line L929. A novel lytic molecule responsible for lysis of tumor L929 cells was identified and isolated as 42-kDa protein called CCF for coelomic cytolytic factor (Bilej et al. 1995a). CCF is localized in the cells of chloragogenous tissue adjacent to the gut wall and in coelomocytes morphologically similar to macrophages (Bilej et al. 1998).

CCF shows homology with Gram-negative bacteria binding protein, with saccharide-binding motif of bacterial and animal β-1,3-glucanases, with β-1,3-glucan-recognition proteins of arthropods and with glucan-sensitive factor G from the horseshoe crab *Limulus polyphemus* (see Table 1., (Beschin et al. 1998)). Although these proteins show high homology in the putative polysaccharide-binding domain and the catalytic sites of the bacterial glucanases, neither CCF nor its invertebrate homologues exhibit glucanase activity. All these invertebrate homologs have been suggested to play a role in invertebrate innate immunity acting as pattern recognition receptors.

Indeed, CCF is a lectin recognizing and binding the components of cell walls of microorganisms that leads to the activation of the phenoloxidase cascade, which is an important defense system both in protostomian and deuterostomian invertebrates (chapter 2.2.5). CCF binds the O-antigen of LPS of Gram-negative bacteria, muramyl dipeptide and muramyl acid of peptidoglycan presented in cell walls of Gram-positive bacteria and β-1,3-glucans and N,N'-diacetylchitobiose of yeast (Beschin et al. 1998; Bilej et al. 2001). The capability of CCF to recognize a broad spectrum of saccharide structures is based on the presence of two lectin domains. The saccharide-binding domain is located in the central part of CCF and shows the homology with the polysaccharide and glucanase motif of β-1,3-glucanases. This domain interacts with LPS and β-1,3-glucans (Bilej et al. 2001). The second, C-terminal, domain is rich in tryptophan and mediates the interaction with N,N'-diacetylchitobiose, muramyl dipeptide and muramic acid (Beschin et al. 2003).
Table 1. CCF and homologous proteins

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>% Identity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eisenia fetida</em></td>
<td>CCF</td>
<td></td>
<td>(Beschin et al. 1998)</td>
</tr>
<tr>
<td><em>Pacifastacus leniusculus</em></td>
<td>LPS and β-1,3-glucan-binding protein</td>
<td>58</td>
<td>(Lee et al. 2000)</td>
</tr>
<tr>
<td><em>Strongylocentrotus purpuratus</em></td>
<td>β-1,3-glucanase</td>
<td>56</td>
<td>(Bachman and McClay 1996)</td>
</tr>
<tr>
<td><em>Bombyx mori</em></td>
<td>β-1,3-glucan-recognition protein</td>
<td>44</td>
<td>(Ochiai and Ashida 2000)</td>
</tr>
<tr>
<td><em>Manduca sexta</em></td>
<td>β-1,3-glucan-recognition protein</td>
<td>43</td>
<td>(Ma and Kanost 2000)</td>
</tr>
<tr>
<td><em>Hyphantria cunea</em></td>
<td>Gram-negative-bacteria-binding protein</td>
<td>42</td>
<td>(Shin et al. 1998)</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Gram-negative-bacteria-binding protein</td>
<td>42</td>
<td>(Kim et al. 2000)</td>
</tr>
<tr>
<td><em>Bombyx mori</em></td>
<td>Gram-negative-bacteria-binding protein</td>
<td>43</td>
<td>(Lee et al. 1996)</td>
</tr>
<tr>
<td><em>Tachypleus tridentatus</em></td>
<td>Factor G subunit α</td>
<td>61</td>
<td>(Seki et al. 1994)</td>
</tr>
<tr>
<td><em>Bacillus circulans</em></td>
<td>β-1,3-glucanase</td>
<td>42</td>
<td>(Yamamoto et al. 1993)</td>
</tr>
<tr>
<td><em>Peneus monodon</em></td>
<td>β-1,3-glucan-binding protein</td>
<td>57</td>
<td>(Sritunyalucksana et al. 2002)</td>
</tr>
<tr>
<td><em>Litopenaeus stylirostris</em></td>
<td>LPS and β-1,3-glucan-binding protein</td>
<td>54</td>
<td>(Roux et al. 2002)</td>
</tr>
<tr>
<td><em>Spisula sachalinensis</em></td>
<td>β-1,3-glucanase</td>
<td>58</td>
<td>(Kozhemyako et al. 2004)</td>
</tr>
</tbody>
</table>

The coelomic fluid of other related earthworm species *L. terrestris* displays distinct biochemical properties as compared with *E. fetida* coelomic fluid. Proteolytic and hemolytic activities are considerably lower in *L. terrestris* than in *E. fetida* coelomic fluid (Tuckova et al. 1986; Leipner et al. 1993). Further, *L. terrestris*\(^\dagger\) the coelomic fluid has no lytic activity against African trypanosomes or murine cell lines (Bilej et al. 2001). Its coelomic fluid contains 42-kDa protein, which reacts with monoclonal anti-CCF antibody and can trigger the proPO cascade by binding β-1,3-glucans and LPS but not peptidoglycan treated with lysozyme, muramic acid, or N,N'‐diacetylchitobiose. The comparison of the amino acid sequence of this CCF homologue with CCF from *E. fetida* revealed 91 % identity. The C-terminal domain shows the highest

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\(^\dagger\) Note: These properties were originally described for *L. terrestris*. Recently we have found that due to a classification error it was *Aporrectodea longa*, but the described data are valid also for *L. terrestris* (see Šilerová et al., chapter 4.4).
divergence, while the region corresponding to the putative polysaccharide-binding motif and glucanase motif have a complete identity in both earthworm species. These two regions are highly conserved in invertebrate pattern recognition molecules homologous to CCF, which binds LPS or β-1,3-glucans (Table 1).

Beside the main properties CCF is involved in the cell-mediated cytotoxic reaction (Bilej et al. 1998), agglutinates Gram-negative bacteria or Gram-positive bacteria (Beschin et al. 1998), has an opsonizing effect facilitating the phagocytosis during earthworm defense reactions (Bilej et al. 1995a), and raises the lytic activity of the coelomic fluid against red blood cells (Bilej et al. 2000). In contrast to fetidins, CCF is not hemolytic (Bilej et al. 1995a) and does not recognize lipids, suggesting a functional and structural nature different from other lytic factors presented in coelomic fluid of E. fetida.

CCF displays functional analogy with mammalian tumor necrosis factor. It was observed that the coelomic fluid lysed TNF-sensitive tumor L929 cells in a proteinase-independent way and that the lysis was caused by 42-kDa protein - coelomic cytolytic factor (Bilej et al. 1995a). In addition to the ability to lyse TNF-sensitive tumor cell lines, CCF exerts a number of biological activities resembling mammalian TNF. CCF is secreted by macrophage-like coelomocytes upon stimulation with lipopolysaccharide, whereas in mammals lipopolysaccharide triggers the production of TNF by macrophages (Aggarwal et al. 1985; Bilej et al. 1998). Both CCF and TNF have opsonizing properties (Lucas et al. 1993; Bilej et al. 1995a) and bind β-1,3-glucan and N,N’-diacetyldichitobioside through a lectin-like interaction (Olson et al. 1996; Beschin et al. 1998). Both proteins share similar β-1,3-glucan and N,N’-diacetyldichitobioside lectin-like activities and domains. Moreover, murine monoclonal antibodies elicited against the lectin-like domain of TNF (TIP domain) that is spatially distinct from the TNF-receptor binding site (Lucas et al. 1994), cross-react with CCF. Furthermore, a monoclonal antibody anti-CCF reacts with TNF. On the other hand, the activity of CCF is not inhibited by anti-TNF monoclonal antibodies suggesting different mechanisms of TNF- and CCF-mediated lysis.
Structure of *Eisenia fetida* CCF. CCF comprises two distinct domains interacting with pathogen-associated molecular patterns. The first domain interacts with LPS and glucans while the second domain binds diacetylchitobiose and muramyl residues. Further, both domains are required for the interaction with trypanosomes and mammalian cells.

(Progress in Molecular and Subcellular Biology: Invertebrate cytokines and the Phylogeny of Immunity, Beschin *et al.*, 2003)

TNF was reported to lyse the bloodstream forms of the African trypanosome *T. brucei brucei* via its TIP lectin domain (Lucas *et al.* 1994; Magez *et al.* 1997). *Trypanosoma brucei brucei* is a protozoan parasite transmitted by tsetse flies that causes African sleeping sickness. Since CCF has a domain and lectin activity similar to that of TNF, the possible trypanolytic activity of CCF was investigated (Beschin *et al.* 1999). The coelomic fluid of *E. fetida* as well as purified CCF display trypanolytic activity that can be inhibited by anti-CCF monoclonal antibodies, by anti-TIP TNF antibodies, and also by N,N'-diacetylchitobiose. The trypanolytic activity is based on the ability of both CCF and TNF to bind N,N'-diacetylchitobiose, which represents an important part of the variant-specific glycoprotein (VSG) that acts as a protective coat on *T. brucei brucei* bloodstream forms. CCF and TNF are able to lyse only bloodstream forms of parasites expressing VSG, but not insect-stage procyclic forms that express procyclin as surface protein (Beschin *et al.* 1999; Bloc *et al.* 2002). Further, a lysate of bloodstream form parasites, isolated VSG and N,N'-diacetylchitobiose can activate the prophenoloxidase cascade.
in contrast to deglycosylated VSG. N,N'-diacetylcchitobiose by itself initiated the proPO cascade in the entire coelomic fluid. In contrast, this saccharide failed to induce proPO cascade in CCF-depleted coelomic fluid and this activity can be restored upon addition of recombinant or purified CCF.

Comparison of the lytic activity of CCF against African Trypanosoma brucei brucei and South-American Trypanosoma cruzi showed that bloodstream form T. cruzi is lysed by coelomic fluid and also by CCF, but in a lower extent that in the case of T. brucei brucei. This lytic effect of coelomic fluid and CCF on T. cruzi can be again partially inhibited in the presence of anti-CCF monoclonal antibody, antibody neutralizing the lectin-like activity of TNF or N,N'-diacetylcchitobiose (Olivares Fontt et al. 2002).

TNF was reported to increase the membrane conductance in some mammalian cells through interaction with ion channels or their associated molecules on mammalian cell surface (Hribar et al. 1999; van der Goot et al. 1999; Fukuda et al. 2001). This effect does not rely on interaction with both types of TNF membrane receptors since it also occurred in cells isolated from TNF receptor-deficient mice. CCF also interacts with ion channels of mammalian cells (Bloc et al. 2002). CCF treatment increases the outward current in macrophages from mice lacking both types of TNF-receptors excluding the interaction of CCF with the TNF receptor. The ion channel gating effect of TNF as well as CCF is mediated by the lectin-like domain of the molecule (Bloc et al. 2002).

Despite the functional analogies of CCF and TNF and cross-reactivity of anti-CCF or anti-TIP monoclonal antibody respectively with TNF or CCF, CCF and TNF do not show gene or amino acid sequence homology, indicating a lack of common evolutionary origin (Beschin et al. 1999).
2.2.5 Prophenoloxidase cascade

The prophenoloxidase cascade is a sensitive and efficient defense system in invertebrates. Non-self molecules, i.e. pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide or peptidoglycans from bacteria and β-1,3-glucans from fungi (Söderhäll and Cerenius 1998), are recognized by endogenous pattern recognition proteins or receptors, they activate zymogenic proteinases that cleave prophenoloxidase (proPO) to phenoloxidase (PO) (Lee and Söderhäll 2002; Cerenius and Söderhäll 2004). This system consists of several different proteins, which are zymogenic proteinases, proteinase inhibitors, proPO, PO and recognition molecules (pattern recognition proteins PRPs).

![Diagram of the prophenoloxidase cascade]

Prophenoloxidase cascade. Upon recognition of cell wall components of bacteria or yeast, serine proteinases cleave inactive proPO to its active state phenoloxidase. Active enzyme catalyzes hydroxylation and oxidation of phenols to quinones, which are subsequently polymerized to melanin, which exhibits various biological properties.

The key component of the system is phenoloxidase (PO; monophenol, dihydroxyphenylalanine: oxidoreductase; EC1.14.18.1). PO, which is also known as tyrosinase, is a bifunctional copper containing enzyme that is usually present in its inactive form, named prophenoloxidase (proPO). Conversion of proPO to its active state is achieved by proteolytic
cleavage and the resulting enzyme catalyses both the o-hydroxylation of monophenols and the oxidation of diphenols to quinones. The quinones are polymerized non-enzymatically to melanin (Ashida and Yamazaki 1990; Söderhäll et al. 1994). Melanin, the final product of PO cascade, exhibits fungistatic, bacteriostatic and antiviral properties and together with other components of proPO cascade is involved in wound healing and defense reactions.

Activation of proPO is dependent upon a cascade of serine proteinases, including so called prophenoloxidase activating enzyme (ppA) and other factors. PpAs have been characterized in various invertebrates and they are all zymogens of trypsin-like serine proteinases. In vitro, proPOs can be activated also after treatment with several detergents, salts or lipids (Ashida and Yamazaki 1990; Sugumaran and Kanost 1993).

Prophenoloxidases and/or phenoloxidases have been isolated and characterized from several invertebrate animals (Table 2.).

Table 2. proPO and/or PO identified in various animal species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Localization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>crayfish <em>Pacifastacus leniusculus</em></td>
<td>hemocytes</td>
<td>(Aspan and Söderhäll 1991)</td>
</tr>
<tr>
<td>black tiger shrimp <em>Penaeus monodon</em></td>
<td>hemocytes</td>
<td>(Aspan et al. 1995)</td>
</tr>
<tr>
<td>horseshoe crab <em>Limulus polyphemus</em></td>
<td>hemolymph</td>
<td>(Nellaiappan and Sugumaran 1996)</td>
</tr>
<tr>
<td>silkworm <em>Bombyx mori</em></td>
<td>hemolymph</td>
<td>(Ashida 1971)</td>
</tr>
<tr>
<td></td>
<td>cuticle</td>
<td>(Asano and Ashida 2001)</td>
</tr>
<tr>
<td>silk moth <em>Hyalophora cecropia</em></td>
<td>hemolymph</td>
<td>(Andersson et al. 1989)</td>
</tr>
<tr>
<td>housefly <em>Musca domestica</em></td>
<td>hemolymph</td>
<td>(Tsukamoto et al. 1986)</td>
</tr>
<tr>
<td>European bluebottle blowfly <em>Calliphora vicina</em></td>
<td>hemolymph</td>
<td>(Naqvi and Karlson 1979)</td>
</tr>
<tr>
<td>yellow mealworm <em>Tenebrio molitor</em></td>
<td>hemolymph</td>
<td>(Lee et al. 1999)</td>
</tr>
<tr>
<td>flesh fly <em>Sarcophaga bullata</em></td>
<td>hemolymph</td>
<td>(Chase et al. 2000)</td>
</tr>
<tr>
<td>fat webworm <em>Hyphantria cunea</em></td>
<td>larvae</td>
<td>(Park et al. 1997)</td>
</tr>
<tr>
<td>cockroach <em>Blaberus discoïdalis</em></td>
<td>hemocytes</td>
<td>(Durrant et al. 1993)</td>
</tr>
<tr>
<td>tobacco horn worm <em>Manduca sexta</em></td>
<td>hemolymph</td>
<td>(Aso et al. 1985)</td>
</tr>
<tr>
<td>wax moth <em>Galleria mellonela</em></td>
<td>hemolymph</td>
<td>(Kopacek et al. 1995)</td>
</tr>
<tr>
<td>mosquito <em>Anopheles stephensi</em></td>
<td>hemocytes</td>
<td>(Cui et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>fat body epidermis</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Fluid</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>mosquito <em>Aedes aegypti</em></td>
<td>larvae</td>
<td>(Taft et al. 2001)</td>
</tr>
<tr>
<td>mosquito <em>Armigeres subalbatus</em></td>
<td>hemocytes</td>
<td>(Cho et al. 1998b)</td>
</tr>
<tr>
<td>mosquito <em>Anopheles gambie</em></td>
<td>hemolymph</td>
<td>(Jiang et al. 1997)</td>
</tr>
<tr>
<td>fruit fly <em>Drosophila melanogaster</em></td>
<td>cDNA library from whole animals</td>
<td>(Fujimoto et al. 1995)</td>
</tr>
<tr>
<td>honey bee <em>Apis mellifera</em></td>
<td>hemocytes</td>
<td>(Zufelato et al. 2004)</td>
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<td></td>
<td></td>
<td>(Lourenco et al. 2005)</td>
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<tr>
<td>scarab beetle <em>Holotrichia diomphalia</em></td>
<td>hemolymph</td>
<td>(Kwon et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Kim et al. 2002)</td>
</tr>
<tr>
<td>mollusk <em>Illex argentinus</em></td>
<td>ink</td>
<td>(Naraoka et al. 2003)</td>
</tr>
<tr>
<td>tobacco budworm <em>Heliothis virescens</em></td>
<td>hemolymph</td>
<td>(Lockey and Orth 1992)</td>
</tr>
<tr>
<td>army worm <em>Spondoptera littoralis</em></td>
<td>hemolymph</td>
<td>(Lee 1995)</td>
</tr>
<tr>
<td>locust <em>Locusta migratoria</em></td>
<td>hemolymph</td>
<td>(Cherqui 1996)</td>
</tr>
<tr>
<td>European spiny lobster <em>Palinurus elephas</em></td>
<td>hemolymph</td>
<td>(Jaenicke and Decker 2003)</td>
</tr>
<tr>
<td>freshwater crayfish <em>Astacus leptodactylus</em></td>
<td>hemolymph</td>
<td>(Jaenicke and Decker 2003)</td>
</tr>
<tr>
<td>wasp <em>Pimpla hypochondriaca</em></td>
<td>venom</td>
<td>(Parkinson et al. 2001)</td>
</tr>
<tr>
<td>crab <em>Charybdis japonica</em></td>
<td>hemolymph</td>
<td>(Liu et al. 2006)</td>
</tr>
</tbody>
</table>

The molecular weight of proPO and PO differs among species and varies usually between 70 and 90 kDa. Primary structure of several proPOs was determined and all of them contain two functional copper-binding sites. Actually, sequences of proPO in different species within and adjacent to the copper-binding sites show 60-70 % of similarity. Another conserved peptide motif, GCGEQNM, which is present in α-macroglobulins both in vertebrates and some invertebrates, such as crayfish (Hall et al. 1989) and lobster (Spycher et al. 1987) and is conserved also in the complement proteins C3 and C4 (Dodds and Day 1993) can be found in the protein sequence of proPOs as well.

The hemolymph of several arthropods and mollusks is characterized by the presence of hemocyanin, another high molecular weight copper binding protein. Its physiological function is to transport oxygen, which is sustained by its capability to reversibly bind dioxygen to a dinuclear copper site (Solomon et al. 1992). It means that hemocyanins and phenoloxidases are equipped with a structurally similar oxygen-binding center. Interestingly, the potential copper-binding sites of proPO are highly homologous to the corresponding sites of hemocyanin of tarantula *Eurypeima californicum*, horse shoe crab *Limulus polyphemus*, crab *Cancer magister* (Decker and Rimke
1998; Decker and Terwilliger 2000; Decker et al. 2001), horseshoe crab *Tachyleus tridentatus* (Nagai and Kawabata 2000) and crayfish *Pacifastacus leniusculus* (Lee et al. 2004). Moreover, hemocyanins of these arthropods were reported to have PO activity after proteolytic cleavage. PO activities have been reported also for some molluscan hemocyanins (Zlateva et al. 1996; Salvato et al. 1998).

Localization of proPO in different species is also ambiguous. In different crustaceans proPO is localized in the semigranular and granular blood cells (Söderhäll and Smith 1983; Johansson and Söderhäll 1985; Sritunyalucksana et al. 1999) and is secreted from these cells in its inactive form. ProPO has been found in blood cells of some insects (Leonard et al. 1985), but in others is detectable only in the plasma (Ashida 1971; Saul and Sugumaran 1986). Most probably, the proPO is stored in the granules of some blood cells, from which it is released to plasma and eventually activated.

To avoid harmful effects, the proPO system is controlled and regulated. The regulation of proPO system supplies inhibition of proPO activation and activity. In the housefly *Musca domestica*, a DOPA-containing peptide with a molecular weight of 4.2 kDa was found to be a competitive inhibitor of the fly phenoloxidase (Daquinag et al. 1995; Daquinag et al. 1999). Similarly, three different proteins inhibiting the proPO activation were identified in *Locusta migratoria* (14 kDa) and two (LCIM I and LCIM II) have been cloned (Brehelin et al. 1991; Kromer et al. 1994). Endogenous proteinase inhibitors preventing undesired activation of proPO were found also in the hemolymph of the insect *Sarcophaga bullata* and the tobacco horn worm *Manduca sexta* (Sugumaran et al. 1985; Saul and Sugumaran 1986), and in hemolymph of *Bombyx mori* (Aso et al. 1994). Another proteinase inhibitor preventing over-activation of ppA (Aspan et al. 1990) and a large molecular weight proteinase inhibitor, pacifastin, with a mass of 155 kDa (Liang et al. 1997), have been reported in crayfish *Pacifastacus leniusculus*. *Drosophila* serpin-27A specifically inhibited the pro-PO-activating enzyme and prevented the melanin synthesis induced by activated PO (De Gregorio et al. 2002). Recently, a 43-kDa novel negative regulator of the melanization reaction was identified in the beetle *Tenebrio molitor* (Zhao et al. 2005).

In annelids, melanization reactions proceed like the cellular defense reactions of the host through the formation of so-called brown bodies around encapsulated invading pathogens (Dales 1983). The origin and function of the brown pigment in capsules was first described in *Nereis*
*diversicolor* and it was suggested that the brown color is due to melanin (Porchet-Hennere and Vernet 1992). Formation of brown bodies containing bacteria, parasites or altered self structures was described in *Eisenia fetida* as well (Valembois *et al.* 1992). Then, PO activity was documented in some other annelids, like *Lumbricus terrestris* (Fischer 1978) and *Eisenia fetida andrei* (Valembois *et al.* 1991).

PO activity was evidenced in the coelomic fluid of *Eisenia fetida* indicating the presence of proPO activating cascade in annelids (Seymour *et al.* 1992; Beschin *et al.* 1998). It was found that proPO cascade can be activated by Gram-negative bacteria and by yeasts within 6 hours upon recognition in *E. fetida* coelomic fluid. Gram-positive bacteria are able to activate the proPO system in *E. fetida* coelomic fluid only after a treatment with lysozyme (Bilej *et al.* 2001). Activation of proPO cascade in *E. fetida* coelomic fluid is triggered by coelomic cytolytic factor (CCF), which acts as a pattern recognition molecule. The importance of CCF in the induction of proPO in *E. fetida* was confirmed showing that when CCF is removed from the coelomic fluid, the activation cascade is blocked and the restoration of recombinant CCF retrieves the L-DOPA oxidation (Beschin *et al.* 1998; Bilej *et al.* 2001).

### 2.2.6. Other active molecules in earthworms

Antimicrobial peptides became an important participant of non-specific host defense for both vertebrates and invertebrates. They represent a heterogeneous group of factors ranging from small peptides (4-7 kDa) to proteins with molecular weight of 20-50 kDa. Based on the structural aspect they are classified into four groups containing cationic peptides, anionic peptides, aromatic dipeptides and peptides derived from oxygen-binding proteins. General conception of effect of antimicrobial peptides is based on the fluctuation of lipids, change of membrane structure and in some cases on internalization of the peptide after the interaction of the peptide with bacterial membrane.

Most of antimicrobial peptides so far found in invertebrates were from insects, crustaceans and nematodes (see database [www.bbcm.univ.trieste.it/~tossi/antimic.html](http://www.bbcm.univ.trieste.it/~tossi/antimic.html)). Besides the antimicrobial protein fetidin identified in *Eisenia fetida* earthworm (Lassegues *et al.* 1997), another antimicrobial peptide named Lumbricin was purified from the earthworm
Lumbricus rubellus (Cho et al. 1998a). Lumbricin is a proline-rich antimicrobial peptide with the molecular weight of 7.2 kDa. It is expressed constitutively in adult animals and is not induced by bacterial infection. It showed antimicrobial activity against a broad spectrum of microorganisms without hemolytic activity. Only a limited number of bioactive peptides has so far been described in annelids. Atrial natriuretic peptide as a possible part of the hormonal system was found in the earthworm Lumbricus terrestis (Vesely and Giordano 1992). A novel antimicrobial short peptide of only 5 residues was described in Eisenia fetida earthworm (Liu et al. 2004). Three myoactive peptides were purified and identified from the gut tissue and whole body of Eisenia fetida earthworm and from the whole body of another earthworm species Pheretima vittata. These peptides consist of 17 or 18 amino acids and they were named GGNG peptides based on their common structure of the C-terminus.

A number of earthworm fibrinolytic enzymes was isolated and described. Earthworm fibrinolytic enzyme is a multi-component proteinase belonging to serine proteinase family. Derivatives of this enzyme have been used as traditional drugs in Far East for a few thousand years and preparations with a strong fibrinolytic activity still have a large biomedical application. They have been widely used in prevention of cardiac and cerebrovascular diseases and as an orally administered drug for thrombosis treatment (Mihara et al. 1991, 1992; Nakajima et al. 1993, 1996; Xiong et al. 1997; Yang and Ru 1997; Wang et al. 2003, 2004b; Wang et al. 2004a).
3 Aims

The thesis is aimed at studying various defense molecules differing in the way of non-self recognition - antigen-binding protein (ABP, protein-protein recognition), coelomic cytolytic factor (CCF, protein-saccharide recognition) and fetidin/lysenin (protein-lipid recognition).

1) The high degree of specificity of protein recognition by antigen binding protein (ABP) described by Laulan’s group (Laulan et al. 1985) was not shown in our experiments. In very first experiments only a limited selection of antigens was used and therefore we followed the production of antigen-binding protein (ABP) as a response to the stimulation of the earthworms with different antigens.

2) Coelomic cytolytic factor (CCF) was described as a pattern recognition protein, but its role in the earthworm defense was demonstrated only indirectly and we therefore focused on determining the effect of experimental challenge with live Gram-negative bacteria, Gram-positive bacteria and with glucan on the expression of CCF in comparison with other earthworm defense molecules.

3) To monitor changes in the coelomic fluid after in vivo stimulation with bacterial antigens we determined changes in the concentration of CCF and levels of hemolytic and lysozyme-like activity in the coelomic fluid as a response to stimulation of the earthworms with Gram-negative bacteria, Gram-positive bacteria and with glucan.

4) The existing nomenclature of different proteins from E. fetida causing the lysis of red blood cells and their identity is confusing. Moreover, during in vivo experiments we observed partial discrepancies in the published sequence of fetidin. We therefore tried to elucidate the relationship between two hemolytic molecules - fetidin and lysenin, and to determine the level of their expression in coelomocytes in individual earthworms.
5) *E. fetida* pattern recognition protein CCF exerts a number of specific properties, based on its structure. To recognize whether also other related earthworms have a corresponding molecule and to compare their characteristics we sequenced and characterized CCF-like molecules in other Lumbricid species.

6) The prophenoloxidase-activating system is an important part of the invertebrate defense system, but until now prophenoloxidases have been isolated and characterized only from arthropod, reports in other animal taxa being rather scarce. We wanted to prove the presence of the prophenoloxidase cascade as a defense mechanism in earthworms.
4 Results

4.1 Protein-antigen recognition in earthworms

The defense strategy of earthworms as well as other invertebrates relies mainly on innate factors. Tuckova et al. (Tuckova et al. 1988, 1991b) have described a 56-kDa molecule, antigen-binding protein (ABP) stimulated by antigenic challenge. They used arsanilic acid as a hapten coupled with human serum albumin and identified an antigen-binding protein consisting of two disulfide-linked polypeptide chains, both of which are participating in the formation of the antigen-binding site. From the kinetics of ABP formation it was evident that the response reached a maximum level between the 4th and 8th day after the stimulation and required the proteolytic processing of administered protein (Tuckova and Bilej 1994; Hanusova et al. 1999).

In our experiments we started from known facts and continued in further characterization of antigen-binding protein formed after the stimulation of earthworms with different proteins. We stimulated animals of the two earthworm species, Eisenia fetida and Lumbricus terrestris, with 6 different proteins - bovine serum albumin, human serum albumin, arsanilic acid coupled to human serum albumin, gliadin, ferritin and insulin. The coelomic fluid from stimulated animals was collected 4, 8, 12, and 16 days after the stimulation. Then, the ABP levels in the coelomic fluid were stimulated by ELISA using anti-ABP monoclonal antibody (Tuckova et al. 1991a) or biotin-labeled proteins used for in vivo stimulation. Since earthworms respond to body injury and to other stress conditions by enhanced protein synthesis, we determined the changes in protein concentration together with the level of formed ABP. Maximum protein concentrations were observed on 4th and 8th day after the stimulation, but there were no differences among animals stimulated with various proteins and a similar increase was detected in sham-stimulated controls (worms stimulated with agar only).

In agreement with previous observations (Tuckova et al. 1988, 1991a) we detected the maximum levels of ABP 8 days after the stimulation, when they reached 157-334 % of the control. Both earthworm species responded to the stimulation with the same time course.

We selected the day with the maximum level of formed ABP, i.e. day 8 after the stimulation, and determined the level of specificity of antigen recognition by binding of antigen in a ligand assays with all biotin-labeled proteins used for the stimulation. We found that ABP
molecules bind preferably the same protein that was used for stimulation. Nevertheless, an increase in the formation of ABPs was also observed in the case of related proteins (BSA, HSA, ARS-HSA) suggesting that the specificity of antigen recognition in earthworms is to a certain extent restricted. No substantial differences were found when *L. terrestris* and *E. fetida* were compared.

Antigen-binding proteins formed in response to the stimulation with different proteins were isolated on affinity column and were subjected to SDS-PAGE, which confirmed the molecular weight of isolated proteins - 56 kDa in *L. terrestris* (31 and 33 kDa under reducing conditions) and 60 kDa in *E. fetida* (dimer of two 30-kDa subunits). N-terminal sequence analysis did not reveal any homology with known proteins.

Characterization of the Limited Specificity of Antigen Recognition in Earthworms*

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ABSTRACT. Parenteral administration of foreign proteins results in earthworms in significantly increased levels of the antigen-binding protein (ABP). The earthworms (Lumbricus terrestris and Eisenia fetida; Oligochaeta, Annelida) were stimulated with different proteins and the ABP response was followed with an anti-ABP monoclonal antibody and in ligand assay with protein conjugates of all proteins used for stimulation. ABP levels are increased after the stimulation with different proteins irrespective of the size and extent of glycosylation. Molar mass of the ABP molecules was always the same, 56 kDa in L. terrestris and 60 kDa in E. fetida. The level of the specificity is considerably lower in comparison with immunoglobulins since ABP reacts not only with the protein used for stimulation but also, though to lesser extent, with related proteins.

Abbreviations
ABP antigen-binding protein
BSA bovine serum albumin
HSA human serum albumin

ARS-HSA arsanic acid coupled to human serum albumin
PBS phosphate buffered saline

Discrimination of self and non-self is one of the features of all animal species but the mechanisms of elimination of non-self differ. Undoubtedly there are no lymphocytes and antibodies in invertebrates and their defense strategy is based mainly on innate factors. However, current data indicate that inductive defense reactions with certain degree of specificity exist also in some invertebrates (for review Kannost and Zhao 1996; Karp 1996).

In the sixties, American and French authors showed that earthworms are able to reject xenogenic allografts and display a short-term immunological memory (recently reviewed by Cooper 1996). Based on these observations comparative immunologists have focused on the question whether earthworms are able to adaptively form specific substances. In addition to an inducible character of agglutinins recognizing non-self saccharide moieties (Wojdani et al. 1982; Stein and Cooper 1983; Stein et al. 1986; Kauschke and Mohrig 1987; Valembois et al. 1993), adaptively synthesized substances in response to protein or hapten-protein stimulation have been described (Laulan et al. 1985). Later, Tučková and co-workers (1988, 1991a) have described an adaptively formed 56-kDa molecule designated as antigen-binding protein (ABP), consisting of two disulfide-linked polypeptide chains (31 and 33 kDa) both of which are involved in the formation of the antigen-binding site. A panel of monoclonal antibodies which react with ABP but do not block the binding site were generated (Tučková et al. 1991b). The ABP response reaches a maximum level between 4 and 8 d after the stimulation and requires proteolytic processing of the administered protein (Tučková and Bilej 1994; Hanušková et al. 1999).

This study represents a logical continuation of our previous paper (Bilej et al. 1995) in which we showed that earthworms parenterally stimulated with different proteins exerted binding properties (both on humoral and cellular levels) to proteins used for stimulation. In this report we stimulated earthworms with different proteins and followed the ABP response both with a monoclonal antibody against ABP and in ligand assay using protein conjugates of all the proteins used for stimulation.

MATERIALS AND METHODS

Animals and method of stimulation. Adult earthworms Lumbricus terrestris (Oligochaeta; Annelida) kept at 15°C in soil and Eisenia fetida (Oligochaeta; Annelida) kept at 20°C in compost

**Corresponding author.
were used in all experiments. Earthworms were stimulated by injecting 10 μg of bovine serum albumin (BSA), human serum albumin (HSA), arsanilic acid coupled to human serum albumin (ARS-HSA; approximately 20:1 ratio), and gladiin, or by injecting 100 μg of ferritin, or 1 μg of porcine insulin. All proteins were administered into the coelomic cavity in 10 μL of 3% agar gel to prevent rapid expulsion through dorsal pores. Different quantities of stimulating proteins were used to respect molar ratios approximately. Ten μL of 3% agar gel was injected into earthworms in control groups.

Harvesting of coelomic fluid. Coelomic fluid containing coelomocytes was obtained by puncturing the coelomic cavity with a glass micropipette 4, 8, 12, and 16 d after the stimulation. The suspension pooled from approximately 20 earthworms was centrifuged (100 g, 10 min), the cell-free coelomic fluid was collected, recentrifuged (5000 g, 5 min), the supernatant was supplemented with serine proteinase inhibitor (Pefabloc; 1 mmol/L; 4-(2-aminoethyl)-benzenesulfonyl fluoride; Boehringer Mannheim Biochemica, Germany) and stored at -70 °C. Protein concentration was determined in all coelomic fluid samples using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Evaluation of coelomic fluid ABP levels. The ABP levels were detected in direct ELISA using anti-ABP monoclonal antibody 6D4 (Tučková et al. 1991b) or in ligand assay with biotin-labeled proteins used for in vivo stimulation. Briefly, the wells were coated with 100 μL of diluted coelomic fluid (200 μg/L in phosphate-buffered saline, pH 7.4; PBS) and after washing with PBS containing 0.05% Tween-20 (T-PBS) saturated with 1% ovalbumin. After repeated washing the wells were filled with 100 μL of 6D4 antibody (40 μg/L in PBS; 2 h, 20 °C), washed and the secondary alkaline phosphatase-labeled rabbit anti-mouse IgG antibody was applied (Sigma; 1:1000 in PBS; 1 h, 20 °C). As substrate, 1 mmol/L disodium salt of 4-nitrophenol phosphate hexahydrate in substrate buffer containing 2% ethanamine ethanol (pH 10.2) was used and color reaction was assessed at 405 nm with Titertek Multiscan MCC/340 ELISA Reader.

The protocol for ligand assay was identical as described above, but biotin-labeled proteins (10 μg/L in PBS; 2 h, 20 °C) were employed instead of 6D4 monoclonal antibody and then the binding was followed with alkaline phosphatase-labeled avidin D (100 U/L in PBS; Vector Laboratories Burlingame, CA).

For the detection the proteins were biotinylated with D-biotinoyl-6-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS; Biotin Labeling Kit, Boehringer Mannheim Biochemica, Germany). Non-reacted biotin-7-NHS was separated on a Sephadex G-25 column.

Isolation of ABP. To isolate ABP after the stimulation with different proteins Affi-Gel Hz Hydrazide Gel (Bio-Rad Laboratories, Hercules, CA) columns with bound proteins used for stimulation were prepared following instructions of the manufacturer. Efficacy of the binding was 40–80%.

Since Affi-Gel is an agarose support which reacts with the aldehydes of oxidized saccharide groups on bound proteins, only significantly glycosylated proteins were used (HSA, ARS-HSA, gladiin, ferritin). Coelomic fluid (1 mL) collected 7 d after the stimulation with a certain protein was subjected to Affi-Gel column with bound corresponding protein. After washing with PBS the bound material was eluted with 18 mmol/L glycine buffer (pH 2.4) and the eluate was immediately neutralized with 1 mol/L Tris buffer (pH 8). The eluate was dialyzed in PBS, concentrated and subjected to SDS–PAGE in 12% polyacrylamide gel. Separated proteins were stained with silver.

RESULTS

The effect of parenteral stimulation on coelomic fluid protein concentration. Earthworms respond to body injury or to other stress conditions by nonspecific changes in protein concentration of the coelomic fluid. Therefore it was necessary to determine the protein concentration in each coelomic fluid sample. The changes in the protein concentration were significant, maximum levels were observed 4–8 d after the stimulation, and in some cases reached three-fold values of nontreated controls. There were no differences among the proteins used for stimulation and a similar increase was detected in sham-stimulated controls.

The kinetics of ABP response. We followed the kinetics of ABP levels after the stimulation in 4-d intervals using the monoclonal antibody 6D4. In agreement with our previous observations (Tučková et al. 1988; 1991a,b), we found that the maximum levels can be detected after 8 d (Table I), reaching 157–334% of the control. In sham-stimulated controls the changes in ABP levels were only negligible. Similar results were achieved in ligand assay with biotin-labeled proteins corresponding to those used for stimulation (Table II). Both earthworm species, i.e. L. terrestris and E. fetida responded to the stimulation with the same time course.
Table I. Kinetics of antigen binding

<table>
<thead>
<tr>
<th>Earthworm</th>
<th>Stimulation with</th>
<th>Detected with the monoclonal antibody&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Detected with biotin-labeled proteins&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>days after stimulation</td>
<td>days after stimulation</td>
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<tr>
<td></td>
<td></td>
<td>4</td>
<td>8</td>
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<tr>
<td><em>L. terrestris</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>102</td>
<td>208</td>
<td>83</td>
</tr>
<tr>
<td>HSA</td>
<td>74</td>
<td>213</td>
<td>74</td>
</tr>
<tr>
<td>ARS-HSA</td>
<td>122</td>
<td>309</td>
<td>118</td>
</tr>
<tr>
<td>ferritin</td>
<td>101</td>
<td>294</td>
<td>105</td>
</tr>
<tr>
<td>gliadin</td>
<td>102</td>
<td>267</td>
<td>106</td>
</tr>
<tr>
<td>insulin</td>
<td>73</td>
<td>295</td>
<td>91</td>
</tr>
<tr>
<td>agar</td>
<td>107</td>
<td>101</td>
<td>123</td>
</tr>
<tr>
<td><em>E. fetida</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>128</td>
<td>334</td>
<td>89</td>
</tr>
<tr>
<td>HSA</td>
<td>101</td>
<td>176</td>
<td>96</td>
</tr>
<tr>
<td>ARS-HSA</td>
<td>84</td>
<td>309</td>
<td>137</td>
</tr>
<tr>
<td>ferritin</td>
<td>101</td>
<td>244</td>
<td>95</td>
</tr>
<tr>
<td>gliadin</td>
<td>123</td>
<td>157</td>
<td>71</td>
</tr>
<tr>
<td>insulin</td>
<td>98</td>
<td>274</td>
<td>95</td>
</tr>
<tr>
<td>agar</td>
<td>81</td>
<td>111</td>
<td>97</td>
</tr>
</tbody>
</table>

<sup>a</sup>Earthworms were stimulated with different proteins. Indicated as the percentage of non-stimulated controls. Agar was injected to follow the nonspecific sham stimulation. Representative data of one of three independent experiments.

<sup>b</sup>Followed in direct ELISA with anti-ABP monoclonal antibody 6D4.

<sup>c</sup>Followed in a ligand assay with corresponding biotin-labeled proteins used for the stimulation.

Table II. Specificity of antigen recognition<sup>d</sup>

<table>
<thead>
<tr>
<th>Detection with</th>
<th>Stimulation with</th>
<th>BSA</th>
<th>HSA</th>
<th>ARS-HSA</th>
<th>ferritin</th>
<th>gliadin</th>
<th>insulin</th>
<th>agar</th>
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<tr>
<td><em>L. terrestris</em></td>
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<td></td>
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<tr>
<td>BSA</td>
<td>237</td>
<td>246</td>
<td>171</td>
<td>118</td>
<td>139</td>
<td>108</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>HSA</td>
<td>160</td>
<td>311</td>
<td>186</td>
<td>102</td>
<td>116</td>
<td>101</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ARS-HSA</td>
<td>181</td>
<td>227</td>
<td>312</td>
<td>123</td>
<td>117</td>
<td>118</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Ferritin</td>
<td>137</td>
<td>121</td>
<td>152</td>
<td>220</td>
<td>125</td>
<td>122</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Gliadin</td>
<td>83</td>
<td>100</td>
<td>161</td>
<td>110</td>
<td>219</td>
<td>105</td>
<td>98</td>
<td></td>
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<tr>
<td>Insulin</td>
<td>111</td>
<td>101</td>
<td>152</td>
<td>121</td>
<td>155</td>
<td>218</td>
<td>102</td>
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<tr>
<td><em>E. fetida</em></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>252</td>
<td>174</td>
<td>171</td>
<td>130</td>
<td>166</td>
<td>146</td>
<td>116</td>
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<td>HSA</td>
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<td>173</td>
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<tr>
<td>Insulin</td>
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<td>125</td>
<td>134</td>
<td>168</td>
<td>235</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>

<sup>d</sup>Earthworms were stimulated with different proteins and the binding was followed in a ligand assay with all biotin-labeled proteins that were used for the stimulation. The binding was assessed on day 8 after stimulation when the levels are maximum (bold and double underlined) for all proteins. Indicated as the percentage of nonstimulated controls. Agar was injected to follow the nonspecific sham stimulation. Representative data of one of three independent experiments.

**Specificity of ABP response.** To determine the level of specificity we selected the maximum values obtained in ligand assays, *i.e.* the values from day 8. The results are summarized in Table II, indicating that optimum binding can be detected when the same protein that had been used for stimulation was employed for detection. This reflects that maximum values are placed diagonally in the
Table II. Changes in sham-stimulated controls were insignificant in all cases. Though there were slightly increased levels in the case of related proteins (BSA, HSA, ARS-HSA), the highest binding was observed when the corresponding protein was used for detection. No differences were found when L. terrestris and E. fetida were compared.

Isolation of ABP. Antigen-binding proteins formed in response to stimulation with HSA, ARS-HSA, gliadin, and ferritin were isolated on Affi-Gel affinity column and analyzed in SDS-PAGE. There are no differences in molar mass of isolated proteins, in L. terrestris the molar mass was estimated to be 56 kDa, in E. fetida 60 kDa (Fig. 1).

DISCUSSION

Inducible agglutinins recognizing non-self saccharide moieties were described in numerous papers (Wojdani et al. 1982; Stein and Cooper 1983; Stein et al. 1986; Kauschke and Mohrig 1987; Valembois et al. 1993). Our knowledge of adaptively synthesized substances in response to protein stimulation is based on the report of Lau lan et al. (1985) and on papers describing antigen-binding protein — ABP (Tučková et al. 1989; 1991a). Particularly, the role of ABP was detailed in some papers so that we can draw a certain hypothesis of the response to protein antigens. The administered protein is rapidly degraded by proteinases both in the cecolic fluid and in free cecolocytes (Bilej et al. 1993; Rejnek et al. 1993). Proteolytic processing is limited, results in peptide fragments of molar mass 700–1100 Da (Hanušová et al. 1999), and seems to be prerequisite for triggering of further response (Tučková and Bilej 1994). Antigen fragments are then bound by the precursor cells in the mesenchymal tissue (Rejnek et al. 1993) inducing their proliferation, differentiation, and migration into the cecolic cavity (Bilej et al. 1992). These predetermined free cecolocytes can undergo further mitotic cycles after secondary challenge with the same antigen.

The level of specificity was discussed in our previous contribution (Bilej et al. 1995). In that paper we showed that ABP molecules formed after the stimulation with different proteins are recognized with the same monoclonal antibody 6D4 and that the binding is highest when the same protein is used for capturing in ELISA. In the present paper we confirm that the monoclonal antibody originally generated against ABP isolated from L. terrestris earthworms after ARS-HSA stimulation recognizes probably a conserved epitope on ABPs both in L. terrestris and E. fetida earthworms. Moreover, to follow the changes more quantitatively we expressed the ABP levels as a percentage of

![Fig. 1. SDS-PAGE analysis of the cecolic fluid proteins and affinity-purified ABP after stimulation with HSA (lane a and e), ARS-HSA (lane b and f), ferritin (lane c), and gliadin (lane d and h). Lane a and e: L. terrestris. Right: E. fetida. The samples were separated under non-denaturing conditions. Lane M — molar mass markers.](image_url)
sham-treated controls when all cœlomic fluid samples were diluted to the same concentration estimated as optimum in ELISA. There were no differences in the kinetics of ABP response to different proteins and when *L. terrestris* and *E. fetida* earthworms were compared. In agreement with previous results (Tučková et al. 1989, 1991a), in all cases the maximum levels were detected after 8 d.

The stimulating proteins were selected to vary in size from low-molar mass insulin (approximately 5 kDa) to high-molar mass ferritin (approximately 900 kDa). The size of stimulating proteins had no significant effect on the ABP response. Though increased ABP levels were detected after the stimulation with glycosylated as well as non-glycosylated proteins the role of glycosylation will be further envisaged.

Elevated levels of bound related albumins confirm the limited specificity (Table II) but the highest binding was displayed by the same protein as was used for stimulation.

The molecular mass of ABPs isolated by affinity chromatography from the cœlomic fluid after stimulation with HSA, ARS-HSA, ferritin, or gladin was the same and corresponded to 56 kDa in *L. terrestris* (31 and 33 kDa under reducing conditions; not shown) and 60 kDa in *E. fetida* (probably 30-kDa homodimer, not shown). Preliminary N-terminal sequence analysis of *L. terrestris* revealed that ABPs isolated from both species are new proteins without any homology with known proteins. There is no similarity as concerns the N-terminal amino acid sequence of ABP polypeptide chains in *L. terrestris* and *E. fetida*. Since N-terminal amino acid sequence of both polypeptides forming ABP in *L. terrestris* (i.e. 31 and 33 kDa) is identical it can be suggested that both polypeptides are related and might differ, for example, in the level of posttranslational modifications.

We can conclude that (i) the monoclonal antibody 6D4 recognizes a conserved epitope on ABP, irrespective of the species and the protein used for stimulation, (ii) ABP levels are increased after the stimulation with different proteins, irrespective of the size and extent of glycosylation, (iii) the level of specificity is considerably lower as compared with immunoglobulins since ABP reacts not only with the protein used for stimulation but also, even if to a lesser extent, with related proteins.

This work was supported by grants no. 310/99/1385 of the Grant Agency of the Czech Republic and no. A7020601 of the Grant Agency of the Academy of Sciences of the Czech Republic. The skilled technical assistance of Mrs. J. Perková is gratefully acknowledged.

REFERENCES


4.2 Effect of experimental microbial challenge on the expression of defense molecules in *E. fetida* earthworms

One pattern recognition protein named coelomic cytolytic factor - CCF (Bilej et al. 1995a; Beschin et al. 1998) and two main groups of antimicrobial factors - lysozyme-like molecule (Ito et al. 1999) and factors with hemolytic activity have been identified and detailed in last two decades in *Eisenia fetida* earthworms (Roch 1979, 1981; Lange et al. 1997, 1999; Lassegues et al. 1997; Sekizawa et al. 1997; Eue et al. 1998; Yamaji et al. 1998).

We monitored the effect of *in vivo* stimulation of *Eisenia fetida* earthworms with microbial antigens on changes of defense activity of the coelomic fluid (lysozyme-like activity, hemolytic activity, production of CCF) and on the expression of defense molecules CCF and fetidin in coelomocytes.

Earthworms were injected with live Gram-negative and Gram-positive bacteria, with β-1,3-glucan and with salt solution (LBSS) as a sham control, and all changes were followed during an interval of 2 hours to 13 days after the infection. All values were compared with values of animals injected with LBSS only to avoid the influence of the non-specific response. We knew that earthworms respond to antigenic stimulation, but also to body injury by non-specific changes in protein concentration of the coelomic fluid. In the case of injection with LBSS alone, the increased protein concentration is caused probably by body injury and returns back to the level of non-injected animals after 2 days post injection. Administration of bacteria and microbial polysaccharides further modulated coelomic fluid protein concentration.

An increase of lysozyme-like activity as a result of administration of live bacteria and β-1,3-glucan occurred between 6 hours and 4 days post-injection, with the highest increase occurring 2 days post-infection. Surprisingly, no significant difference was found between animals infected with Gram-negative or Gram-positive bacteria.

Hemolytic activity was expressed as the protein concentration of the coelomic fluid that results in the lysis of 50 % of erythrocytes. Thus, an increase in protein concentration causing 50 % hemolysis reflects a relative decrease in the hemolytic activity of the coelomic fluid. The coelomic fluid of animals challenged with Gram-negative bacteria showed a reduced hemolytic activity 12 hours and 2 days after the infection. With Gram-positive bacteria, the hemolytic
activity of the coelomic fluid decreased 2 days after the infection. No significant modulation of the hemolytic activity was observed in β-1,3-glucan-infected individuals.

Injection of live bacteria or β-1,3-glucan results in an increase of concentration of CCF in the coelomic fluid. A significant increase in CCF protein level was observed both in Gram-negative and Gram-positive bacteria-injected worms between 6 and 24 hours. CCF concentration in the coelomic fluid also increased between 6 and 12 hours following the administration of β-1,3-glucan.

Further, levels of mRNA coding for CCF and fetidin in *E. fetida* coelomocytes were determined in stimulated worms within 2 to 70 hours post-injection. We found that the level of CCF mRNA increased from 2 to 40 hours after all microbial stimulations and the maximum increase was detected 17 hours post-injection. Induction of CCF mRNA was lower in *B. subtilis*-injected animals as compared to *E. coli*-infected or β-1,3-glucan-treated worms. Seventy hours post-treatment, CCF mRNA level in all experimental groups dropped below the levels observed in non-infected animals. Interestingly, the level of mRNA coding for fetidin was not modulated at any time interval, suggesting its constitutive expression.

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

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**Abstract**

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

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

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**1. Introduction**

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

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

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

In E. foetida, hemolytic activity is connected to the antimicrobial activity of the coelomic fluid. Accord- ingly, numerous hemolytic factors that exert aggluti-nation and antibacterial activities against pathogenic soil bacteria have been described [7,14–22]. Du Pasquier and Duprat [17] were the first to evidence a hemolytic activity against erythrocytes of various vertebrates in the coelomic fluid of E. foetida andrei. Further experiments revealed that the hemolytic factors constitute a polymorphic system [7]. Roch et al. [8,18] described an E. foetida hemolytic factor, with two isoforms of 40 and 45 kDa differing in the extent of glycosylation. These molecules are secreted by chloragocytes or eleocytes and exhibit antibacterial activity. E. foetida hemolytic factors were named fetidins and one of them was later on cloned [6,19]. Independently, a 41 kDa hemolytic protein, produced by coelomocytes and causing the contraction of rat vascular smooth muscle, was cloned and described as lysenin by Sekizawa et al. [11,20]. Simultaneously, two 42 kDa lysenin-related proteins were identified. Lysenin and lysenin-related proteins reveal a high sequence homology with fetidins, suggesting that these molecules are isoforms [11,12,21,22].

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

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

2. Materials and methods

2.1. Animals and their stimulation

Adult E. foetida earthworms (Oligochaeta; Annelida) kept at 20 °C in compost were transferred 2 days before experiments on filter paper soaked with isotonic Lumbricus balanced salt solution (LBSS) [26]. They were injected post-citellum with insoluble β-1,3-glucan preparation from Saccharomyces cerevisiae (kindly provided by Dr Novak, Institute of Microbiology, Prague), live Gram-negative bacteria Escherichia coli K12 and live Gram-positive bacteria Bacillus subtilis W23. Fifty micrograms of β-1,3-glucan were given in 25 μL of LBSS. Cultures of bacteria at OD of 1 (50 ml of culture in LB broth, Fluka, Buchs, Switzerland) were centrifuged (3000g, 20 min) and resuspended in 10 ml LBSS. Twenty-five microliters (approximately 10^5 CFU) of resuspended cultures were injected into earthworms. Non-stimulated earthworms and earthworms injected with LBSS were used as controls.

2.2. Harvesting of the coelomic fluid and coelomocytes

Coelomic fluid was obtained by puncturing post-citellum segments of the coelomic cavity with a Pasteur micropipette and kept at 4 °C. At different time intervals post-challenge (0, 6, 12, 18, and 24 h and 2, 3, 4 and 6 days after the stimulation) and for each experimental group (non-stimulated animals, animals injected with LBSS only, animals
stimulated with *E. coli*, *B. subtilis* and *β-1,3-glucan*, equal volumes of coelomic fluid suspension from 15 earthworms were pooled and centrifuged (500g, 10 min, 4 °C). Supernatant was centrifuged again (7000g, 10 min, 4 °C), and stored at −20 °C.

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

2.3. Evaluation of protein concentration

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

2.4. Evaluation of lysozyme-like activity

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

2.5. Evaluation of hemolytic activity

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

2.6. Determination of CCF level

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

2.7. Statistical analysis

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

2.8. RNA isolation and cDNA synthesis

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

Each PCR cycle consisted of denaturation at 94 °C for 40 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 90 s. PCR primer pairs were: for CCF (accession number AF030028), sense (5'-ATCAAT ATCACATCGTCTGGCAG-3') and anti-sense (5'-GTCGATCTCGCCCGATCGAG-3') giving an amplicon of 0.510 kb; for fetidin (accession number U02710), sense (5'-AAGCATGCGAGCAGAGG AGTAT-3') and anti-sense (5'-GCGGAAACGAATA TGTGTAATGCTT-3') giving an amplicon of 0.443 kb; for myosin (accession number AF537290), sense (5'-GCGGAAATCGAGGATGTTGCT-3') and anti-sense (5'-CTACTTCTCGGAGGTCCACG-3') giving an amplicon of 0.453 kb. Amplifications were performed for 26 cycles. The amount of template cDNA and number of PCR cycles were optimized so that the analysis of the PCR products could be carried out within the linear range of amplification. Myosin was used as a control to ensure that differences in the expression levels of each gene in different samples were not due to differences in the amounts of template cDNA.

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

3. Results

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

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

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

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

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

Hemolytic activity was investigated in coelomic fluids of E. foetida up to 6 days post-LBSS, β-1,3-glucan, Gram-negative and Gram-positive bacteria administration. This activity was expressed as the protein concentration of the coelomic fluid that lyases
Fig. 1. Protein concentration in the coelomic fluid of *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with live Gram-negative bacteria *E. coli*, live Gram-positive bacteria *B. subtilis* and β-1,3-glucan. Sham-stimulated earthworms received LBSS. At different time intervals post-administration, protein concentration (mg/ml) in the coelomic fluid was determined. Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of protein concentration in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control (*P* < 0.05).

Fig. 2. Lysozyme-like activity in the coelomic fluid of *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with LBSS, live Gram-negative bacteria *E. coli*, live Gram-positive bacteria *B. subtilis* and β-1,3-glucan. At different time intervals post-administration, lysozyme-like activity was determined and expressed (U/ml) using chicken-egg white lysozyme as a standard. Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of lysozyme-like activity in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control (*P* < 0.05).
50% of erythrocytes (LD50). Thus, an increase in protein concentration causing 50% hemolysis reflects a relative decrease in the hemolytic activity of the coelomic fluid.

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

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

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

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

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

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

Fig. 3. Hemolytic activity in the coelomic fluid of E. foetida challenged in vivo with bacteria and β-1,3-glucan. Earthworms were stimulated with live Gram-negative bacteria E. coli, live Gram-positive bacteria B. subtilis and β-1,3-glucan. Sham-stimulated earthworms received LBSS. At different time intervals post-administration, hemolytic activity of coelomic fluid, was expressed as protein concentration (μg) causing 50% hemolysis (LD50). Data are expressed as mean ± SD of triplicates of three independent experiments. Horizontal lines represent the range of hemolytic activity in non-stimulated earthworms. Dashed line indicates LBSS sham-stimulated control. Asterisks indicate significant difference as compared to sham-stimulated control (P < 0.05).
stimulations as compared to non-stimulated and sham-stimulated earthworms, maximum level being observed 17 h post-injection (Fig. 5). The induction of CCF mRNA was lower in B. subtilis-infected individuals as compared to E. coli-infected or β-1,3-glucan-treated animals, in particular 2 and 5 h post-stimulation. At 40 h post-inoculation, CCF expression level was still twice as high in E. coli, B. subtilis and β-1,3-glucan-injected worms as compared to non-injected and LBSS-injected E. foetida. Finally, it can be mentioned that LBSS-treatment marginally increased CCF mRNA level at 5 and 17 h post-injection as compared with untreated animals. Seventy hours post-treatment, CCF mRNA level in the four experimental groups dropped below the levels observed in non-injected worms.

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

4. Discussion

The coelomic cavity of annelids is not aseptic and typically contains bacteria, protozoans and fungi entering from the outer environment mainly via dorsal pores. Dales and Kalac [30] reported that the concentration of naturally occurring bacteria was commonly of the order of $6 \times 10^5$ per ml of coelomic fluid, or $0.9 \times 10^5$ per worm of an average size. Because the number of potentially phagocytic cells in the coelomic fluid is more than 10 times higher than the number of naturally occurring bacteria, the excess of phagocytic cells and other factors can prevent the detrimental multiplication of coelomic microflora. The amount of bacteria injected in the present study exceeded approximately by 10 times the number of naturally occurring phagocytic cells, yet it did not affect the viability of E. foetida and was sufficient to activate defense mechanisms. We did not estimate in vitro the number of colony forming units in the collected coelomic fluids in the course of infection with live bacteria for several reasons. First, it is worthwhile remembering that the coelomic cavity of annelids is metameric, segments being separated by
transversal septa. The transport of coelomic fluid and cells between neighboring segments is regulated by sphincters within the septa [31]. As such, the metamerization restrains the homogeneous distribution of bacteria and other corpuscular antigens, but not of soluble factors, in the coelomic cavity in the early stage of infection. Second, agglutination occurring in the coelomic fluid of *E. foetida* [2,32], may further impair the spreading of bacteria through the coelomic cavity via channels of the septa. Therefore, the precise assessment of bacteria number through puncturing the coelomic cavity is not obvious.

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

Cotuc and Dales [4] have reported that the lysozyme-like activity in coelomic fluid of *E. foetida* is low, yet the enzyme may be released from coelomocytes as a part of an internal defense mechanism against particular bacterial species. Accordingly, we have shown that the in vitro induction of phenoxodase activity in *E. foetida* coelomic fluid by Gram-positive bacteria required lysozyme-like activity [24]. Therefore, it was of interest to investigate the modulation of lysozyme-like activity in earthworms challenged in vivo with Gram-positive bacteria as well as Gram-negative bacteria and β-1,3-glucan.

Maximum increase of lysozyme-like activity in the coelomic fluid was observed 2 days after microbial stimulation, returning back to control levels on day 6. A significant increase in lysozyme-like activity was also recorded on day 2 in sham-stimulated as compared to non-stimulated earthworms, but did not reach the values observed in animals challenged by microbial injections. Lysozyme degrades peptidoglycan, an activity mainly considered to be directed against Gram-positive bacteria. Surprisingly, we did not observe a principal difference in the maximum lysozyme-like activity in Gram-positive bacteria infected worms as compared to animals stimulated with Gram-negative bacteria or β-1,3-glucan raising the question of the specificity of the increased lysozyme-like activity recorded in Gram-negative bacteria and β-1,3-glucan-challenged earthworms.

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

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

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Fig. 5. mRNA levels of CCF and fetidin in *E. foetida* challenged in vivo with bacteria and β-1,3-glucan. CCF and fetidin mRNA expression in coelomocytes of non-challenged earthworms and of earthworms challenged with LBSS, live Gram-negative bacteria *E. coli* (E.c.), live Gram-positive bacteria *B. subtilis* (B.s.) and β-1,3-glucan was analyzed by semi-quantitative RT-PCR. At different time intervals post-administration, levels of expression of CCF and fetidin genes were normalized to the expression of myosin and expressed as fold induction as compared to non-stimulated earthworms. Data are representative of two independent experiments.
that both cellular and humoral levels of CCF significantly increased within 24 h after parenteral injection of LPS, then dropped down to control values.

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

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

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

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References


4.3 Relationship between hemolytic molecules in *E. fetida* earthworms

*E. fetida* proteins causing the lysis of red blood cells represent a heterogeneous set of proteins described by different groups and comprises *Eisenia fetida andrei factor* (EFAF) (Roch et al. 1981, 1984), fetidin (Lassegues et al. 1997; Milochau et al. 1997), lysenin (Sekizawa et al. 1996, 1997), eiseniapore (Lange et al. 1997), and hemolysins isolated either from coelomic fluid or from cell lysate (Eue et al. 1998; Koenig et al. 2003). All these proteins are hemolytic, but their nomenclature, identity and relationship are unclear.

Our major task was to elucidate whether fetidin and lysenin are either protein isoforms encoded by alleles of one gene, or products of two related genes.

Both cDNA and amino acid sequences of fetidin and lysenin display high homology, with differences in 5'UTR. We designed lysenin- and fetidin-specific primers used and them in PCR. We found that lysenin-specific primer is not specific for lysenin, but amplifies also fetidin cDNA due to errors in the published 5'UTR sequence of fetidin. Based on our findings we designed a new set of specific primers based on the presence of deletions in the sequences of lysenin and fetidin cDNA. The use of these specific primers together with stringent annealing temperature in PCR provides a simple tool for amplifying only the desired sequence (either fetidin or lysenin). By using these specific primers we performed PCR in individual earthworms and proved that the coelomocytes of each individual *E. fetida* contain mRNA for both fetidin and lysenin.

Furthermore, isolated genomic DNA of individual *E. fetida* specimens was used again in PCR with lysenin- and fetidin-specific primers. Partial DNA sequences of genes coding for both proteins in every individual was amplified, suggesting that each protein is encoded by a different gene.

To see the quantitative differences in the expression of the genes encoding these two proteins, real-time PCR with cDNA of several individual worms was performed. We found that fetidin was expressed to similar levels in all tested animals, while the expression of lysenin was in some individuals 23-26 times higher than in others.

The coelomic fluids of individual tested animals were further separated in native PAGE and the gels were applied on sheep erythrocyte suspensions in agarose. After some hours of
incubation it was possible to distinguish four different patterns of hemolytic proteins of coelomic fluids. In parallel, the hemolytic activity of individual tested animals was quantified by incubating coelomic fluids with sheep erythrocyte suspensions. The distinct hemolytic patterns correlated with differences in the level of hemolytic activity of the coelomic fluids; all animals exerting higher hemolytic activity developed the same hemolytic pattern consisting of a higher number of bands on agarose-embedded erythrocytes, reflecting the presence of more hemolytic proteins in the coelomic fluids of these animals.

Interestingly, we proved the presence of cDNA sequence of neither fetidin nor lysenin in other earthworm species (Lumbricus terrestis, Aporrectodea caliginosa, Aporrectodea icterica, Aporrectodea longa, Aporrectodea rosea, Dendrobaena veneta, Lumbricus rubellus) by PCR using lysenin- and fetidin-specific primers. This suggested the unique occurrence of these hemolytic molecules in Eisenia fetida earthworms.

Relationship between hemolytic molecules in *Eisenia fetida* earthworms

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Abstract

The coelomic fluid of the earthworm *Eisenia fetida* has been reported to contain a variety of proteins causing the lysis of red blood cells—EFAF (*Eisenia fetida andrei* factor), fetidin, lysenin, eiseniapone, and hemolysins isolated either from coelomic fluid (H1, H2, H3) or from cell lysate (CL\(_{09}\) and CL\(_{41}\)). We document the presence of two distinct genes with a high level of homology. These genes encode fetidin and lysenin but their level of expression differs in individual *E. fetida andrei* animals. © 2005 Elsevier Ltd. All rights reserved.

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

1. Introduction

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

Hemolytic *E. fetida* proteins were described for the first time in 1968 by Du Pasquier and Duprat [5]. Later on, they were named EFAF (*Eisenia fetida andrei* factors), and characterized as two glyco-proteins of 40 and 45 kDa, secreted by chloragocytes and eleocytes, which differ in the extent of glycosylation [1,6,7] and constitute a polymorphic system. Accordingly, the 45-kDa protein was characterized as having a pI of 6.0 and, possibly, being encoded by one
nonpolymorphic gene, while the 40-kDa protein was defined as being encoded by a gene having four alleles encoding four isoforms with pl of 6.3, 6.2, 5.95 and 5.9. All animals possess either 2 or 3 isoforms of the 40-kDa molecule, with one isoform, with pl of 6.0, invariably present. As a result of the polymorphism, six different hemolytic patterns were found in E. fetida individuals [1,8].

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

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

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

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

In 2003, Koenig et al. [22] have isolated native hemolytic proteins both from coelomocyte lysate (CL-10 and CL-41) and from the coelomic fluid (H1, H2, H3) of E. fetida earthworms. Using mass spectrometric analyses, they have demonstrated the identity of CL-10 with fetidin and CL-41 with lysenin, and the probable identity of H1-3 hemolyzins with fetidin. Hence, different proteins from E. fetida cause lysis of red blood cells, but there is no consensus concerning their nomenclature, identity, and relationships. The major task in this respect is therefore to convincingly document that fetidin and lysenin are either protein isoforms encoded by alleles of one gene or, alternatively, they are products of two related genes. Here, we show that fetidin/CL-10 and lysenin/CL-41 are encoded by two individual genes with high homology, whose level of expression differs from individual to individual.

2. Material and methods

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

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

2.2. Hemolysis assay

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

2.3. Lysis of SRBC in agarose after native PAGE

To visualize differences in the pattern of hemolytic proteins, 8 μl samples of coelomic fluid were run for 5 h at 300 mA constant current at 4 °C on native 10 % PAGEs with SDS (16 × 20 cm gel). After separation, the gels were washed in 145 mM NaCl, pH 7.4 and applied on plates containing 1% low melting agarose (Invitrogen) and 0.9% NaCl with 1% sheep erythrocyte suspension. The lysis was observed after a 3-h incubation at room temperature as transparent bands in front of the reddish background.

2.4. Genomic DNA isolation

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

2.5. RNA isolation, cDNA synthesis, PCR and cloning

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Direction</th>
<th>Occurrence in fetidin (nucleotide pos.)</th>
<th>Occurrence in lyisenin (nucleotide pos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys1up</td>
<td>ACG GCT CAT AGT ATT GGT TCT</td>
<td>sense</td>
<td>256–279</td>
<td>300–323</td>
</tr>
<tr>
<td>Lys1low</td>
<td>CCT TCC TGT CCG CAT GCT TTA TCC</td>
<td>antisense</td>
<td>622–599</td>
<td>666–643</td>
</tr>
<tr>
<td>Lys2up</td>
<td>AAG CAT GCG GAC AGG GAG GAT TAT</td>
<td>sense</td>
<td>604–627</td>
<td>648–671</td>
</tr>
<tr>
<td>Lys2low</td>
<td>GCG GAA GCA AAT GTG TAA TGG TG</td>
<td>antisense</td>
<td>1042–1021</td>
<td>1090–1069</td>
</tr>
<tr>
<td>Lys3up</td>
<td>CAG CCG TGG GCA ATC AAT AAG TCA</td>
<td>sense</td>
<td>820–843</td>
<td>864–887</td>
</tr>
<tr>
<td>Lys3low</td>
<td>CGG CGG CGC AGA ACA ATA GG</td>
<td>antisense</td>
<td>1473–1454</td>
<td>1510–1491</td>
</tr>
<tr>
<td>Lysen4up</td>
<td>CTC AGA TGC AGT TAT GTA TCT A</td>
<td>sense</td>
<td>–</td>
<td>1–24</td>
</tr>
<tr>
<td>VAV1</td>
<td>TGT GAG CGA TGT CTT GCA GAG CAG GAA TCG</td>
<td>sense</td>
<td>80–109</td>
<td>–</td>
</tr>
<tr>
<td>VAV2</td>
<td>CCC CAT TCC AGG GGT TAA CCA ACC A</td>
<td>antisense</td>
<td>–</td>
<td>1045–1021</td>
</tr>
<tr>
<td>VAV3</td>
<td>TGG CTA TTA CAA TCT ACA CCG C</td>
<td>antisense</td>
<td>–</td>
<td>1154–1133</td>
</tr>
<tr>
<td>VAV4</td>
<td>TGG TGT TAC ACT TGG TAA AG</td>
<td>antisense</td>
<td>1310–1291</td>
<td>–</td>
</tr>
</tbody>
</table>
0.2 mM dNTP. 1 × company-supplied buffer, 1.5 mM MgCl₂ and 0.4 μM primer pairs were added. PCR conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55-63 °C for 40 s, 72 °C for 120 s, and 72 °C for 7 min.

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

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

2.6. Relative quantification of fetidin and lysenin gene expression

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

2.7. Sequencing

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

2.8. Statistical analysis

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

3. Results

3.1. Comparison of fetidin and lysenin sequences

Both cDNA and amino acid sequences of fetidin and lysenin display high homology, with differences being found in 5'UTR (Figs. 1–3). To elucidate the relationships between the two molecules we designed lysenin-specific primers based on the published nucleotide sequences of fetidin and lysenin cDNA (Fig. 1). RNA from coelomocytes of 25 adult E. fetida andrei earthworms was isolated, used in One-Step RT-PCR with Lys2low and Lysen4up primers and all amplified fragments were sequenced. Unexpectedly, we found that primer Lysen4up is not specific for lysenin, but amplifies also fetidin cDNA due to errors in the published 5'UTR sequence of fetidin [9]. The sequence in 5'UTR fetidin cDNA we have amplified corresponded rather to the published sequence of lysenin cDNA. Comparison of 5'UTR nucleotide sequences published for fetidin and lysenin cDNA
with fetidin cDNA found in 20 samples tested here is shown in Fig. 3.

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

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

<table>
<thead>
<tr>
<th>Lysenup</th>
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<tbody>
<tr>
<td>Lys: 1</td>
</tr>
<tr>
<td>Lys: 56</td>
</tr>
<tr>
<td>Lys: 111</td>
</tr>
<tr>
<td>Lys: 12</td>
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<tr>
<td>Lys: 42</td>
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<tr>
<td>Lys: 72</td>
</tr>
<tr>
<td>Lys: 825</td>
</tr>
<tr>
<td>Lys: 29</td>
</tr>
<tr>
<td>Lys: 66</td>
</tr>
</tbody>
</table>

Fig. 1. Comparison of nucleotide sequences of fetidin (GenBank accession no. U02710) and lysenin cDNA (GenBank accession no. D85846). The start (atg) codons are boxed and the stop (tga, taa) codons are indicated with asterisks. Oligonucleotides used as primers specific for the fetidin and lysenin mRNA are underlined and their names are in bold. Nucleotides of fetidin cDNA that match with the lysenin cDNA are indicated by a dash, gaps are indicated by a slash.
primers together with stringent annealing temperature in PCR provides a simple tool for amplifying only the desired sequence (either fetidin or lysenin). Using specific primers for fetidin (VAV1, VAV4) or lysenin (VAV2, VAV3) we could amplify cDNA of both fetidin and lysenin in the 25 earthworms tested, suggesting that the coelomocytes of each individual *E. fetida andrei* contain mRNA for both fetidin and lysenin.

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

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

3.3. Different expression of fetidin and lysenin

To see the quantitative differences in the expression of genes encoding lysenin and fetidin, the cDNA of four individual worms was used in realtime PCR with fetidin-specific forward primers Lys2up or Lys3up and reverse primer VAV4 and with lysenin-specific forward primer Lys2up and reverse primers VAV2 or VAV3 (Table 1). Relative expression of fetidin and lysenin was determined as fold expression as compared to individual no. 1 that was chosen as a reference value. As summarised in Fig. 5, there was a significantly higher expression of lysenin in CDNA from individual no. 3 and than no. 1 and 2. Expression of lysenin gene in individuals no. 3 and 4 was 23–26 times higher than in individuals no. 1 and 2 while the expression of fetidin gene was similar in all individuals. The fact that individuals no. 3 and 4 contain a higher level of lysenin mRNA corresponds to the results of sequencing, with these
Fig. 2. Amino acid sequence alignment of fetidin and lysenin proteins. Identical amino acids are indicated by dash (—), missing amino acids are indicated by slash (/). Similar residues identified by the ClustalW program are indicated by plus (+).

| Lysenin   | 1 | ctcagatgca gttattgtat ctaataggca ttctggttt cctggcagata ctcacaaga |
| Fetidin   | 1 | gagatcc cggccgtgaggg |
| 5'UTR     | 1 | ctcagatgca gttattgtat ctaataggca ttctggttt cctggcagata ctcacaaga |

Fig. 3. Comparison of nucleotide sequence of 5'UTRs of published sequences of lysenin and fetidin cDNA with the sequence of fetidin cDNA found in 20 individuals tested (5'UTR). The start (atg) codons are in bold. Nucleotides identical in both sequences are indicated with colon (:); nucleotides corresponding to nucleotide sequence of lysenin cDNA are indicated with plus (+), nucleotides corresponding to nucleotide sequence of fetidin cDNA are indicated with slash (/).
Table 2
Comparison of amino acid sequences of fetidin and/or lysenin proteins of 25 individual *E. fetida* earthworms with published amino acid sequences of fetidin (GenBank accession no. U02710) and lysenin (GenBank accession no. D85846) protein

| Lysenin | Fetidin | No. 1 | SRA | GI | E | K | R | L | K | L | S | L | AT | D | Y | E | L | K | L | E | Y | D | Y | M | S | Y | D | KP |
| 3-4     | 3-4     | 37   | 41 | 47 | 55 | 60 | 65 | 69 | 70 | 73 | 76 | 78 | 83 | 102 | 145 | 152 | 157 | 164 | 167 | 168 | 176 | 200 | 207 | 210 | 240 | 276 | 282 | 299 | 0  |
| 4       | 6.7     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 2       | 3       |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 1       | 2       |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 10      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 11      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 12      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 13      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 14      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 15      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 16      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 17      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 18      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 19      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| 20      | SRA     |       |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |

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

To address the relationships between hemolytic activity and expression of fetidin and lysenin, coelomic fluids of 25 individual earthworms were separated in native PAGEs and gels were applied on sheep erythrocyte suspensions embedded in agarose. Patterns of hemolytic proteins of coelomic fluids were observed as transparent bands in front of the reddish background (Fig. 6). In this way, we determined four different hemolytic patterns. Pattern no. 1 consists of one clear hemolytic band, pattern no. 2 is formed by at least five faint bands including the one present in pattern no. 1, pattern no. 3 consisted of 2 bands that are most likely present also in pattern no. 2, and pattern no. 4 did not develop any visible hemolysis.

Out of the 25 tested worm coelomic fluids, three generated pattern no. 1, twelve pattern no. 2, three pattern no. 3 and seven did not develop any visible hemolytic pattern (no. 4). In parallel, the hemolytic activity of the 25 samples was quantified by incubating coelomic fluids with sheep erythrocyte suspension (Fig. 7). The distinct hemolytic patterns correlated with differences in the level of hemolytic activity of the coelomic fluids. The coelomic fluids of animals developing hemolytic pattern no. 2 have higher hemolytic activity (0.42–3.0 O.D.) than coelomic fluids from animals displaying pattern no. 1, 3 or 4. Also, the hemolytic pattern no. 2 showed a higher number of bands on agarose-embedded erythrocytes, reflecting probably the presence of more hemolytic proteins in the coelomic fluids of these earthworms.

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

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

4. Discussion

Many E. fetida proteins of that cause lysis of red blood cells were described and even though their terminology differs (for review see [28,29]), their further characterization indicates that they are the same or similar molecules. Indeed, all hemolytic molecules described so far share biochemical analogies, having a similar molecular mass around 40 kDa, similar pI and ability to bind sphingomyelin and create pores in lipid membranes. The controversy concerning the nomenclature and identity of hemolytic proteins of E. fetida was partially elucidated by Koenig et al. [22], who characterized hemolysins from coelomocyte lysate (CL39 and CL41) and coelomic fluid (H1-3). Using mass spectrometry, they demonstrated the identity of CL39 and CL41 with fetidin and lysenin, respectively. Moreover, they reported the identity of H1-3 with fetidin. On the other hand, another hemolytic protein named eiseniapore, which has not yet been sequenced, shows common characteristics with lysenin [19,20]. Thus, when all the data are viewed together, it is most likely that E. fetida earthworms have two main hemolytic proteins fetidin and lysenin, although it has not yet been convincingly shown whether these proteins are isoforms or encoded by two independent genes.

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

By testing the hemolytic activity in the coelomic fluid of 25 individual E. fetida andreii, we found three different hemolytic patterns plus one group of seven animals with hemolytic pattern below the detection limit. The absence of the hemolytic pattern in the fourth group is in a good agreement with the very low hemolytic activity assessed in suspension (Fig. 7). However, Roch [11] found six different hemolytic patterns based on the presence of four isoforms of the 40-kDa hemolysin. The difference in the number of hemolytic patterns can arise from the use of different techniques used by the two groups. Whereas we separated coelomic fluid proteins in native PAGE before evaluating the hemolytic activity, Roch separated these proteins according to their isoelectric points. Most coelomic fluids tested in our study (12 out of 25) generated a
hemoletic pattern (no. 2) formed by at least five bands. However, the identity of the proteins in hemoletic spots could not be determined by proteome techniques due to the low amount of coelomic fluid, which can be obtained from one individual earthworm and loaded on the native PAGE. Seven samples did not reveal any significant hemoletic pattern on agarose with red cells; this was in accordance with the measurement of hemoletic activity, which was very low in these samples.

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

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

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References


4.4 Characterization of CCF-like molecules in Lumbricids

Coelomic fluid of *Eisenia fetida* earthworms contains a 42-kDa pattern recognition protein named coelomic cytolytic factor (CCF) - (Bilej et al. 1995a; Beschin et al. 1998). CCF binds cell wall components of Gram-negative and Gram-positive bacteria and yeast. It also triggers the activation of the prophenoloxidase cascade, which is an important invertebrate defense mechanism. CCF comprises two distinct lectin-like domains, each responsible for binding different motifs (Bilej et al. 2001). Moreover, it was found that CCF lyses TNF-sensitive tumor cell line and is able to kill African and American trypanosomes (Beschin et al. 1999; Olivares Fontt et al. 2002).

Our study aimed at identifying and characterizing CCF-like molecules of seven other Lumbricids: *Aporrectodea caliginosa, Aporrectodea icterica, Aporrectodea longa, Aporrectodea rosea, Dendrobaena veneta, Lumbricus rubellus* and *Lumbricus terrestris*.

Using western blot analysis we showed that monoclonal antibody elicited against *E. fetida* CCF reacts with CCF-like proteins in coelomic fluids of all tested species. Further, coelomic fluids of all eight earthworm species were tested for the ability to lyse TNF-sensitive L929 cell line and *Trypanosoma brucei brucei* parasites. We observed that only the coelomic fluid of *E. fetida* was able to lyse tumor cells and parasites. We were not able to detect either cytolytic or trypanolytic activity in the coelomic fluid of other species.

Also the ability of coelomic fluids of tested earthworm species to trigger the prophenoloxidase cascade upon binding laminarin and N,N’-diacetylchitobiose, i.e. sugar compounds that are recognized by distinct CCF domains in *E. fetida* earthworms (Bilej et al. 2001), was examined. The coelomic fluid of all tested earthworm species was able to activate the prophenoloxidase cascade in the presence of laminarin (β-1,3-glucan), whereas only the one of *E. fetida* could trigger the cascade in the presence of N,N’-acetylchitobiose suggesting a broader pattern recognition specificity of *E. fetida* CCF than in other earthworm species.

Further, the CCF-like cDNA of all other species was sequenced; their comparison showed 80-90% sequence identity. They are nearly identical in the region considered as a lectin-like domain, which binds β-1,3-glucans; this is in accordance with the findings that CCF and all CCF-like molecules are able to bind laminarin and initiate activation of the
prophenoloxidase cascade. C-terminal part of all CCF-like molecules is the most variable as compared to the C-terminal part of *E. fetida* CCF, which is in keeping with the fact that *E. fetida* CCF is the only one recognizing N,N′-diacetylchitobiose.

Phylogenetic comparison based on the amino acid sequences of CCF-like molecules and CCF revealed that earthworms of the genus *Lumbricus* fall into a single well-supported group having the same progenitor. On the other hand, CCF-like molecules of the genus *Aporrectodea* are more heterogeneous and they form a paraphyletic group. *E. fetida* CCF is closely related to the CCF-like molecule of *D. veneta* although its saccharide recognition specificity differs.

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

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

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Abstract

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

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1. Introduction

Defense strategies of invertebrates, which naturally lack the adaptive immune system comprising antibodies and lymphocytes, are based on innate mechanisms including wound healing, clotting and coagulation responses, phagocytosis, and encapsulation reactions. Lectin-like molecules and pattern recognition receptors contribute to non-self-recognition processes involved in host innate defense not by discriminating between individual antigens but by recognizing surface determinants common to potential pathogens [1]. Coelomic fluid (CF) of Eisenia fetida earthworms (Oligochaeta, Annelida) contains a 42-kDa pattern recognition protein named coelomic cytolytic factor (CCF; [2]). CCF binds, via lectin-like interactions, cell wall components of Gram-negative bacteria (O-antigen of lipopolysaccharide), Gram-positive bacteria (the
peptidoglycan constituents muramic acid and muramyl dipeptide) and yeast (β-1,3-glucans and N,N'-diacetylchitobiase). It also triggers the activation of the prophenoloxidase cascade [3,4], a major invertebrate defense pathway resulting in the production of melanin that exerts antimicrobial and cytotoxic activities [5].

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

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

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

2. Materials and methods

2.1. Animals, isolation of CF and coelomocytes

Adult earthworms (*A. caliginosa* (Savigny), *A. ictertia* (Savigny), *A. longa* (Ude), *A. rosea* (Savigny), *D. veneta* (Rosa), *E. fetida* (Savigny), *L. rubellus* (Hoffmeister) and *L. terrestris* (Linne); Oligochaeta, Annelida) were collected from their natural habitats with an electrical expeller [14] and maintained in soil at 4°C. They were transferred on moist paper towels 2 days before experiment. CF containing coelomocytes from 10 earthworms of each species was harvested by puncturing post-citelleum segments of the coelomic cavity with a Pasteur micropipette. Coelomocytes were separated by centrifugation (500 g, 10 min, 4°C) and the coelocyte pellet was washed twice with Lumbricus balanced salt solution (71.5 mM NaCl, 4.8 mM KCl, 3.8 mM CaCl2, 1.1 mM MgSO4, 0.4 mM KH2PO4, 0.4 mM NaH2PO4, 4.2 mM NaHCO3, pH 7.3; [15]) and then used for RNA isolation. The cell-free CF was centrifuged again (7000 g, 10 min, 4°C) and the supernatant was stored at −20°C after having determined protein concentration according to Bradford [16] using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories).

2.2. Polyacrylamide gel electrophoresis and Western blot analysis

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

2.3. Cytotoxic L929 assay

L929 fibrosarcoma TNF-sensitive cell line was cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 × 10−6 U/l of penicillin, 100 mg/l of streptomycin and 250 μg/l of amphotericin B. One hundred microitters of 4 × 105 cells/ml cell suspension was
adhered in 96-well flat-bottomed culture plates for 1h at 37 °C and 5% CO₂. Then 100 μl of serially diluted CF was added and after 18 h of incubation at 37 °C cell viability was assessed by crystal violet uptake; cells were stained for 10 min with 100 μl of a 0.5% solution of crystal violet dissolved in 22% ethanol and 8% formaldehyde. The plates were rinsed in water, 100 μl of 30% acetic acid was added and dye uptake was measured at 620 nm.

2.4. Trypanosomes and trypanolytic assay

*Trypanosoma brucei brucei* (AnTat 1.1E) was maintained by intraperitoneal inoculations in BALB/c mice. Trypomastigotes were obtained from the blood (supplemented with 10 U of heparin/ml) of infected mice by ion-exchange chromatography on DEAE-cellulose equilibrated with phosphate-buffered saline (PBS) supplemented with 1.5% d-glucose, pH 8.0. Parasites were centrifuged (1800g, 10 min) and resuspended at 2 x 10⁹/ml in glucose-supplemented PBS. Parasite suspension (100 μl) was mixed with 100 μl of serially diluted CF and incubated at 30 °C and 5% CO₂ for 3 h. Trypanolysis was quantified by counting the remaining intact parasites under a light microscope.

2.5. Prophenoxidase-activating assay

The level of prophenoxidase activation was assessed as described previously [3,4]. Briefly, 10 μl of CF (with or without 1 mM serine protease inhibitor Pefabloc (4-(2-aminoethyl)benzene sulfonyl hydrochloride, Boehringer Mannheim), 65 μl of 0.1 M Tris, pH 8.0, containing 50 mM CaCl₂, and 10 μl of 15 mM L-DOPA (3-(3,4-dihydroxyphenyl)-L-α-lanine; Fluka) were incubated at room temperature for 6 h in the absence or presence of laminarin or N,N-diacetylcysteine (1 μg/ml). Proteolytic enzymes are necessary for activating the cascade; therefore the level of the prophenoxidase activation was estimated as the difference of L-DOPA oxidation in the absence and presence of the serine protease inhibitor. The oxidation of L-DOPA was measured at 492 nm and expressed as the difference between the values with or without Pefabloc.

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

Total RNA was isolated from 5 x 10⁶ coelomocytes using 1 ml of TRIZOL reagent (Gibco BRL) according to the manufacturer’s protocol. Three micrograms of DNase I-treated total RNA was reverse-transcribed using Oligo(dT)₁₂₋₁₈ primer and Superscript II RNase H⁻ Reverse Transcriptase (Gibco BRL). Primers for *E. fetida* CCF used in the polymerase chain reaction (PCR) were as follows: sense 5'-TTCAGACTGGATCGACGACCC-3' and antisense 5'-ATGCGTTTGTGACTCGCAG-3'. An approximately 1100 bp fragment was amplified using the following cycling parameters: 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 58 °C and 90 s at 72 °C and a final extension for 7 min at 72 °C. The PCR product was ligated in pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The remaining 3’ end of CCF-like cDNA was obtained using 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). Total RNA (3 μg) was reverse-transcribed using an adapter primer (5’-GCCCAGCGCTGAGACTGACTTGGG-3’) and a CCF-specific internal sense primer (5’-CTGACCACATGCTTCCGAAAACA-3’) were used in the subsequent PCR reaction. Similarly, the 5’ end of CCF-like cDNA was obtained using 5’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). Reverse transcription was carried out with 3 μg of total RNA using an antisense CCF internal primer (5’-TCAGTTGCCGTTGAGACTCGG-3’). Terminal deoxynucleotidyl transferase was used to add homopolymeric oligo-dC tails to the 3’ end of purified cDNA. Primers used in subsequent PCR were: sense abridged anchor primer (5’-GCCCAGCGCTGAGACTGACTTGGG-3’) and antisense CCF specific primer (5’-CTGACCACATGCTTCCGAAAACA-3’). Both 3’ and 5’ RACE products were cloned in pCR2.1-TOPO and sequenced.

2.7. Sequencing

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

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

3. Results and discussion

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

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

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

We therefore examined the ability of CFs of *A. caliginosa*, *A. ictericus*, *A. longa*, *A. rosea*, *D. veneta*, *E. fetida*, *L. rubellus* and *L. terrestris* to trigger the prophenoxinidase pathway upon binding laminarin and N,N′-diacetylchitobiose as these sugar compounds are recognized by spatially distinct CCF domains in *E. fetida* earthworms [4]. We found that the CFs of all tested earthworm species were able to induce the oxidation of L-DOPA, i.e. to initiate the prophenoxinidase cascade in the presence of laminarin, whereas the CF of *E. fetida* is the only one triggering the cascade in the presence of N,N′-diacetylchitobiose (Table 1). This indicates that the pattern recognition specificity of CCF in *E. fetida* earthworms is broader than in other earthworm species. We already reported [4] that recognition of lipopolysaccharide and β-1,3-glucans mediated by the central part of CCF encompassing the polysaccharide-binding and glucanase motif leads to the activation of the prophenoxinidase cascade both in *E. fetida* and *L. terrestris* earthworms. Yet, N,N′-diacetylchitobiose and peptidoglycan trigger prophenoxinidase cascade only in *E. fetida* earthworms. Sequence differences in the C-terminal carbohydrate-binding domain were suggested to account for the inability of *L. terrestris* CCF to bind N,N′-diacetylchitobiose and peptidoglycan [4]. Therefore, we sequenced the CCF-like gene in all other species.
Using specific primers designed from the nucleotide sequence of *E. fetida* CCF, approximately 1100 bp fragments were obtained in PCR from the cDNAs of all eight earthworm species (Fig. 1b). In order to assemble the full-length cDNA sequence of CCF-like genes, RACE amplifications of the 5' and 3' cDNA ends were performed. Resulting PCR products were cloned and sequenced. Deduced amino acid sequences of CCF-like molecules of all species are shown in Fig. 2a. CCF-like molecules display 80 90% sequence identity; they are nearly identical in the polysaccharide-binding motif (with an exception in *A. ictericus* where histidine residue 150 is substituted by serine, and in *A. rosea* where histidine 150 is replaced by arginine and methionine 165 by leucine) and glucanase motif (except for one threonine residue 186 which is in endoergic earthworms replaced by either serine in *A. caliginosa* and *A. ictericus* or by alanine in *A. rosea*). A high level of homology in this central part of CCF, which is considered as a lectin-like domain capable of binding β-1,3-glucans, is in coincidence with the ability of CCF and all CCF-like molecules to bind laminarin and initiate activation of the propheino-oxidase cascade. All molecules contain a high number of tryptophan residues (5-7%) located mainly in the C-terminal part of the molecules. However, this part of all CCF-like molecules is the most variable as compared to the C-terminal part of *E. fetida* CCF, which is in accordance with the fact that *E. fetida* CCF is the only one recognizing N,N-diacetylchitobiose. Nucleotide sequences of CCF-like molecules were submitted to the GeneBank database and can be found under these accession numbers: *A. caliginosa* DQ017143; *A. ictericus* DQ018721; *A. longa* DQ018722; *A. rosea* DQ018723; *D. veneta* DQ018724 and *L. rubellus* DQ018725. We found that the sequence of CCF homologue described previously for *L. terrestris* [4] is, due to a taxonomic error, a sequence of *A. longa*, a species macroscopically similar.

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

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

Both *E. fetida* and *D. veneta* are epigeic earthworms which prefer the loose upper topsoil layer where they feed on decaying organic matter. They are adapted to the high predation pressure and the widely variable moisture and temperature conditions at the soil surface. The composition of microflora varies in different parts of the soil and can play an important role in molecular evolution of CCF and CCF-like molecules. It was documented that the soil microbial diversity is dependent on a vast number of environmental factors such as water and substrate availability, temperature, pH, mineral components as well soil structure and pore size [22,23]. Thus each earthworm group living in a different part of the soil is subjected to different microbial conditions. The highest microbial activity is definitely found in the places where the organic matter is the most abundant. It is therefore obvious that *E. fetida* living in decaying organic matter, in compost and in mold appears to be best equipped to
Fig 2. (a) Alignment of amino acid sequences of *E. fetida* (GenBank accession no. AI038402), *A. caliginosa* (DQ018721), *A. icterica* (DQ018722), *A. rovesc* (DQ018723), *D. vederi* (DQ018724), *L. rubellus* (DQ018725) and *L. terrestris* (AF39805) CCF and CCF-like molecules. Only mutated amino acids as compared with *E. fetida* CCF are indicated. Polysaccharide-binding motif is underlined; glucamoct motif is double-underlined, signal peptide is dotted line underlined. Asterisks indicate missing amino acids. (b) Neighbor-joining phylogenetic tree relating CCF and CCF-like proteins in selected Lymnaeidae. The numbers near the nodes represents bootstrap values.
resist microbial activity, as reflected by the broader CCF pattern recognition specificity. This can explain why the CCF-like molecule of *D. veneta* that lives in a similar but usually less aggressive environment is so similar to *E. fetida* CCF.

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

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**References**


4.5 Phenoloxidase activity of *E. fetida* coelomic fluid

The prophenoloxidase (proPO) cascade represents one of the most important defense mechanisms in invertebrates. Non-self molecules are recognized by endogenous pattern recognition molecules, and then they cause activation of the proPO activating system. The active form of proPO, phenoloxidase, is produced by a serine proteinase, which cleaves inactive prophenoloxidase to its active state. Phenoloxidase is a key enzyme responsible for catalyzing melanization reactions whose final product melanin is involved in wound healing and immune responses in invertebrates. Prophenoloxidase was isolated and characterized in numerous crustaceans, insects and some mollusks.

We detected phenoloxidase activity in the coelomic fluid of *E. fetida* by using a most common substrate for phenoloxidase (PO) - L-DOPA (L-β-3,4-dihydroxyphenylalanine). When we added L-DOPA to the coelomic fluid, we detected L-DOPA oxidation suggesting PO activity. The level of spontaneous L-DOPA oxidation was marginal, occurring after 8 hours of incubation. However, in the presence of an activator such as LPS or β-1,3-glucan, the L-DOPA oxidation started after 2 hours and reached a maximum level between 6 and 10 hours of incubation. In arthropods, PO activity is detectable within minutes after the incubation of body fluid with L-DOPA. This suggests very low level of PO in earthworms.

Different substrates were used (L-DOPA, dopamine, N-acyldopamine, 4-methylcatechol, tyrosine) to further characterize the proPO cascade in *E. fetida* coelomic fluid. Moreover, different non-specific activators of proPO such as detergents (SDS, CPC, CHAPS) were employed. The highest activation of proPO was obtained by combining CPC as an activator and L-DOPA as a substrate. L-DOPA was producing the highest values of PO activity in combination with the three activators, whereas tyrosine used as a substrate and the coelomic fluid without any substrate/activator of PO/proPO did not reveal significant PO activity.

To identify PO enzyme, coelomic fluid content was separated in native SDS-free PAGE and incubated with L-DOPA as a substrate and CPC as an activator. In these conditions only one band showing oxidase activity appeared. Such oxidizing activity was completely abolished when coelomic fluid was preincubated with irreversible proteinase inhibitor Pefabloc or boiled before separation, which suggests an enzymatic nature of the earthworm material exhibiting PO activity.
Bands showing detectable oxidation were cut from the native SDS-free PAGE, and
electroeluted for further analyses. (i) First, when separated in SDS-PAGE and stained with
Coomassie blue, the electroeluted material revealed a high molecular-weight band, a strong band
of approximately 90 kDa, and a few weak bands of molecular weight lower than 40 kDa. The high
molecular-weight band is assumedly formed by precipitated material that did not enter the gel. (ii)
Second, the electroeluted material was concentrated and separated by gel filtration. The \( A_{475} \)
detecting the oxidation of L-DOPA to dopachrome and \( A_{280} \) as an estimation of protein content
were recorded by UV spectrophotometer during elution. (iii) Third, the 90 kDa protein band was
further subjected to amino acid sequence analysis. Edman degradation was performed three
times and revealed only an N-terminal peptide sequence of 8 amino acids, due to repeated
blocking of N-terminus. The sequencing of internal peptides yielded four peptide sequences. N-
terminal amino acid sequence of one peptide showed homology with the endogenous inhibitor of
PO from the housefly *Musca domestica*.

Two other peptide sequences (15 and 17 amino acids long), shared partial homologies
with the sequences of phenoloxidase and/or hemocyanin of different invertebrate species. The two
remaining earthworm peptide sequences (17 and 8 amino acids long, respectively) did not display
homology with known proteins.

The obtained data indicate that peptides having a partial homology with PO and/or
hemocyanin originated from the active fraction from *E. fetida* coelomic fluid proteins exhibiting
proPO oxidizing properties. Therefore, it can be suggested that phenoloxidase and related
inhibitors exist in *E. fetida* earthworms, but the level of PO activity is lower than in other
invertebrates. Moreover, PO activation is slower as compared to other invertebrate species and
probably does not represent the main defense system in earthworms.

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Evidence for prophenoloxidase cascade in the coelomic fluid of *Eisenia fetida* earthworms

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Abbreviations:

CF, coelomic fluid; LBSS, *Lumbricus* balanced salt solution; PBS, phosphate buffered solution; proPO, prophenoloxidase; PO, phenoloxidase; L-DOPA, L-β-3,4-dihydroxyphenylalanine; ppA, prophenoloxidase activating enzyme; CPC, cetylpyridium chloride; Hc, hemocyanin;

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Abstract:

The prophenoloxidase cascade represents one of the most important defense mechanisms in invertebrates. Following the recognition of microbial saccharides by pattern recognition molecules, proteinases cleave inactive prophenoloxidase to its active form, phenoloxidase. Phenoloxidase is a key enzyme responsible for catalysis of melanization reaction. Final product melanin is involved in wound healing and immune responses in invertebrates. As far, prophenoloxidase cascade was widely described in arthropods; data in other invertebrate groups are less frequent. In this article we show the presence of proteins having phenoloxidase activity in the coelomic fluid of *Eisenia fetida* earthworms.

Keywords:

Innate immunity; Phenoloxidase; *Eisenia*; Earthworm; L-DOPA
Introduction
The phenoloxidase-activating system is a sensitive non-self-recognizing cascade triggered by components of microbial cell wall such as lipopolysaccharides, peptidoglycans, and β-1,3-glucan (Cerenius and Söderhäll 2004, Söderhäll and Cerenius 1998). One of component of the system is phenoloxidase (PO; EC 1.14.18.1, EC 1.10.3.1), an enzyme that is usually present in an inactive form, named prophenoloxidase (proPO), in cells or body fluid of different invertebrate species. Current evidence suggests that proPO is stored in the granules of some blood cells, from which it is released to body fluid and eventually gets activated. Conversion of proPO to its active state is achieved by proteolytic cleavage that depends on a cascade of serine proteases, so-called the prophenoloxidase activating enzymes (ppA), and other factors. The resulting phenoloxidase (PO) catalyses both the o-hydroxylation of monophenols and the oxidation of diphenols to quinones. Then the quinones non-enzymatically polymerize to melanin (Ashida and Yamazaki 1990, Söderhäll et al. 1994). Melanin, as final product of proPO cascade, has fungistatic, bacteriostatic and antiviral properties and together with its intermediates is involved in the innate immune response of certain invertebrates, especially arthropods. Melanin also serves as a structural component in the wound healing and is important for encapsulation of foreign materials.

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

Prophenoloxidases and phenoloxidases were characterized in numerous crustaceans, insects and some mollusks (for review see (Cerenius and Söderhäll 2004)). Their molecular weight differs among species, and varies usually between 70 and 90 kDa. The primary structure of known proPOs reveals that they contain two functional copper-binding sites. Actually, sequence of proPO in different species within and adjacent to the copper-binding sites shows 60-70 % of similarity. Another conserved peptide motif, GCGEQNM (Armstrong and Quigley 1999, Dodds and Law 1998), which is present in α-macroglobulins both in vertebrates and invertebrates, can be found in the sequence of proPOs as well. This motif is present also in the vertebrate thiolester-containing complement proteins (C3, C4) and in the complement-related proteins of some invertebrates (Dishaw et al. 2005, Levashina et al. 2003, Marino et al. 2002, Zhu et al. 2005).

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

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

Material and methods
Earthworms and harvesting of the coelomic fluid (CF) and coelomocytes
Adult E. fetida earthworms (Oligochaeta; Annelida) were kept in compost and 3 days prior to experiments were transferred on filter paper soaked with isotonic Lumbricus balanced salt solution.
Coelomic fluid containing coelomocytes was obtained by puncturing post-citellum segments of the coelomic cavity of the earthworms with a Pasteur micropipette and kept on ice. Samples were centrifuged (500 g, 10 min, 4 °C). The supernatant containing the cell-free coelomic fluid was centrifuged again (7000 g, 10 min, 4 °C) and the supernatant was stored at -20 °C.

**Activation of proPO cascade**
The level of proPO activation was assessed as described previously (Beschin et al. 1998, Bilej et al. 2001). Briefly, 10 μl of the coelomic fluid (with or without 1 mM serine proteinase inhibitor Pefabloc (Boehringer Mannheim), 90 μl of 100 mM Tris, pH 8, containing 50 mM Ca²⁺ and 10 μl L-DOPA (L-β-3,4-dihydroxyphenylalanine; Fluka); final concentration 1.5 mM) was incubated at room temperature up to 12 h in the absence or presence of LPS (E. coli 055:B5 S strain, Sigma) or β-1,3-glucan (laminarin, Sigma), at final concentration of 1 μg/ml. The oxidation of L-DOPA to dopachrome was measured every two hours at 475 nm and evaluated as the difference between the A₂₅₇₅ values with or without Pefabloc.

To determine the substrate specificity of PO and to test non-specific activation of proPO, different activators of PO and substrates were tested. Coelomic fluid (10 μl) was diluted in 50 mM sodium phosphate buffer (pH 6.5) in a total volume of 100 μl containing one of the substrates for PO (final concentration 1 mM): L-DOPA (Fluka), dopamine (Sigma), acyldopamine (Sigma), 4-methylcatechol (Sigma), tyrosine (Fluka). Then, one of the non-specific activators of proPO (final concentration 0.02 % SDS; cetylpyridium chloride (CPC); CHAPS, all from Sigma) was added to the reaction mixture. The reaction was allowed to proceed at room temperature for 6 hours, which is the time period when a maximum L-DOPA oxidation occurs using LPS and β-1,3-glucan to activate the proPO cascade, and then the A₂₅₇₅ was measured. Absorbance was calculated as the difference between the values of samples with and without the coelomic fluid. Control samples were prepared without activator of the proPO or without the substrate for PO.

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

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

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

**Protein purification by chromatography**
The concentrated electroeluted sample (100 μl) was applied to a pre-equilibrated (150 mM Tris-HCl, pH 7.5) Superdex S75 column (Pharmacia), run at 100 μl/min and eluted in 25 mM Tris, 20 mM glycine, 0.01 % SDS, 0.1 M NaCl, 10 mM β-mercaptoethanol, pH 7.5. Besides the A₂₅₀ (corresponding to protein content) the A₂₅₇₅ (reflecting the oxidation of L-DOPA to dopachrome) of eluted fractions was directly measured during elution. Collected fractions exhibiting PO activity were pooled, concentrated on Vivaspin (Vivascience) concentrator with a cut-off 15 kDa and subjected to SDS-PAGE and Edman degradation.
Edman degradation

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

Results and Discussion

Activation of proPO cascade in the coelomic fluid of E. fetida earthworms

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

To further characterize the proPO cascade in E. fetida coelomic fluid, different substrates (L-DOPA, dopamine, N-acyldopamine, 4-methylcatechol, tyrosine) were used. Moreover, different non-specific activators of proPO like detergents (SDS, CPC, CHAPS) were envisaged (Ashida and Yamazaki 1990, Sugumaran and Kanost 1993). As shown in Table 1, the highest activation of the proPO was obtained by combining CPC as an activator and L-DOPA as a substrate. L-DOPA was giving the highest values of PO activity in combination with the three activators. Tyrosine used as a substrate and the coelomic fluid without any substrate/activator of PO/proPO did not reveal significant PO activity. It should be mentioned, that the name phenoloxidase encompasses two similar enzymes, which only differ in their enzymatic properties: tyrosinase (EC 1.14.18.1) and catecholoxidase (EC 1.10.3.1). Tyrosinase catalyzes the hydroxylation of monophenols (i.e. tyrosine, considered as the natural substrate of the enzyme) and the oxidation of diphenols to quinones, whereas catecholoxidase catalyzes only the oxidation of diphenols. The latter enzyme is almost indistinguishable from the different kinds of tyrosinases by sequence and properties other than the enzymatic activity (Decker and Jaenicke 2004, Sanchez-Ferrer et al. 1995). This may be the reason why the term phenoloxidase is often used for tyrosinases and catecholoxidases of invertebrates in the literature without discrimination between them. A number of phenoloxidases are reported for arthropods, but only a few were demonstrated as having tyrosinase activity i.e. using the monophenol tyrosine as substrate (Aspan et al. 1995, Chase et al. 2000, Fujimoto et al. 1993, Jaenicke and Decker 2003). Since we did not detect PO activity in the coelomic fluid after the incubation with any of activators and tyrosine as a substrate together, we suggest the presence of catecholoxidase rather than tyrosinase in E. fetida earthworms.

Identification of proteins having PO activity

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

Bands showing detectable oxidation were cut from the native SDS-free PAGE, and electroeluted for further analyses. (i) First, when separated in SDS-PAGE and stained with Coomassie blue, the electroeluted material revealed a high molecular-weight band, a strong band of approximately 90 kDa, and few weak bands of molecular weight lower than 40 kDa (Fig. 2c, lane a). The high molecular-weight band is assumedly formed by precipitated material that did not enter the gel.

(ii) Second, the electroeluted material was concentrated and separated by gel filtration. The A_{275} detecting the oxidation of L-DOPA to dopachrome (Harisha 2005) and A_{280} as an estimation of protein content were recorded (Fig. 2b) by UV spectrophotometer during elution. Between the two main protein peaks
(A\textsubscript{250}) showing PO activity (A\textsubscript{475}) only one (fraction 3-4) contained enough material to be further analyzed in SDS-PAGE, revealing in Coomassie blue a protein band of approximately 90 kDa (Fig. 2c, lane b). (iii) Third, the 90 kDa protein band was further subjected to amino acid sequence analysis. Edman degradation was performed three times and revealed only an N-terminal peptide sequence of 8 amino acids, due to repeated blocking of N-terminus. We proceeded to sequencing of internal peptides, hereby obtaining four peptide sequences. Sequences of the identified earthworm peptides as well as those of homologous peptides are shown in Table 2. N-terminal amino acid sequence (peptide no. 1) showed homology with the endogenous inhibitor of PO from housefly Musca domestica. In the latter animal, this competitive inhibitor of the PO was found to be a DOPA-containing peptide with a molecular weight of only 4.2 kDa (Daquinag et al. 1995, Daquinag et al. 1999). It is known, that proPO system involves also inhibitors of proPO activation and PO activity. They can prevent undesired activation of proPO, or prevent over-activation of ppA (Aspan et al. 1990, De Gregorio et al. 2002, Liang et al. 1997). The endogenous inhibitor of PO from Musca domestica was described as a DOPA-containing peptide, wherein DOPA is a modified tyrosine residue. The presence of such modified tyrosine residue in phenoloxidase inhibitor that could form a complex with phenoloxidase in earthworms, might explain the correlation of activity and absorbance of 475 nm of fractions obtained during gel filtration of our proteins (Fig. 2b). From observed data we can suggest the existence of inhibitor of PO in earthworms, which might probably be forming a complex with PO.

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

Efforts to identify whole proPO/PO sequence including (i) PCR with degenerated primers designed based on conserved regions in published phenoloxidases and with degenerated primers designed in accordance with identified peptide sequences, and (ii) the screening of E. fetida cDNA library with a probe containing a partial sequence of the gene coding for phenoloxidase in crayfish Pacifastacus leniusculus (kindly provided by Prof. Söderhäll, Uppsala), were so far unsuccessful. One of the plausible reasons for our failure may be the very low level of mRNA for phenoloxidase in E. fetida earthworms.

Together these data indicate that peptides having a partial homology with PO and/or hemocyanin originated from the active fraction from E. fetida coelomic fluid proteins exhibiting proPO oxidizing properties. Therefore, it can be suggested that phenoloxidase and related inhibitor exist in E. fetida earthworms, but the level of PO activity is lower than in other invertebrates. Moreover, it is clear that proPO activation is slower as compared to other invertebrate species. We can presume that proPO cascade does not represent the main defense system in earthworms.

**Acknowledgements**

This work was supported by the Czech Science Foundation (310/04/0806; B500200613), Institutional Research Concept (AVOZ50200510) and a bilateral international scientific and technological cooperation grant of the Ministry of the Flemish Community (BOF-BWS 03/06) and was performed within the frames of an Interuniversity Attraction Pole Program. All experiments comply with the current laws of the Czech Republic and Belgium.
Fig. 1. Activation of proPO cascade in the coelomic fluid of *E. fetida* earthworms by β-1,3-glucan (laminarin) and LPS. Coelomic fluid (CF) levels of L-DOPA oxidation are expressed as the mean of A₄₇₅ values difference of the sample with and without proteinase inhibitor ± SD calculated from 3 independent experiments (* significant at p<0.05).

Fig. 2. A) Native PAGE of *E. fetida*. coelomic fluid. Coelomic fluid run on acrylamid gel was incubated in a mixture of sodium phosphate buffer, L-DOPA and CPC for 12 hours at room temperature to detect L-DOPA oxidation. Lane 1: 20 μl of the coelomic fluid (approx. 200 μg of proteins) boiled before separation. Lane 2: 20 μl of the coelomic fluid preincubated with proteinase inhibitor Pefabloc. Lane 3: 2 μl of the coelomic fluid (approx. 20 μg of proteins). Lane 4: 20 μl of the coelomic fluid.

B) Purification of proteins showing oxidase activity on a Superdex S75 column. Material present in bands detected after native PAGE and incubation with L-DOPA and CPC was electroeluted and separated by gel filtration. The absorbance at 280 nm for estimation of protein content and at 475 nm corresponding to the oxidation of L-DOPA to dopachrome was monitored. The fractions with highest PO activity (fraction 3-4) eluted after gel chromatography were collected.

C) Material exhibiting PO activity obtained in native SDS-free PAGE (lane a) and the fractions with highest PO activity (fraction 3-4) eluted after gel chromatography (lane b) were electroeluted and analyzed on SDS PAGE. Proteins were stained with Coomassie blue.
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<th>activators</th>
<th>substrates</th>
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Table 1. Substrate specificity of *Eisenia fetida* proPO. Detergents that can non-specifically activate proPO and different substrates for PO were tested for their ability to trigger the proPO cascade in the coelomic fluid. Activation of proPO after 6 hours of incubation is expressed as the mean of ΔA_{275} value difference of the sample with and without coelomic fluid ± SD calculated from three independent experiments. In control samples, coelomic fluid was incubated without activator of proPO or without the substrate for PO. (* significant at p<0.05).

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<td>proPO sub. 1 of <em>Anopheles gambiae</em></td>
<td>LGTVIRAD---QCPPVPADT</td>
<td>CAC69246</td>
</tr>
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<td></td>
<td>proPO of <em>Pacifastacus leniusculus</em></td>
<td>LGDVVEASLTPNAQLY-GSLHN</td>
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<tr>
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<td>LGDAFEAD---AQLSPNLYF</td>
<td>CAA58471</td>
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<tr>
<td>Peptide no. 4</td>
<td></td>
<td>PPPAEAGGEGHYYLQV</td>
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<tr>
<td>Peptide no. 5</td>
<td></td>
<td>LRFPGFAST</td>
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</table>

Table 2. Amino acid sequence of peptides derived from a 90 kDa *E. fetida* coelomic fluid protein with oxidase activity obtained by N-terminal and internal sequencing. Peptides exhibiting sequence homology found by a search in NCBI Blast database are listed below the obtained earthworm peptides (no. 1-5). Amino acids identical within the different sequences are in bold.
References:


5 General discussion

For better understanding of the function and principles of human defense system, various model organisms are used for biomedical research. Our laboratory uses earthworms as representatives of protostomian invertebrates. Earthworms belong to a large phylum, Annelida, to the class Oligochaeta, and they are segmented animals with a true coelom of mesenchymal origin.

The main defense of earthworms is hiding in their burrows in the soil. On detecting a predator they quickly crawl down into the ground. But their defense against enemies on the cellular level is more complicated. It is now generally accepted that the adaptive immune system arose in the jawed vertebrates. It is most clearly described in the mammalian system and is characterized by the ability of the immune system lymphoid cells to recognize and form a "memory" of foreign invaders. This system is characterized by the presence of T and B lymphocytes, their antigen-specific receptors (T and B cell receptors), and MHC proteins for the presentation of antigen to T cells. T and B cell subpopulations are present in fish, amphibians, reptiles, birds and mammals. Vertebrates and invertebrates also possess non-specific cellular and humoral defenses, components of the innate immune response. These non-specific defenses are based upon phagocytic cells, natural killer cells and an extensive array of soluble molecules, and it is these that present the main mechanisms of the defense of earthworms.

The main scope of this work was to study the proteins involved in various forms of recognition of antigens in earthworms, such as protein-protein recognition, protein-saccharide recognition and protein-lipid recognition.

Earthworms as well as other invertebrates do not possess adaptive immunity. Nevertheless, they respond to the administration of the antigen by producing adaptively synthesized substance, which binds the antigens - antigen-binding protein - ABP (Laulan et al. 1985; Tuckova et al. 1988, 1991b; Bilej et al. 1995b). The administered protein is rapidly degraded by proteinases both in the coelomic fluid and in free coelomocytes (Bilej et al. 1993; Rejnek et al. 1993). This proteolytic processing is limited and results in peptide fragments of molecular weight of 700-1000 Da (Hanusova et al. 1999). The level of specificity of protein recognition by ABP was discussed in our previous manuscript (Bilej et al. 1995b), but the high degree of specificity of recognition determined in previous experiments performed by Laulan’s
group was not reached in our experiments. These very first experiments included only a limited selection of antigens and we therefore followed the production of ABP as a response of the earthworms to stimulation with different antigens. Monoclonal antibodies that react with ABP but do not block the binding site, were generated to study the binding properties of ABP (Tuckova et al. 1991a). New experiments confirmed that the monoclonal antibody originally generated against ABP isolated from *L. terrestris* earthworms after ARS-HSA stimulation recognizes probably a conserved epitope on ABPs in both *L. terrestris* and *E. fetida* earthworms. Additionaly, this recognition is independent of the protein used for the stimulation, because the stimulating proteins were selected to vary in size from low molecular weight protein of 5 kDa (insulin) to high molecular weight protein of 900 kDa (ferritin), and the size of these proteins used for stimulation had no significant effect on the ABP response.

In agreement with previous results, the ABP response reached in all cases the maximum level on day 8 after the stimulation (Tuckova et al. 1991b; Bilej et al. 1995a). There were no differences in the kinetics of ABP response to different proteins and when both earthworm species were compared. We found that ABP molecules formed in response to the stimulation with different proteins bind preferably the same protein that was used for stimulation, but react also to a lesser extent with related proteins, suggesting only a limited level of specificity of ABPs.

To determine the molecular weight of ABPs, we isolated them by affinity chromatography from the coelomic fluid after the stimulation with different proteins. Molecular weight was the same in all tested coelomic fluids and corresponded to 56 kDa in *L. terrestris* and 60 kDa in *E. fetida*. ABP of *E. fetida* is formed by two polypeptides of 30 kDa, which could present a homodimer; however, this was not proved by sequencing. ABP of *L. terrestris* is formed by two subunits of 31 and 33 kDa, the N-terminal amino acid sequences of which are identical, and it can be suggested that both polypeptides are related and might differ, for example, in the level of posttranslational modifications. Nevertheless, there is no similarity of ABPs with other known proteins listed in protein databases. Some features of immune memory in earthworms were observed in transplantation experiments showing an increase in the number of coelomocytes in association with the graft and their infiltration into the graft section. The infiltration of coelomocytes into the graft section occurs regardless of whether it is xenograft
or allograft, but it is markedly lower in the case of autograft (Cooper 1970; Hostetter and Cooper 1973). If the second-set grafts were transplanted, rejection occurred in an accelerated mean time and also the number of coelomocytes was 20 - 30% higher in comparison with first grafts (Hostetter and Cooper 1973). Further, experiments demonstrating the adoptive transfer of memory (Bailey et al. 1971; Valembois 1971) support a role of coelomocytes in graft rejection. This memory is apparently related only to surviving coelomocytes, but is not based on the existence of specific clones of cells. This is supported also by the fact that the proliferative activity of free coelomocytes is not potentiated by first contact with antigens, but the coelomocytes increase their DNA synthesis as the result of a second contact with the stimulating protein (Bilej et al. 1992). It seems probable that after antigenic stimulation the precursor cells in the coelomic lining proliferate and differentiate, and only after the second contact with antigen can this subpopulation undergo further mitotic cycles.

Probably the most important way of recognition is based on pattern recognition proteins (PRP), which bind saccharide motifs on the cell wall components of microorganisms. One of PRP, coelomic cytolytic factor (CCF), was identified and described in Eisenia fetida earthworms (Bilej et al. 1995a; Beschin et al. 1998). Until now, the role of CCF in the earthworm defense was demonstrated only indirectly and we therefore sought to determine the effect of experimental challenge with live bacteria and with glucan on the concentration and expression of CCF in comparison with other earthworm defense molecules. It is known that the coelomic cavity of earthworms is not aseptic and contains bacteria from the outer environment, and that the coelomic fluid contains 10 times more potentially phagocytic cells than these bacteria. In our experiments we injected approximately a 10-times higher amount of bacteria than is the number of naturally occurring phagocytic cells, but we took care not to affect the viability of animals and to use the bacteria in a quantity sufficient to activate the defense mechanisms.

We stimulated earthworms with Gram-negative bacteria E. coli, Gram-positive bacteria B. subtilis and with glucan, measured the changes in concentration of CCF and, for comparison, changes in hemolytic and lysozyme-like activity of the coelomic fluid were followed. The level of expression of CCF and fetidin, one of the hemolytic proteins of earthworms, was estimated. Since the earthworms respond to antigenic stimulation or body injury by a nonspecific increase in coelomic fluid protein concentration (Tuckova et al. 1988), we followed also the changes in
earthworms injected with LBSS (*Lumbricus* balanced salt solution) alone. All detected changes passed till the 6th day after the stimulation and then the levels of all assessed parameters returned back to the level of non-injected animals.

It was reported that the coelomic fluid of *E. fetida* exhibits a low lysozyme-like activity and the enzyme is released from coelomocytes as part of an internal defense mechanisms against particular bacterial species (Cotuc and Dales 1984). We investigated the modulation of lysozyme-like activity in animals challenged with different antigens; an increased lysozyme-like activity was found 2 days after the stimulation in all injected individuals. Because lysozyme degrades peptidoglycan, the activity should be directed mainly against Gram-positive bacteria. Surprisingly, any principal difference in the maximum lysozyme-like activity in worms infected with Gram-positive bacteria as compared to animals stimulated with Gram-negative bacteria and glucan was not observed. This raises the question of specificity of the increased lysozyme-like activity detected in animals challenged by other antigens. It might reflect the fact that also Gram-negative bacteria contain peptidoglycan in their cell walls, under the thick layer of lipopolysaccharides. This outer membrane comprising lipids, proteins and in particular lipopolysaccharides, could be primarily predigested, the peptidoglycan layer becoming accessible for lysozyme-like proteins.

*E. fetida* coelomic fluid displays also hemolytic activity due to various hemolytic proteins, such as fetidins (Milochau *et al.* 1997). Although these proteins were considered to participate in the antimicrobial activity of the coelomic fluid (Lassegues *et al.* 1997), we did not observe any significant modulation of fetidin mRNA levels in coelomocytes of stimulated earthworms. Moreover, hemolytic factor protein levels did not change upon challenge while the concentration of other proteins increased. Different behavior was observed in the case of CCF. Earthworms contained a rather low level of CCF (15.0 ± 2.5 µg/ml, i.e. 0.1 % of the coelomic fluid protein content). CCF protein level increased between 6 hours and 3 days after the stimulation with different antigens. Whereas CCF acts as agglutinin strongly binding pathogen-associated molecular patterns (Beschin *et al.* 1998; Bilej *et al.* 2001), the observed increase in CCF concentrations may represent only the free molecules and thus may be underestimated. This increase further correlated with an increase in CCF expression as a result of microbial challenge. The mRNA level in coelomocytes from *E. coli* and glucan-injected animals increased.
as early as 2 hours post-challenge, reaching the maximum after 17 hours, whereas in *B. subtilis*-infected animals, maximum mRNA level was lower or reached later, suggesting the requirement of lysozyme pre-digestion for recognition of peptidoglycan compounds by CCF. Interestingly, mRNA synthesis reached a maximum when humoral CCF level started to decrease; this might mean that CCF was at first released from intracellular deposits and de novo synthesis was required to recharge the stock in the granules. From our data it is clear that lysozyme-like activity and synthesis of CCF are upregulated upon *in vivo* microbial stimulation in *E. fetida* while fedin expression is not modulated. CCF is released within the first 24 hours following microbial challenge; this reinforces the idea that this pattern recognition molecule is an important initiator of the innate immune reaction in earthworms.

Many proteins that cause lysis of red blood cells were described in *E. fetida* earthworms. However, the existing nomenclature and identity of these proteins are confusing. All hemolytic molecules reported so far share biochemical analogies, have similar molecular weight around 40 kDa, similar pI and are capable of binding sphingomyelin and form pores in lipid membranes. A heterogeneous group of these proteins comprises *Eisenia fetida andrei factor* (EFAF) (Roch et al. 1981, 1984), fedin (Lassegues et al. 1997; Milochau et al. 1997), lysenin (Sekizawa et al. 1996, 1997), eiseniapore (Lange et al. 1997, 1999), and hemolysins isolated either from coelomic fluid or from cell lysate (Eue et al. 1998; Koenig et al. 2003). The confusion in the nomenclature and identity of *E. fetida* hemolytic proteins was partially clarified by Koenig (Koenig et al. 2003), who characterized hemolysins from coelomocyte lysate (CL-39 and CL-41) and coelomic fluid (H-1,3). Using mass spectrometry they demonstrated the identity of CL-39 and H-1,3 proteins with fedin and CL-41 with lysenin. Another hemolytic protein named eiseniapore shows some concordant characteristics with lysenin (Lange et al. 1997, 1999).

From all these data, when viewed together, it is likely that *E. fetida* earthworms have two main hemolytic proteins - fedin and lysenin.

All reported *E. fetida* hemolytic proteins interact with sphingomyelin, which is a major component of sphingolipid, cholesterol-rich plasma membrane microdomains called “lipid rafts” (Brown 1998; Rietveld and Simons 1998; Ostermeyer et al. 1999). Lipid rafts are believed to play an important role in a variety of cellular functions, such as signalling, adhesion, motility and membrane trafficking (Brown and London 1998; Simons and Toomre 2000). In mammals,
trafficking dysfunctions lead to disease development, such as Niemann-Pick disease. This disease is a lipid storage disorder that results from the deficiency of a lysosomal enzyme, acid sphingomyelinase, and hence leads to intracellular accumulation of sphingomyelin. Since lysenin specifically binds to sphingomyelin, it makes this protein an additional useful tool for examining the organization of lipid rafts, molecular motion and function of sphingomyelin in various human genetic disease conditions or during lytic processes in earthworms.

During in vivo experiments we observed partial discrepancies in the published sequence of fetidin and therefore tried to elucidate the relationship between these two hemolytic molecules - fetidin and lysenin, to find out whether these proteins are isoforms or encoded by two independent genes, and to determine the level of their expression in coelomocytes in individual earthworms. We examined 25 experimental animals; all of them were found to express mRNA for both fetidin and lysenin. Moreover, the presence of DNA encoding both proteins at the genomic level was proved in six selected animals, suggesting that fetidin and lysenin do not result from posttranscriptional splicing or other modification of the transcript. There could still exist the possibility that both proteins are products of different alleles of one gene, but it is not probable, since all used experimental animals would have to be heterozygotes. Therefore, our data confirm that fetidin and lysenin are two different proteins encoded by two distinct genes, which have very high sequence and functional homology.

Further, the hemolytic activity in the coelomic fluid of these 25 E. fetida individuals we tested and 3 different hemolytic patterns and one group of seven animals with a hemolytic pattern below the detection limit were found. All samples of coelomic fluids of these seven specimens had also very low hemolytic activity, which was determined in suspension with sheep blood cells. In contrast to our findings, Roch (Roch 1979) found six different hemolytic patterns, which result from four isoforms of the 40-kDa hemolysin described by his group. The difference in the number of hemolytic patterns can arise from the use of a different technique used by our and their group. Whereas we separated coelomic fluid proteins in native PAGE before evaluating the hemolytic activity, Roch (Roch 1979) separated coelomic fluid proteins according to their isoelectric points.

Fetidin and lysenin differ in their location within the earthworm, since fetidin is considered as an intracellular and secreted protein while lysenin is proposed to be solely
intracellular (Lassegues et al. 1997; Milochnau et al. 1997; Sekizawa et al. 1997). Moreover, we found that the expression of fetidin and lysenin in coelomocytes might differ from individual to individual. By determining the relative expression of the two genes in real-time PCR, we showed that fetidin was expressed to similar levels in all tested animals, while the expression of lysenin varied from animal to animal.

Interestingly, the expression of fetidin or lysenin mRNA in any other earthworm species tested so far except the very same *Eisenia fetida* was not detected (*Lumbricus terrestris, Aporrectodea caliginosa, Aporrectodea icterica, Aporrectodea longa, Aporrectodea rosea, Dendrobaena veneta, Lumbricus rubellus*); in addition, only the coelomic fluid from *E. fetida* exerts detectable hemolytic activity. These findings suggest the unique appearance of fetidin and/or lysenin in *E. fetida* earthworms. On the other hand, it is conceivable that the gene sequences coding for these proteins may differ so that any primer designed for fetidin and lysenin mRNA of *E. fetida* would be ineffective for other species. The fact that fetidin and lysenin occur only in *E. fetida* species could reflect their living conditions. This species of earthworms lives in the compost and hence could have developed distinct defense mechanisms to better tolerate a wide range of environmental conditions and fluctuations. This presumption confirms also the comparative study of CCF and CCF-like molecules in other Lumbricid species.

To recognize whether also other related earthworms have corresponding molecules and to compare their characteristics we collected, classified, sequenced and characterized CCF-like molecules of additional seven Lumbricid species different from *Eisenia fetida: Aporrectodea caliginosa, Aporrectodea icterica, Aporrectodea longa, Aporrectodea rosea, Dendrobaena veneta, Lumbricus rubellus* and *Lumbricus terrestris*. Their coelomic fluids and RNA isolated from coelomocytes were then used in our experiments.

CCF exhibits number of specific properties, which arise from its structure. We knew that *E. fetida* CCF displays functional analogy with mammalian tumor necrosis factor and is also able to lyse fibrosarcoma TNF-sensitive cell line L929 and bloodstream forms of trypanosomes (Bilej et al. 1995a; Beschin et al. 1999). All other species were tested for the ability to lyse this tumor cell line and *Trypanosoma brucei brucei* parasites. Interestingly, we did not detect either cytolytic or trypanolytic activity in the coelomic fluid of any other earthworms. On the other hand, a monoclonal antibody, that was elicited against *E. fetida* CCF, cross-reacted with a 42-kDa
protein in coelomic fluids of other earthworm species using western blot analysis. The data suggested that other tested earthworm species contain CCF-like proteins, but they might differ from *E. fetida* CCF.

It was reported that the coelomic fluid of *E. fetida* triggers the prophenoloxidase cascade, which can be activated by various microbial compounds such as lipopolysaccharide, saccharides bearing β-1,3-glucosidic link (laminarin, curdlan, zymosan), N,N′-diacetylchitobiose or peptidoglycan constituents muramyl dipeptide and muramic acid (Beschin *et al.* 1998). In our experiments we used laminarin and N,N′-diacetylchitobiose, since these sugar compounds are recognized by spatially distinct *E. fetida* CCF domains. The central part of CCF constitutes the saccharide-binding domain and interacts with LPS and β-1,3-glucans, while another, C-terminal, domain mediates the interaction with N,N′-diacetylchitobiose, muramyl dipeptide and muramic acid. All tested earthworm species were able to initiate the prophenoloxidase cascade in the presence of laminarin, but not in the presence of N,N′-diacetylchitobiose. Hence, these findings indicate that the pattern recognition specificity of CCF in *E. fetida* earthworms is broader than in other earthworm species.

To find closer distinctions we sequenced the CCF-like gene in all other species. Interestingly, the sequencing of our samples revealed that the sequence of CCF homologue described previously for *L. terrestris* (Bilej *et al.* 2001) is, due to a taxonomic error, a sequence of *A. longa*, a species macroscopically similar. Comparison of the obtained sequences showed that CCF-like molecules display 80-90 % sequence identity. All molecules are nearly identical in the central part, which contains the polysaccharide-binding and glucanase motif and is considered as a lectin-like domain. This observed high level of homology is in accordance with the ability of CCF and all CCF-like molecules to bind laminarin and trigger the activation of prophenoloxidase cascade. In contrast, C-terminal part of the molecules is variable as compared to *E. fetida* CCF, which explains the fact that *E. fetida* CCF is the only one recognizing N,N′-diacetylchitobiose. All putative CCF-like sequences were subjected to phylogenetic analysis, which distributed different species into several groups. Earthworms of genus *Lumbricus* have 94 % identity and fall together into a group having the same progenitor. CCF-like molecules of the genus *Aporrectodea* are more heterogeneous, display 80 % identity and form a paraphyletic group. It is not surprising that *E. fetida* CCF is closely related to the
CCF-like molecule of *D. veneta*, because of their similar appearance. Moreover, both *E. fetida* and *D. veneta* are epigeic earthworms that live in the superficial soil layers and feed on undecomposed plant litter. These worms are usually small and produce new generations rapidly. Epigeic earthworms do not burrow into the soil and are therefore more easily contained within vermicomposting systems than other types of earthworms. Due to their living environment they are adapted to a high predation pressure and to variable moisture and temperature conditions. Because different parts of the soil contain miscellaneous composition of microflora, each earthworm group living in a different part of the soil is subjected to distinct microbial conditions. The compost is the place with the highest microbial activity. It is therefore obvious that *E. fetida* living in compost is the best equipped to resist microbial activity, as reflected by the broader CCF pattern recognition specificity. This can explain why the CCF-like molecule of *D. veneta* that lives in a similar but usually less aggressive environment is so similar to *E. fetida* CCF.

As mentioned above, CCF is capable of initiating the prophenoloxidase cascade. The prophenoloxidase-activating system is an important part of the invertebrate defense system, but until now, prophenoloxidases were isolated and characterized only from arthropods, the reports in other animal taxa being rather scarce. Therefore, we wanted to prove the presence of prophenoloxidase cascade as a defense mechanism in earthworms.

In arthropods, PO activity is detected within minutes after the incubation of body fluid with L-DOPA and microbial cell wall constituents (Cerenius and Söderhäll 2004). Our experiments with earthworm coelomic fluid revealed that it takes at least 2 hours to detect L-DOPA oxidation after incubation with L-DOPA as a substrate for PO and some activator, and maximum level was found between 6 and 10 hours of incubation. Our observation and results from previous experiments (Bilej et al. 2001) indicate the presence of proPO system in annelids, but our data suggest that level of PO or ints activity is lower in earthworms than in arthropods.

It should be mentioned that the name phenoloxidase encompasses two similar enzymes, which only differ in their enzymatic properties: tyrosinase (EC 1.14.18.1) and catecholoxidase (EC 1.10.3.1). Tyrosinase catalyzes the hydroxylation of monophenols (i.e. tyrosine, considered as the natural substrate of the enzyme) and the oxidation of diphenols to quinones, whereas catecholoxidase catalyses only the oxidation of diphenols. The latter enzyme is almost
indistinguishable from the different kinds of tyrosinases by sequence and properties other than the enzymatic activity (Sanchez-Ferrer et al. 1995; Decker and Jaenicke 2004). This may be the reason why the term phenoloxidase is often used in the literature indiscriminately for tyrosinases and catecholoxidases of invertebrates. A number of phenoloxidases are reported for arthropods, but only a few were demonstrated as having tyrosinase activity i.e. using the monophenol tyrosine as substrate (Fujimoto et al. 1993; Aspan et al. 1995; Chase et al. 2000; Jaenicke and Decker 2003). We tested different substrates in combination with different non-specific activators of proPO for the ability to trigger the proPO activation. In accordance with our presumption, the coelomic fluid without substrate/activator of PO/proPO did not reveal significant PO activity. Since we did not detect PO activity in the coelomic fluid after the simultaneous incubation with any of the activators and tyrosine as a substrate, we suggest the presence of catecholoxidase rather than tyrosinase in *E. fetida* earthworms.

PO activity can also be detected in the coelomic fluid separated in native SDS-free PAGE when the resulting gel is incubated with L-DOPA as a substrate and an activator (we used cetylpyridinium chloride - CPC). After 12 hours of incubation only one band showing oxidase activity appeared. No spontaneous oxidation was visible after the incubation of the gels in the absence of the L-DOPA substrate and/or the CPC activator. This could reflect that the *E. fetida* coelomic fluid contains a minimal amount of PO.

Bands showing detectable oxidation were cut from the native SDS-free PAGE and electroeluted for further analyses and finally prepared for sequencing. The sequencing yielded 5 peptide sequences. The N-terminal amino acid sequence of one peptide showed homology with the endogenous inhibitor of PO from the housefly *Musca domestica*. In *Musca* this competitive inhibitor of the PO was found to be a DOPA-containing peptide with a molecular weight of 4,2 kDa (Daquinag et al. 1995, 1999). It is known that the proPO system involves also inhibitors of proPO activation and PO activity. They can prevent undesired activation of proPO, or prevent over-activation of ppA (Aspan et al. 1990; Liang et al. 1997; De Gregorio et al. 2002). The endogenous inhibitor of PO from *Musca domestica* was described as a DOPA-containing peptide, wherein DOPA is a modified tyrosine residue. The presence of such modified tyrosine residue in phenoloxidase inhibitor, which could form a complex with phenoloxidase in earthworms, might explain the correlation of activity and absorbance at 475 nm of the fractions obtained during gel
filtration of our proteins. The observed data imply the existence of an inhibitor of PO in earthworms, which might probably be forming a complex with PO. Two other peptides (15 and 17 amino acids long, respectively) shared partial homologies with the sequences of phenoloxidase and/or hemocyanin of different invertebrate species. The two remaining earthworm peptide sequences did not display homology with known proteins.

Efforts to identify whole proPO/PO sequence including (i) PCR with degenerated primers designed based on conserved regions in published phenoloxidases and with degenerated primers designed in accordance with identified peptide sequences, and (ii) the screening of *E. fetida* cDNA library with a probe containing a partial sequence of the gene coding for phenoloxidase in the crayfish *Pacifastacus leniusculus* (kindly provided by Prof. Söderhäll, Uppsala), have so far been unsuccessful. One of the plausible reasons for our failure may be the very low level of mRNA for phenoloxidase in *E. fetida* earthworms.

Together these data indicate that peptides having a partial homology with PO and/or hemocyanin originated from the active fraction from *E. fetida* coelomic fluid proteins exhibiting proPO oxidizing properties. Therefore, it can be suggested that phenoloxidase and a related inhibitor exist in *E. fetida* earthworms, but the level of PO activity is lower than in other invertebrates. Moreover, it is clear that PO activation is slower as compared to other invertebrate species. Therefore we can presume that the proPO cascade does not represent the main defense system in earthworms.
6 Conclusions

1) Earthworms respond to antigenic stimulation by formation of antigen-binding protein (ABP), the increase in ABPs level being independent of the size or extent of glycosylation of proteins. Formed ABPs bind preferably the same protein as that used for stimulation, suggesting the presence of partially specific adaptive immunity in annelids. Nevertheless, the specificity of antigen recognition is considerably lower as compared to immunoglobulin since ABP reacts not only with the protein used for stimulation but also to a lesser extent with related proteins.

2) *E. fetida* earthworms respond to antigenic stimulation, body injury or stress conditions by a marked non-specific increase in coelomic fluid protein concentration, but in contrast to antigenic stimulation, the increased level of the protein concentration caused by body injury as a result of wounding returns back to the level of non-injected animals after 2 days post injection. Stimulation of earthworms by microbial antigens leads to distinct changes: lysozyme-like activity in the coelomic fluid of earthworms challenged with bacteria and glucan increases within the first 4 days; hemolytic activity of the coelomic fluid of earthworms challenged with Gram-negative bacteria decreases 12 hours and 2 days after the infection, with Gram-positive bacteria 2 days after the infection; protein level of the coelomic cytolytic factor (CCF) in the coelomic fluid increases within the first 24 hours, most likely due to the release of CCF from intracellular stocks after the infection with bacteria and glucan. Expression of CCF in coelomocytes significantly increases from 2 to 40 hours upon experimental administration of microbial compound, with the maximum level at 17 hours post-injection, while the expression of fetidin is not affected in *E. fetida* earthworms.

3) Two *E. fetida* hemolytic proteins, fetidin and lysenin, are encoded by two separate genes with a high homology and their level of expression in coelomocytes differs from individual to individual. The two hemolytic proteins, fetidin and lysenin, are most probably unique for *E. fetida* earthworms.
4) Earthworm species *D. veneta*, *L. rubellus*, *A. icterica*, *A. rosea*, *A. caliginosa*, *L. terrestris*, *A. longa* contain in their coelomic fluid CCF-like molecules having 80-90 % homology with *E. fetida* CCF, and the monoclonal antibody against *E. fetida* CCF reacts with CCF-like proteins in coelomic fluids of all tested species. CCF from *E. fetida* is unique in that it has a broader saccharide binding specificity and is the only one recognizing N,N'-diacetylchitobiose and peptidoglycan constituents as well as having the ability to lyse tumor TNF-sensitive cell line and *Trypanosoma* parasites. Phylogenetic relationship between the original *E. fetida* CCF and the CCF-like molecules of other Lumbricid species shows that earthworms of the genus *Lumbricus* belong to a group having the same progenitor, whereas the CCF-like molecules of the genus *Aporrectodea* from a paraphyletic group, and *E. fetida* CCF is closely related to the CCF-like molecule of *D. veneta*.

5) *E. fetida* coelomic fluid displays phenoloxidase activity; however, the level of PO activity is lower than in other invertebrates. ProPO activation is also slower as compared to other invertebrate species, suggesting that the proPO cascade does not represent the main defense system in earthworms.
7 Original publications


8 Book sections and abstracts


9 Bibliography


Ashida M. Purification and characterization of pre-phenoloxidase from hemolymph of the silkworm \textit{Bombyx mori}. \textit{Arch Biochem Biophys.} \textbf{144}: 749-62 (1971).


Keilin ND. Parasitic autotomy of the host as a mode of liberation of coelomic parasites from the body of the earthworm. Parasitology. 17: 170-172 (1925).


