4. Results

4.1. Relationship between hemolytic molecules

The coelomic fluid of *E. fetida* earthworms contains various proteins causing the lysis of red blood cells. These proteins, often described independently by different research groups, comprise *Eisenia fetida andrei* factor (EFAF) (Roch *et al.* 1981; Roch 1984), fetidin (Lassegues *et al.* 1997; Milochau *et al.* 1997), lysenin (Sekizawa *et al.* 1996; Sekizawa *et al.* 1997), eiseniapore (Lange *et al.* 1997) and hemolysins isolated either from the coelomic fluid (H_1, H_2 and H_3) or from the cell lysate (CL_{39} and CL_{41}) (Eue *et al.* 1998; Koenig *et al.* 2003). Even though their nomenclature and terminology differ, their further characterization indicates that they are the same or similar molecules. All hemolytic proteins 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 target lipid membranes.

The aim of our study was to clarify whether fetidin and lysenin are either protein isoforms encoded by different alleles of one gene, or products of two related genes.

Both cDNA and amino acid sequences of fetidin and lysenin display high homology. We took advantage of differences between their sequences in 5'UTR regions to design primers specific for each molecule. However, using primers specific for lysenin in PCR we amplified also fetidin. Subsequent sequencing revealed errors in the published 5'UTR sequence of fetidin. A new set of primers designed based on the presence of different deletions in the sequence of fetidin and lysenin in combination with stringent annealing temperature in PCR reaction provided a reliable tool for amplifying the desired sequence only. Using these specific primers we proved that the coelomocytes of each individual *E. fetida* contain mRNA for both fetidin and lysenin. Moreover, PCR reactions with *E. fetida* genomic DNA as a template

resulted in amplification of both molecules, fetidin and lysenin, suggesting that they are encoded by two distinct genes.

Real-time PCR experiments were performed to see the differences in the expression of genes encoding fetidin and lysenin in individual earthworms. We found out that, while the expression of fetidin is similar in all individuals, the expression of lysenin strongly varies. The expression of lysenin was up to 26 times higher in some individuals than in others.

To address the relationships between hemolytic activity and expression of fetidin and lysenin, coelomic fluids of tested animals were separated in native PAGE and then the gels were applied on an erythrocyte suspension embedded in agarose. After several hours of incubation, four different patterns of hemolytic proteins were observed. In parallel, the hemolytic activity of coelomic fluids of tested animals was quantified. The distinct hemolytic patterns correlated with differences in the level of hemolytic activity of the coelomic fluids. All animals exhibiting higher hemolytic activity share the same hemolytic pattern consisting of several bands on agarose-embedded erythrocytes, reflecting the presence of more hemolytic proteins in the coelomic fluids of these earthworms.

Surprisingly, we did not prove the presence of either fetidin of lysenin in other earthworm species (*Aporrectodea caliginosa*, *Apporectodea icterica*, *Apporectodea longa*, *Apporectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus*, *Lumbricus terrestris*) as tested by PCR using fetidin and lysenin specific primers. This finding suggests a unique occurrence of these hemolytic molecules in *Eisenia fetida* earthworms.

In conclusion, we document that fetidin and lysenin are encoded by different genes with high homology and their expression differs in individual *E. fetida* earthworms.

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