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Eisenstasin, new antistasin family inhibitor from the earthworm

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Abstract: A couple of new antistasin family serine protease inhibitors have been isolated from the non-hematophagous earthworm, *Eisenia andrei*. These novel inhibitors have been designated as eisenstasin I and II. Similar to other antistasin family inhibitors, eisenstasin I and II feature 3 and 4 internal repeats, respectively, of a 24–29 amino acid sequence, both of which exhibit a conserved pattern of 6-cysteine/2-glycine at an identical position between the third and fourth cysteine residues. This suggests that the eisenstasins isolated from the earthworm are members of the antistasin family. The eisenstasins are 82% similar with regard to amino acid sequences and exhibit over 70% similarity with the antistasins from the earthworm *Lumbricus rubellus*, while also displaying less than 40% sequence similarity with the leech antistasins. Earthworm eisenstasins are basic proteins, primarily due to the frequent occurrence of arginine residues in their structure, especially at the C-terminal region. As arginine is a key residue for the substrate specificity of some serine proteases including FXa, it is thought that these multiple arginine residues may play a role in the inhibitory characteristics of the eisenstasins. Considering the structure and number of the internal repeats derived from a variety of animal species, the deletion as well as the duplication of all or part of an internal repeat may be implicated in the evolution of the structure and function of the antistasin family inhibitors.

Key words: earthworm; antistasin family serine protease inhibitors; eisenstasin; primary structure

Introduction

A serine protease inhibitor evidencing anticoagulant as well as antimetastatic activity, was initially isolated from the Mexican leech, *Haementeria officinalis* de Filippi, 1849, and was designated as an antistasin according to its inhibitory activities (Tuszynski et al. 1987). Antistasin is a potent anticoagulant, due to its ability to inhibit coagulant factor Xa (FXa) (Holstein et al. 1992). As FXa is positioned at the convergence point of the intrinsic and extrinsic pathways in the coagulant cascade, antistasin has been the focus of many years of study, toward the development of a safety and effective anticoagulant to replace conventional agents (Danalev et al. 2004). Moreover, several lines of evidence suggest that FXa elicits numerous and profound cellular events, including cytokine release (Papapetropoulos et al. 1998), the expression of adhesion molecules (Senden et al. 1998), tissue factor gene expression (Camerer et al. 1999), and cell proliferation (Herbert et al. 1998). These non-haemostatic effects of FXa may contribute to diseases including arterial restenosis, venous graft disease, acute inflammation, sepsis, and cancer (Leadley et al. 2001). Many antistasins from hematophagous animals have been identified and characterized (Salzet 2001; Atanassov & Tchorbanov 2009). Amino acid sequence analysis revealed that antistasin is a cysteine-rich peptide, featuring internal repeats which contain the conserved 6 cysteine and 2 glycine residues. This suggests that the antistasin gene has evolved via gene duplication (Moser et al. 1998). The number of internal repeats depends, however, on the species. Antistasins from the leech feature two-fold internal repeats while an antistasin from primitive metazoan hydra contains six internal repeats. These internal repeats appear to play a possible role in inhibition specificity and potential of the antistasin family protease inhibitors.

We here report the cloning and sequencing of a couple of new antistasin genes from the earthworm, *Eisenia andrei* Bouché, 1972, a non-hematophagous animal. We have designated these novel genes eisenstasin I and II. Here, we also report the characteristics of the nucleotide sequence and primary structure of these eisenstasins.

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Material and methods

Experimental animals
Sexually mature *Eisenia andrei* were reared in plastic containers. Each container harbored approximately 60 earthworms, and all were placed in a darkened incubator at 22–24 ℃. Powdered cow manure was supplied daily as a food material.

Construction of midgut cDNA library and sequencing
A directional cDNA library was constructed using the pCMV-script XR cDNA Library Construction Kit (Stratagene). About 1,500 bacterial clones were randomly taken up from these plates, and then allowed to grow overnight in 1.5 ml of LB medium. The plasmid DNA was prepared using Plasmid Spin kits (Genemed) and was stored at −20 ℃. Sequencing reactions were then conducted in a MJ Research Gradient Cycler, using a Big-Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq®DNA polymerase (FS enzyme), according to the manufacturer's recommendation. Single-pass sequencing was performed on each template, using the T3 primer with an ABI 3700 sequencer (Applied Biosystems).

Rapid amplification of cDNA end (RACE)-PCR
In order to obtain the full-length antistasin sequences from the cDNA fragments, 5' and 3' RACE was performed using the SMART™ RACE cDNA amplification kit, according to the manufacturer's protocols (Clontech). Gene-specific primers for the 5' and 3' RACE reactions were designed from the EST fragments. The RACE-PCR products were then cloned into pGEM-T cloning vector (Promega) for sequencing.

Comparative and phylogenetic analysis
The obtained sequences were compared with the NCBI protein database via BLASTX search. The expressed sequence tags (ESTs) were searched by using TBLASTN program with eisenstasin II amino acid sequence as a query to identify potential antistasin homologs from other earthworms including *Lumbricus rubellus*. Alignments of the antistasin proteins were carried out using the ClustalW. A molecular phylogenetic analysis was performed by using the neighbor-joining (NJ) method implemented in the MEGA 4 program.

Results and discussion

During an EST project performed on a cDNA library from the midgut of the earthworm, *Eisenia andrei*, we identified two contigs which exhibited some similarity to leech antistasins, and designated them eisenstasin I and II (Fig. 1A). In order to obtain the full-length cDNA, we conducted 3'/5' RACE-PCR. As shown in Fig. 1A, the eisenstasin I and II proteins are 156 and 187-amino acids long, respectively, and feature a predictable signal peptide, which is composed of 22 amino acids at the N-terminal (PSORT: www.psort.org). Both eisenstasin I and II exhibit two possible N-X-S/T glycosylation sites. In the 5'-flanking region of the first repeat, there is a hydrophilic consensus motif, DSSN. Similarly to the other antistasin family inhibitors, eisenstasin I and II feature 3 and 4 internal repeats, respectively, of a 24–29 amino acid sequence. The internal repeat, exhibiting a highly conserved pattern of either the 5-cysteine/2-glycine as in most leech antistasins (Moser et al. 1998) or the 6-cysteine/2-glycine as in hydra antistasin (Holstein et al. 1992), is a striking feature of the antistasin family. Although the repeats are not identical with regard to length or number, the internal repeats in eisenstasin I and II show a conserved pattern of 6-cysteine/2-glycine, at identical positions between the third and fourth cysteine residues (Fig. 1B). This clearly indicates that the eisenstasins isolated from the earthworm are members of the antistasin family. The eisenstasins share an 82% amino acid sequence similarity, and over a 70% similarity with the putative antistasins identified from ESTs of another earthworm, *Lumbricus rubellus* Hoffmeister, 1843. However, they exhibit less than 40% sequence similarity with the antistasins from the leech, even though the leech is closely related to the earthworm phylogenetically.

In the eisenstasins, we were unable to locate the multiple homologous reactive site domains, which are a characteristic of many protease inhibitors, including the hydra antistasin. Comparative analysis, by means of the alignment of the amino acid sequences of the antistasin family inhibitors, allows us to recognize a possible scissile reactive-site in the eisenstasins, between the second and third cysteines of the last internal repeat (Fig. 2A). It should be also noted that both eisenstasins are very basic proteins, exhibiting isoelectric points of over 8.4. This is thought to be attributable to the frequent occurrence of arginine residues, particularly at the C-terminal region. As arginine is a key residue for the substrate specificity of some serine proteases including FXa, it is thought that these multiple arginine residues may play a role in the inhibitory effects of the eisenstasins. Phylogenetic analysis reveals that the eisenstasins can be grouped into a clade with the antistasins from *L. rubellus*, and appear to be more closely related with the antistasin from the hydra than with those from the leech (Fig. 2B).

The antistasin expressed in the primitive metazoan hydra features six internal repeats, (Holstein et al. 1992) while the antistasins from the leech comprise a two-fold internal repeated structure (Nutt et al. 1988; Blankenship et al. 1990). The earthworm, *E. andrei*, is the first species to exhibit the antistasins with the different numbers of internal repeats. Although eisenstasin II appeared to comprise a four-fold internal repeated structure, the deletion of the underlined portion between the second and third repeat in eisenstasin II (Fig. 1B) results in the two eisenstasins displaying higher amino acid sequence similarity. A sequence comparison implies that eisenstasin I and II were formed by duplication of an ancestral eisenstasin and that the deletion occurred in eisenstasin I subsequently.

The structural organization of the hydra and earthworm antistasins suggests that the antistasin family serine protease inhibitors may have evolved via the duplication of small ancestral genes, corresponding to the basic repeat unit (Holstein et al. 1992). Considering the structure and number of the internal repeats in the

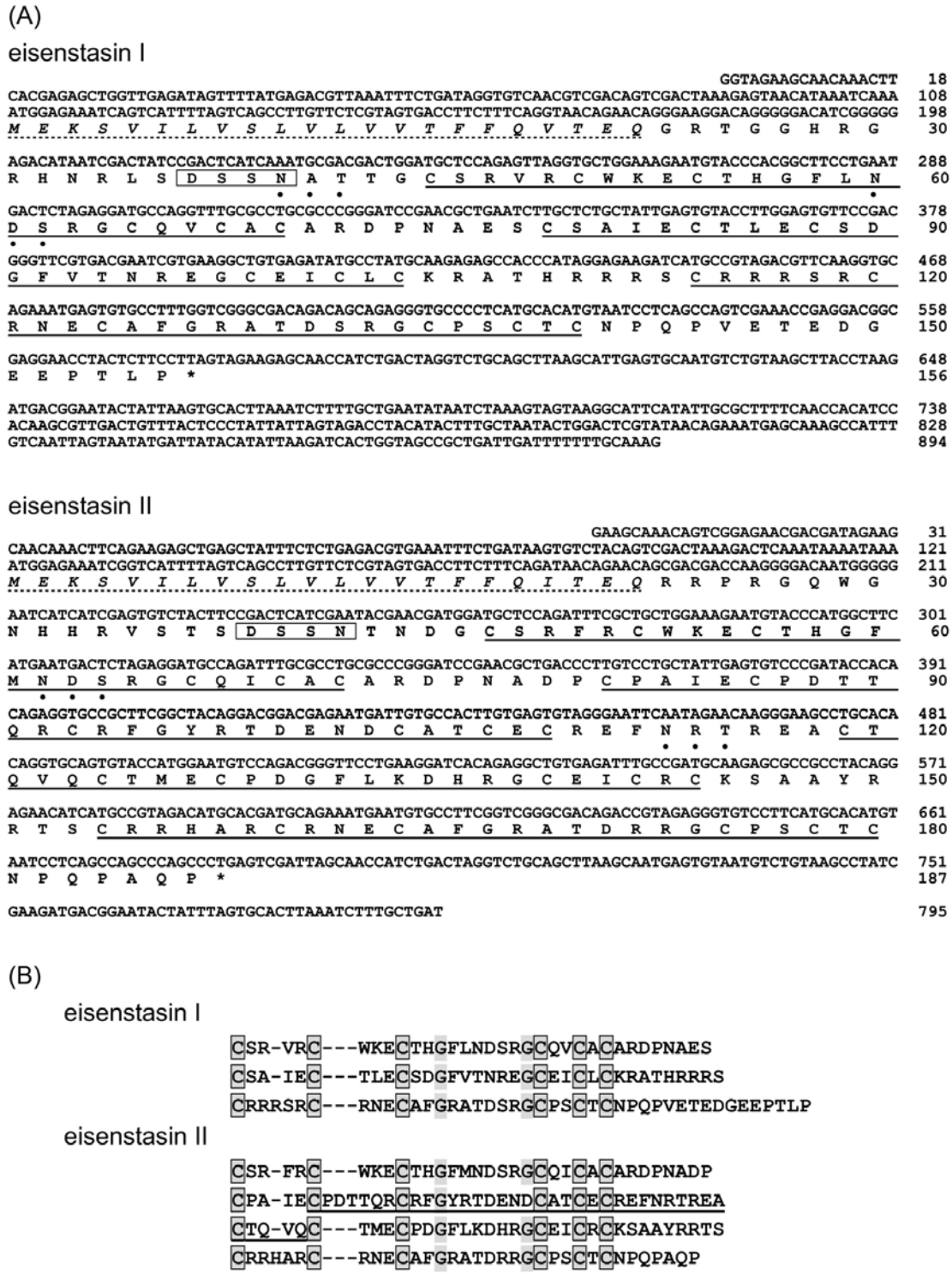

Fig. 1. A – Nucleotide and deduced amino acid sequences of eisenstasin I and II. The predicted signal peptide and internal repeats are dotted and solid underlined, respectively. The hydrophilic consensus motif (DSSN) in the 5'-flanking region and the possible glycosylation sequences are indicated by a box and dots, respectively. B – Internal similarities between repeats. Both eisenstasin I and II exhibit 3 and 4 internal repeats, respectively, and feature a conserved pattern of 6-cysteine (C, shaded and boxed) and 2-glycine (G, shaded) at identical positions between the third and fourth cysteine residues. The region of eisenstasin II which is missing in eisenstasin I is underlined.

antistasins isolated from a variety of animal species, the deletion of all or part of an internal repeat may be implicated in the evolution of the structures and functions of the antistasin family inhibitors. However, there remains an ambiguous relationship between the structures and numbers of the internal repeats and the inhibitory specificity and capability of the antistasin family inhibitors. Therefore, further study is warranted to determine the influences exerted by the structures and numbers of internal repeats on the characteristics

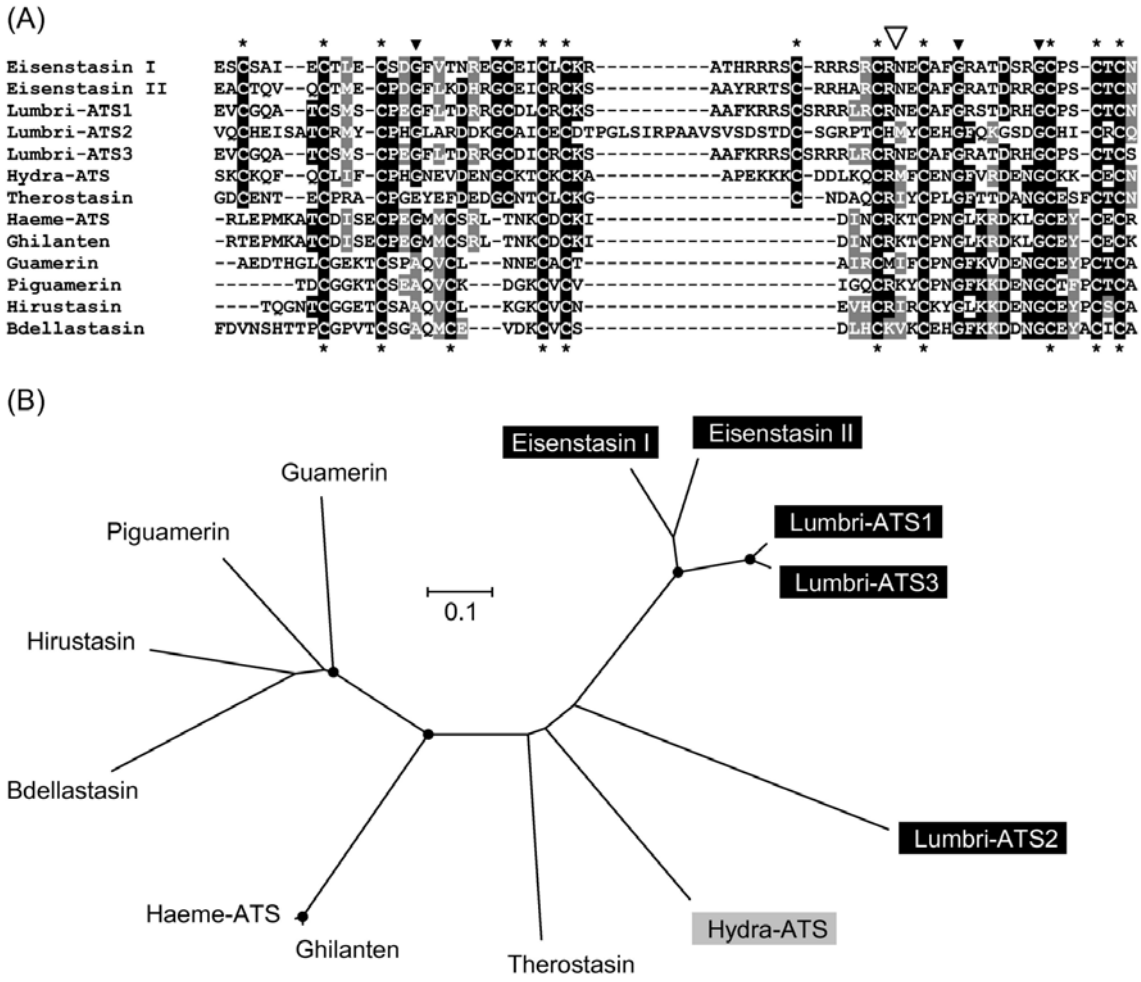

Fig. 2. A – Alignment of amino acid sequences and phylogenetic analysis of antistasin family inhibitors. The representative two consecutive repeats of selected antistasins from leech, earthworm, and hydra were multiply aligned. Residues identical and conserved in 6 or more proteins are highlighted on black and gray, respectively. The conserved 6-cysteine/2-glycine patterns are indicated by asterisks and closed upside-down triangles, respectively, at the top. The asterisks at the bottom indicate the 5-cysteine pattern of the leech antistasins except Therostasin which has 6 cysteines. The assumed scissile reactive-site peptide bond is designated by an open upside-down triangle. Database numbers and position coordinates are: Eisenstasin I, AAX11349:77–141; Eisenstasin II AAX11350:117–181; Lumbri-ATS1, BG269944(EST):427–624; Lumbri-ATS2, CO047510(EST):197–427; Lumbri-ATS3, CO058509(EST):429–626; Hydra-ATS, P38977:118–181; Therostasin, Q9NBW4:19–76; Haeme-ATS, AAA29193:71–126; Ghilanten, P16242:54–109; Guamerin, P46443:5–57; Piguamerin, P81499:1–48; Hirustasin, P80302:1–51; Bdellastasin, P82107:1–55. ATS, antistasin. B – Evolutionary relationship among antistasins. The tree was constructed by using the NJ method based on the alignment shown in A. The molecular phylogenetic analysis confirms that the eisenstasins are grouped into a clade with the antistasins from *Lumbricus rubellus*. The earthworm and the hydra antistasins are highlighted on black and gray background, respectively. Note the 6-cysteine-containing Therostasin and hydra antistasin are placed closer to the earthworm homologs than the other 5-cysteine-bearing leech antistasins are. The nodes with bootstrap values greater than 800 from 1000 replicates are indicated by closed circles.

of inhibitory activities, and to ascertain whether an independent internal repeat is capable of inhibiting its target protease.

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