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Localization and characterization of sulfated glycosaminoglycans in the body of the earthworm *Eisenia andrei* (Oligochaeta, Annelida)

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ABSTRACT

The aim of this study was to characterize the compartmental distribution of sulfated glycosaminoglycans (S-GAGs) in adults and their occurrence during the development of the earthworm *Eisenia andrei*. S-GAGs were extracted from the body of earthworms to identify their composition and the time of their appearance and disappearance in embryonic, newborn, juvenile, and adult earthworms. S-GAGs were also analyzed in earthworm tissue using histochemical metachromatic staining. Purified S-GAGs obtained from the whole body of adult earthworms were composed of chondroitin sulfate (CS) and heparan sulfate (HS). In addition, an unknown, highly sulfated polysaccharide (HSP) was detected. In order to characterize specifically the S-GAG composition in the integument, earthworms were dissected and as much as possible of their viscera was removed. HS and CS were the predominant sulfated polysaccharides in the dissected integument, whereas in viscera, CS, HS and the HSP were found in proportions similar to those identified in the body. The qualitative S-GAG composition in juveniles was similar to that obtained from adult earthworms. CS was the predominant S-GAG in newborn earthworms, accompanied by lesser amounts of HS and by tiny amounts of the HSP. This study provides a detailed descriptive account of the pattern of S-GAG synthesis during development, and also the characterization of the tissue distribution of these compounds in the body of earthworms.

Introduction

Sulfated glycosaminoglycans (S-GAGs) are large complex carbohydrate molecules that are linear, negatively charged, and composed of disaccharide repeating units (Gandhi and Mancera, 2008). These molecules are sometimes known as sulfated mucopolysaccharides because of their viscous, lubricant properties, as found in mucous secretions. S-GAGs are present on all animal cell surfaces and in the extracellular matrix (Medeiros et al., 2000). Owing to the variability in sulfate substitution, all GAGs display considerable sequence heterogeneity, and it is believed that structural differences are responsible for highly specific interactions of GAGs with other macromolecules (Coombe and Kett, 2005; Gandhi and Mancera, 2008). Their strategic location and highly charged nature make them important biological players in cell–cell and cell–matrix interactions that take place during normal and pathological events, including organogenesis in embryonic development, cell recognition, adhesion, migration, regulation of growth factor action, wound repair, lipid metabolism, neural development and regeneration, and initiation and modulation of inflammation (Kresse and Schonherr, 2001; Sugahara et al., 2003; Laabs et al., 2005; Whitelock and Iozzo, 2005; Taylor and Gallo, 2006).

S-GAGs include chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin and heparan sulfate (HS) (Nader et al., 2004; Volpi, 2006; Yamada and Sugahara, 2008; Nadanaka and Kitagawa, 2008). Their disaccharide repeating units are composed of hexosamine (D-glucosamine or D-galactosamine) and either hexuronic acid (D-glucuronic or L-iduronic acid) or galactose (as in KS).

Abbreviations: S-GAGs, sulfated glycosaminoglycans; CS, chondroitin sulfate; HS, heparan sulfate; DS, dermatan sulfate; KS, keratan sulfate; HSP, highly sulfated polysaccharide; DMB, 1,9-dimethylmethylene blue.

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DS, HS, and heparin contain both glucuronic acid and iduronic acid units, whereas CS has glucuronic acid as the only hexuronic acid. In the tissue, S-GAGs are covalently bound to a protein core forming a structure knowing as proteoglycan (Didraga et al., 2006).

Several studies have shown by histochemical methods the presence of sulfated mucopolysaccharides in mucous secretions and in the clitellar and non-clitellar epithelium of earthworms (Varute and Nalawade, 1970; Varute and More, 1972; Richards, 1974; Dall Pai et al., 1981; Morris, 1983, 1985; Licata et al., 2002a). However, little is known regarding their biochemical composition. Until now, only two biochemical studies have been performed to identify the S-GAG composition in earthworms. According to these studies, KS and HS/heparin were the only S-GAGs present in extracts from the whole body of *Lumbricus* sp. and *Pheretima hawayana*, respectively (Rahemtulla and Lovtrup, 1975; Cássaro and Dietrich, 1977). However, very recently, Im et al. (2010) reported the extraction and purification of S-GAGs from the whole body of earthworm *Eisenia andrei*, and the authors identified them as CS/DS and HS.

The aim of the present study was to extend the findings on the S-GAG composition in *E. andrei*, by characterizing the localization of these molecules in the body of adult earthworms by means of biochemical and histochemical analyses. We also characterized their occurrence during development.

Materials and methods

Earthworms and cocoons

Adult and juvenile earthworms and cocoons of *E. andrei* were purchased from Minhobox (Juiz de Fora, Minas Gerais, Brazil). Freshly hatched (newborn) earthworms were collected daily in the laboratory, soon after hatching.

Chemicals and reagents

Chondroitinase AC (EC 4.2.2.5) from *Arthrobacter aurescens*, and Chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris* were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Metachromatic staining of S-GAGs in earthworms

Tissues from adult, juvenile, and newborn earthworms were fixed overnight in Bouin's liquid. After fixation and washing, the tissues were dehydrated in ethanol and embedded in paraffin. Tissue sections (7 μm) were collected on polylysine-coated slides and stained with the cationic dye 1,9-dimethylmethylene blue (DMB) (Farndale et al., 1986) in 0.1 M HCl, containing 0.04 mM glycine and 0.04 mM NaCl (Souza et al., 2007). The sections were then examined and photographed using an optical microscope (Zeiss, Axioskop 2). The positive reaction reveals S-GAGs as metachromatic structures (stained purple).

Isolation, purification, and characterization of S-GAGs from the whole body and body portions of adult earthworms

Around 300 adult earthworms were dissected into three portions, the anterior and posterior ends and the clitellum. Tissues from the whole body and body portions of adult earthworms were incubated with acetone for 24 h at room temperature and dried. The tissues were suspended in the 0.1 M sodium acetate buffer, pH 5.5, containing papain (10 mg/g of dry tissue) in the presence of 5 mM EDTA and 5 mM cysteine, and incubated at 60 °C for 24 h. The incubation mixture was centrifuged at 2000 × *g* for 10 min at room temperature, and the supernatant, which contained the GAGs, was retained. A 10% cetylpyridinium chloride solution was added to the supernatant for a final concentration of 0.5%, and the mixture left to stand at room temperature for 24 h. The solution was centrifuged at 2000 × *g* for 30 min at room temperature and the pellet was collected. This pellet, a GAG-cetylpyridinium complex, was dissolved with a solution of 2.0 M NaCl/absolute ethanol (100:15, v/v) and the GAGs were precipitated with the addition of 3 volumes of absolute ethanol. After 24 h at 4 °C, the precipitate was collected by centrifugation. The final pellet, which constitutes the total tissue GAG preparation, was dissolved in distilled water, dialyzed, lyophilized, and dissolved in distilled water (Silva, 2002).

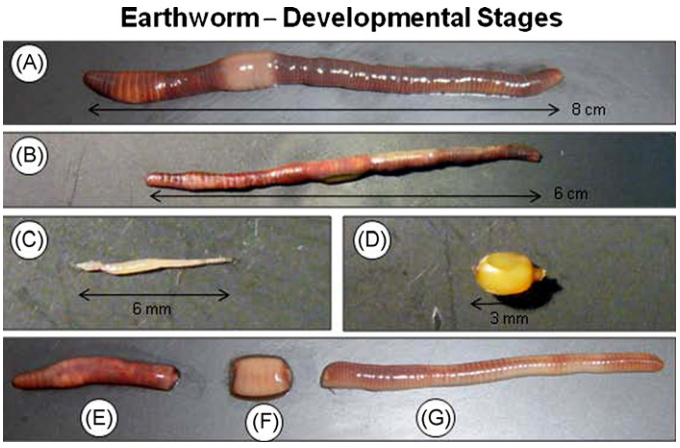

Fig. 1. Photographs of the earthworm *Eisenia andrei* at different developmental stages, adult (A), juvenile (B), newborn (C), and cocoons at the early embryonic stage (D). In (E)–(G) are shown photographs of adult dissected body parts: anterior end (E), clitellum (F), and posterior end (G).

The partially purified GAGs were applied to a Mono Q-FPLC column (HR 5/5), equilibrated with 20 mM Tris:HCl (pH 8.0). A few protocols were performed with different chromatographic elution conditions. The best protocol was selected for use and was as follows. The column was washed with 20 ml of 0.6 M NaCl in Tris buffer, in order to remove pigments and charged contaminants. Then, the column-bound S-GAGs were eluted in stepwise degrees with 1.0 M NaCl, 1.5 M NaCl, and 3.0 M NaCl in Tris buffer. Fractions of 0.5 ml were collected, and the elution was monitored by the metachromatic property of the fractions using 1,9-dimethylmethylene blue (Farndale et al., 1986; Vieira et al., 2004). The GAGs eluted from the column were exhaustively dialyzed against distilled water, lyophilized, and dissolved in distilled water.

S-GAGs were characterized by agarose gel electrophoresis, enzymatic digestion with chondroitinases AC and ABC, and deaminative cleavage with nitrous acid as described below. Agarose gel electrophoresis was carried out as previously described (Silva, 2006). Approximately 10 μg of S-GAGs, before and after chondroitinase AC or ABC digestions, or deaminative cleavage with nitrous acid (see below) were applied to 0.5% agarose gels in 0.05 M 1,3-diaminopropane: acetate (pH 9.0). After electrophoresis, S-GAGs were fixed in the gel with 0.1% *N*-acetyl-*N*,*N*,*N*-trimethylammonium bromide in water, and stained with 0.1% Toluidine blue in acetic acid:ethanol:water (0.1:5:5, v/v/v).

Digestions with chondroitinases AC or ABC were carried out according to Saito et al. (1968). S-GAGs were incubated with 0.3 units of chondroitinases AC or ABC for 8 h at 37 °C in 100 μl 50 mM Tris:HCl (pH 8.0) containing 5 mM EDTA and 15 mM sodium acetate.

Deamination by nitrous acid at pH 1.5 was performed as previously described (Shively and Conrad, 1976). Briefly, S-GAGs were incubated with 200 μl freshly generated HNO_2 at room temperature for 10 min. The reaction mixture was then neutralized with 1.0 M Na_2CO_3.