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Glycolipoprotein G-90 obtained from the earthworm *Eisenia foetida* exerts antibacterial activity

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ABSTRACT

G-90 is a glycolipoprotein mixture obtained from the tissue homogenate of earthworm *Eisenia foetida* (Annelida, Lumbricidae) which exhibits pleiotropic biological functions. The antibacterial activity of G-90 was investigated *in vitro* and *in vivo*. Concentrations of 10 ng/mL and 10 µg/mL of G-90 exhibited an inhibitory effect on *in vitro* growth of non-pathogenic and facultative-pathogenic bacteria. The bacteriostatic effect of the G-90 was 21% stronger for facultative-pathogenic bacteria than that observed for non-pathogenic bacteria. The analysis of CFU/mL values following swabbing of surgical wounds in mice demonstrated that G-90 used in either 10 ng/mL or 10 µg/mL concentrations inhibited *in vivo* growth of the bacteria obtained from these wounds. The antibacterial activity of G-90, as well as the biological characteristics exhibited by the preparation, may be of interest for clinical investigations in veterinary and human medicine.

Key words: earthworm, *Eisenia foetida*, G-90, antibacterial activity

Introduction

Invertebrates have developed innate immune mechanisms that detect pathogens by recognizing conserved molecular patterns. The recognition molecules for foreign material have been named as pattern-recognition proteins (PRPs) (MEDZHITOV and JANEWAY, 1997) because the host's primitive effector cells would recognize molecular patterns rather than particular structures of the invading microorganisms. Examples of pathogen-associated

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molecules, which are not found in other multicellular organisms, are lipopolysaccharides (LPS) or peptidoglycans from bacterial cell walls, β-1, 3-glucan of fungal cell walls, and double stranded RNA of viruses.

Immune mechanisms of earthworm *Eisenia foetida* include both cellular and humoral components. Earthworm coelomocytes respond to the presence of pathogens by phagocytosis, encapsulation/brown body formation, and NK-cells activity (COOPER et al., 2001). Humoral components include lectins, antimicrobial peptides, pore-forming proteins, phenoloxidases and proteases. They induce sequestration of antigenic material by agglutination, cytotoxicity and antibacterial activity. Cytolisin of *E. foetida* coelomic fluid, named Eiseniapore, was found to cause pore–like structures at the target membranes. Formation of the pore proteins is the result of oligomerization of Eiseniapore monomers (LANGE et al., 1999).

G-90 is a glycolipoprotein mixture (Patent: "Glycolipoprotein mixture from tissue homogenates of the Lumbricidae family earthworm obtained from the tissue homogenate of earthworm *E. foetida* (Annelida, Lumbricidae) – Procedure for its obtaining and its application in medicine" - P920481A, Republic of Croatia, State Patent Office 1992) (HRŽENJAK et al., 1992).

Numerous biological activities of G-90 were established, such as haemolysis, agglutination and the capability of incorporation into nucleic acids (HRŽENJAK et al., 1992). HRŽENJAK et al. (1992) also showed that G-90 is neither mutagenic nor cancerogenic. A G-90, depending on the protein concentrations, is capable of either increasing (at a concentration of 10 ng/mL) or decreasing (at a concentration of 10 μg/mL) the proliferation of the normal or transformed cells in the culture and of slowing down mouse melanoma growth *in vivo* (HRŽENJAK et al., 1993). The insulin-like growth factor, the adhesins of the immunoglobulin superfamily and the serine proteases of the tripsine code (PI, PII) were isolated from G-90 (POPOVIĆ et al., 1996; HRŽENJAK et al., 1998a; POPOVIĆ et al., 1998). G-90 has strong anticoagulative and fibrinolytic activities in vitro (HRŽENJAK et al., 1998b). When added to the culture of mouse fibroblasts (L929) and monkey epithelial cells (GMK), G-90 showed antioxidative activity during the treatment of cells with H_2O_2 (GRDIŠA et al., 2001).

Further studies of the biological properties of G-90 demonstrate that it exerts an inhibitory activity against non-pathogenic and facultative pathogenic bacteria. In this report we describe its in vitro and in vivo antibacterial activity.

Materials and methods

Preparation of G-90. Glycolipoprotein extract G-90 was prepared from the tissue homogenate of earthworm *E. foetida* (Annelida, Oligocheta, Lumbricidae) according to the method described by HRŽENJAK et al. (1992).

Mice and experimental design. One hundred and sixty-five clinically healthy male mice of NHI breed (Immunological Institute, Brezje, Croatia), aged 3 weeks and weighing an average 20 ± 0.23 g were used. They were maintained under standard conditions, with feed and water ad libitum, but without antibiotics.

Anaesthesia of mice was induced by short-acting inhalation and maintained by ether (Kemika, Zagreb, Croatia) inhalation. The lumbo-sacral region of mice was shaved and disinfected. Five mm-long surgical excisions of skin at lumbo-sacral region were performed in the form of a small square measuring approximately 23.5 mm^2.

The mice were divided into 5 groups and their skin wounds were treated every 24 hours over a 28-day period as follows:

Group A: mice with wounds which healed without any treatment (negative control group);

Group B: mice with wounds treated with 25 μL of the physiological solution (the solvent for G-90);

Group C: mice with wounds treated with D-Panthenole ointment (1 g contains 50 mg of dexapanthenole; Jadran, Rijeka, Croatia) (positive control group);

Group D: mice with wounds treated with 25 μL of G-90 per wound in a concentration of 10 μg/mL;

Group E: mice with wounds treated with 25 μL of G-90 per wound in a concentration of 10 ng/mL.

All laboratory mice were handled and treated in accordance with "The European document of keeping and handling with the laboratory animals" (GREEVE et al., 1993).

Pure bacterial cultures. Non-pathogenic bacterial cultures of Bradyrhizobium japonicum, Rhizobium leguminosarum, Azotobacter chroococcum (kindly donated by the Faculty of Agronomy, University of Zagreb, Croatia) were prepared by standard bacteriological methods (ATLAS, 1995).

Cultures of facultative-pathogenic bacteria such as *Salmonella* Enteritidis, *Staphylococcus aureus, Streptococcus pyogenes* (Faculty of Veterinary Medicine, University of Zagreb, Croatia) were also prepared.

Measuring of G-90 antibacterial activity in vitro. The bacterial cultures (non-pathogenic or facultative-pathogenic) were grown in Petri dishes and grown on Columbia agar (Biolife, Milan, Italy) using the usual sterile laboratory technique and incubated at 37 °C for 15 minutes in a variable atmosphere incubator (Don Whitley Scientific Ltd, Yorkshire, UK). The sterile test discs (made of 7 mm diameter filter paper) were placed on the agar surface and used for testing of the G-90. Before use (2-3 days), the test discs were immersed in 10 μL of G-90 (at concentrations of 10 μg/mL and 10 ng/mL) or 10 μL of horse serum (at a concentration of 10 μg/mL), which served as positive control, and dried at room temperature

in sterile conditions. The Petri dishes with test discs were incubated at room temperature for 15-20 min, and additionally for 24 hours at 37 °C in a variable atmosphere incubator. The number of colony-forming units of bacteria growing around the test discs per millilitre (CFU/mL) was determined according to method described by ATLAS (1995).

Measuring of G-90 antibacterial activity in vivo. Every 24 hours swabs were taken from the surgical wound area of all mice in the experiment, and inoculated in Petri dishes with Columbia agar by the usual sterile laboratory technique. Following 24 hours of incubation at 37 °C in a variable atmosphere incubator, the CFU/mL for grown mixed bacterial cultures was determined.

Staphylococci sp. bacteria were used to estimate the antibacterial activity of G-90. The colonies of Staphylococcus sp. bacteria were identified by routine methods from the mixed bacterial culture grown from the swabs taken from the wounds of mice in group A on the third day post-surgery. These bacterial colonies were cultured in Petri dishes with Columbia agar and incubated at 37 °C for 15 minutes in a variable atmosphere incubator. Subsequently, the sterile test discs were placed on the surface of the agar, as follows: a) the test discs immersed in 10 µL of G-90 of different concentrations (10 ng/mL and 10 µg/mL); b) the commercial test discs specified for Staphylococci sp., such as Gentamicin (10 µg GN-10), Enrofloxacin (20 µg ENR-5) (Oxoid Limited, Hampshire, England) which served as positive controls. After 24 hours of incubation at 37 °C the inhibitory zones around the test discs were measured.

Statistics. Statistical analyses were performed with Man-Whitney and *t*-test.

Results

The influence of G-90 on the in vitro growth of the bacterial cultures was evaluated by non-pathogenic (such as *Bradyrhizobium japonicum*; *Rhizobium leguminosarum*; *Azotobacter chroococcum*) and facultative-pathogenic bacterial cultures (such as *Salmonella* Enteritidis, *Staphylococcus aureus*, *Streptococcus pyogenes*). The best inhibitory effect of G-90 on the growth of non-pathogenic and facultative-pathogenic bacteria was obtained with concentrations from 10 ng/mL to 10 µg/mL (Fig. 1). However, at 10 mg/mL concentration, G-90 stimulated the growth of the same bacterial species. Interestingly, the antibacterial effect of G-90 was 21% stronger for facultative-pathogenic bacteria than that observed for the non-pathogenic species.

The CFU/mL values obtained for all bacterial colonies grown from swabs taken from the surgical wounds were demonstrated as the percentage of bacterial proliferation for the 28-day period of the experiment (Fig. 2). The analysis of values obtained for the bacterial proliferation indicated that G-90 used in concentrations of either 10 µg/mL or 10 ng/mL inhibited the growth of bacteria in the surgical mouse wounds. The bacterial growth obtained from the surgical wounds treated once with G-90 in a concentration of

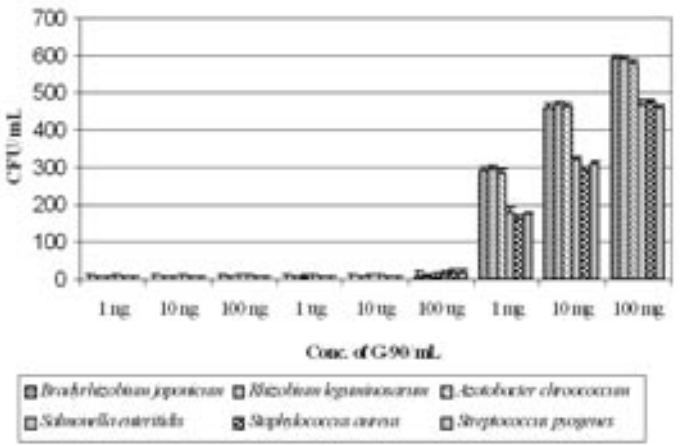

Fig.1. Influence of G-90 on *in vitro* growth of either non-pathogenic bacteria (*Bradyrhizobium japonicum*; *Rhizobium leguminosarum*; *Azotobacter chroococcum*) or pathogenic bacteria (*Salmonella* Enteritidis, *Staphylococcus aureus*, *Streptococcus pyogenes*) during a 24-hour period.

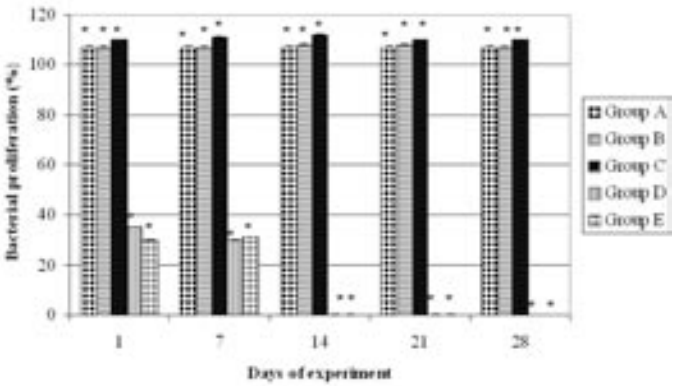

Fig. 2. Influence of G-90 on the *in vivo* growth of bacteria cultured from the swabs taken from the surgical wounds of mice during the 28-day period of the experiment

* Significantly different bacterial proliferation ($P<0.05$) between that in normal mice skin (100%) and those in mice surgical wounds treated with various substances (Group A; B; C; D; E). Group A: mice with wounds healed without any treatment; Group B: mice with wounds treated with 25 µL of physiological solution; Group C: mice with wounds treated with D-Panthenole ointment; Group D: mice with wounds treated with 25 µL of G-90 per wound in a concentration of 10 µg/mL; Group E: mice with wounds treated with 25 µL of G-90 per wound in a concentration of 10 ng/mL.

10 µg/mL (Group D) or 10 ng/mL (Group E) decreased by 65-70% in comparison with that obtained from the normal mouse skin (100%). However, after 14 days of treatment of the surgical wounds with G-90 in both tested concentrations, the bacterial growth of the treated wounds ceased completely and remained unchanged throughout the experimental period. During the experiment the growth of bacteria from the samples taken from surgical wound of the mice in groups A and B increased by 7%, and in group C by 10%, compared with the bacterial growth obtained for normal mouse skin. These findings indicated that the growth of the mixed bacterial cultures was within physiological values, as well as a mild bacterial infection of the surgical wounds. The surgical wounds of mice in group C, which were treated with D-Panthenole ointment, showed an increase in bacterial growth by 3% compared with the values obtained from the surgical wounds of mice in groups A and B. This could be explained by the fact that D-Panthenole is a commercial ointment which only encourages the regeneration of the damaged tissue without any antibacterial effect and therefore could be a suitable nutrient base for bacterial culture growth.

In a pure bacterial culture of Staphylococcus sp. isolated from the swab of the mixed bacterial cultures taken from the surgical wound that healed without treatment, the inhibitory activity of G-90 was compared with the antibacterial activity of the commercial antibiotics specific for Staphylococci sp., such as: GN-10 (10 µg) and ENR-5 (20 µg). Based on the

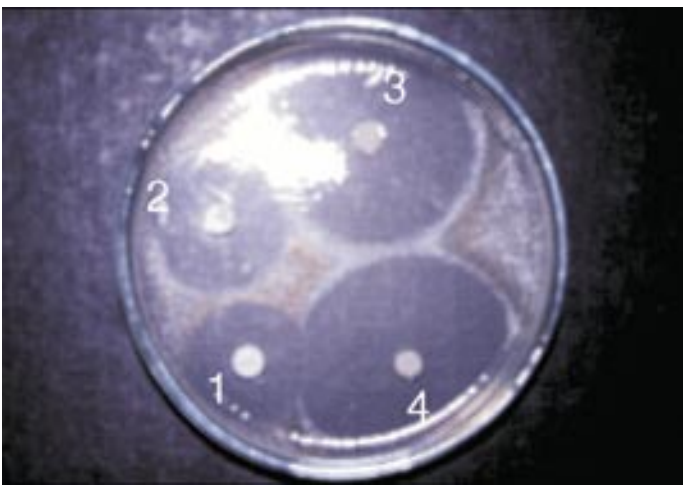

Fig. 3. Comparison of antibacterial activity of G-90 and commercial antibiotics on the *in vitro* growth of *Staphylococcus* sp. *Staphylococcus* sp. bacteria were taken from the mixed bacterial culture grown from the swabs taken from the wounds of mice in Group A (wounds healed without treatment) on the third day post-surgery: 1. Gentamicin (10 µg); 2. Enrofloxacin (20 µg); 3. G-90 (10 ng/mL); 4. G-90 (10 µg/mL)

diameters of inhibitory zones, Staphylococci exhibited 17 ± 0.43% higher sensitivity for G-90 in both tested concentrations in comparison with GN-10 and ENR-5 (Fig. 3)

Discussion

The quoted results imply that earthworms, or the products of their metabolism, applied in the certain concentrations, could decrease the growth of the facultative-pathogenic bacteria and thus may promote growth of non-pathogenic agriculturally useful bacteria, such as *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, *Azotobacter chroococcum*.

In clinical experience, the infection of skin wounds often occurs by staphylococci, causing a decrease in the proliferation of epithelial cells and fibroblasts in the wounds (CHANG et al., 1997). The treatment of infected wounds with commercial antibiotics additionally retard the proliferation of epithelial cells and fibroblasts and consequently retard the healing process by 5 % compared to the physiological time of wound healing without treatment (MESZAROS et al., 1993). The obtained results imply that G-90 applied on in vivo grown bacteria present in the process of wound healing, exerts some antibacterial effect.

The antibacterial effect similar to G-90 influences some plant extracts (in concentrations higher than 10 mg/mL) such as: *Agrimonia eupatoria*, *Calendula officinalis*, *Chemolaena odorata*, *Petroselinum crispum*, *Plantago major*, *Prunella vulgaris* (IMEGWU et al., 1997).

Parenteral administration of the protein antigen to earthworms induces elevated levels of antigen-binding proteins (ABP), which recognized the protein used for stimulation. The molecular masses of these ABP were 56 kDa in Lumbricus terrestris and 60 kDa in *Eisenia foetida* (HANUSOVA et al., 1999b; KOHLEROVA et al., 1999). A protein of 60 kDa was identified as a ubiquitous PLA2-like enzyme, which might be involved in the immune reaction of earthworms, such as anti-bacterial mechanisms (HANUSOVA et al., 1999a).

By inoculation of bacteria into the coelomic cavity of earthworm, the coelomocytes (cells of coelomic fluid) initiate the process of connecting with each other by their adhesive structures around the bacteria and form so-called "brown bodies" (VALEMBOIS et al., 1992; COOPER et al., 1999). At the same time the celomocytes intensively synthesize and secrete proteins that attach to the bacteria, forming aggregations, and may inhibit their further proliferation. One of these proteins is agglutinin of 56 kDa molecular mass, which attaches to the lectin-like monosaccharides of the cellular membrane of the bacteria. REJNEK (1991), TUCKOVA (1991) and VALEMBOIS et al. (1993) demonstrated that significant antibacterial activity, besides celomocytes, have chloragocytes, i.e. cells from the intestinal tract of the earthworms. The chloragocytes secrete two proteins with a molecular mass of 40 and 45 kDa, which share a 35% similarity with immunoglobulins. These proteins adhere to the

bacteria like opsonins, making them suitable for phagocytosis by celomocytes in coelomic fluid (LASSEGUES et al., 1997). Also, the chloragocytes synthesize and secrete the protein lysenin (33 kDa), which binds specifically to phospholipids of the cell membrane and causes cytolysis (KOBAYASHI et al., 2000; OHTA et al., 2000). Coelomic cytolytic factor 1 (42 kDa) isolated from the coelomic fluid of *Eisenia foetida* is involved in the activation of prophenoloxidase cascade via recognition of Gram-negative bacterial cell wall molecules, such as glucan and lipopolysaccharide (BESCHIN et al., 1998).

At the present level of knowledge it is difficult to define which molecules of G-90 are responsible for its antibacterial activity. Theoretically, such activities could be ascribed to some of the following molecules of molecular masses: 33, 40, 42, 45 and 60 kDa, which were detected by SDS-PAGE in G-90 (HRŽENJAK et al., 1992). By immunochemical analyses these proteins were shown to belong to the immunoglobulin superfamily (POPOVIĆ et al., 1998).

Considering the demonstrated biological activities of G-90, we can assume that the macromolecules in the G-90 mixture are present in biologically balanced proportions and that they act pleiotropically. Our findings confirm the suggestions by COOPER et al. (2002) that certain molecules of the earthworm's immune system may be exploited as natural antibiotics.

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SAŽETAK

G-90 je glikolipoproteinska smjesa dobivena iz tkivnog homogenata gujavica *Eisenia foetida* (Annelida, Lumbricidae) koja pokazuje brojne biološki značajne aktivnosti. U ovom radu istražena je antibakterijska aktivnost G-90 *in vitro* i *in vivo*. G-90 u koncentracijama od 10 ng/mL i 10 µg/mL inhibira rast nekih nepatogenih i patogenih bakterija *in vitro*. Inhibicijski učinak G-90 na rast bakterijskih kultura uvjetno-patogenih vrsta bio je za 21% jači u odnosu na nepatogene bakterija. Analizom vrijednosti CFU/mL, dobivenih iz obriska kožnih rana miševa, pokazano je da G-90 i *in vivo* u koncentracijama od 10 ng/mL i 10 µg/mL inhibicijski djeluje na rast bakterija u tretiranim ranama. U ovom radu opisana antibakterijska aktivnost G-90, uz ranije utvrđene biološke aktivnosti, upućuje da bi pripravak mogao biti od interesa za klinička istraživanja u veterinarskoj i humanoj medicini.

Ključne riječi: gujavica, *Eisenia foetida*, G-90, antibakterijski učinak