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Ability of fucoidan to prevent muscle necrosis induced by snake venom myotoxins: Comparison of high- and low-molecular weight fractions ☆

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Abstract

Fucoidan, a natural polysaccharide extracted from brown seaweed, inhibits the myotoxic phospholipases A_2 present in the venoms of crotalid snakes. This study evaluated the influence of molecular weight on the ability of fucoidan to prevent muscle necrosis when rapidly administered after injection of a purified myotoxin or crude venom of *Bothrops asper*, in a mouse model. It was hypothesized that smaller fucoidan fragments, being of higher diffusibility to tissues, might have a better neutralizing efficiency *in vivo*. Fucoidan was subjected to acid hydrolysis to obtain low-molecular weight fragments (F_L), or to gel filtration to isolate its high-molecular weight fraction (F_H). These two preparations were standardized to the same neutralizing potency by preincubation assays, and subsequently tested *in vivo*, by independent administration assays. Local i.m. administration of either F_H or F_L, immediately after i.m. injection of myotoxin II, prevented nearly 50% of muscle necrosis, albeit with no difference between the two preparations. Muscle necrosis was not reduced when either F_H or F_L was administered by i.v. route, immediately after i.m. toxin injection. When tested against crude venom, which contains several myotoxin isoforms, the immediate *in situ* i.m. injection of F_H still inhibited myonecrosis by nearly one-half of the effect recorded in the untreated group, whereas F_L was ineffective. It is concluded that, in this model, and in contrast to expectations, the use of smaller fucoidan fragments to prevent muscle damage induced by snake venom myotoxins is not advantageous, when compared with larger fucoidan molecules.

Keywords: Myotoxin; Myonecrosis; Fucoidan; Phospholipase A_2; Venom

1. Introduction

A diversity of snake venom components, including phospholipases A_2 (PLA_2), PLA_2 homologs, cardiotoxins, and small basic peptides, are responsible for direct damage to skeletal muscle fibers, or myonecrosis (Lomonte et al., 2003). Muscle damage is a frequent consequence of snakebite envenomations, which may lead to significant tissue loss and

☆ *Ethical statement*: The work has been performed in compliance with the ethical requirements of the Institutional Committee for Use and Care of Research Animals at the University of Costa Rica. Also, the authors state that they do not have conflicts of interest in this manuscript.

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disability (Warrell, 1996). Due to the fact that muscle necrosis induced by such toxins develops very rapidly, its prevention, or at least its minimization, represents a major challenge for the improvement of current therapeutic strategies against snakebites. Antivenoms are not always efficient in preventing or limiting myonecrosis. In some cases, the ineffectiveness of antivenoms has been explained by the scarcity of antibodies against the main venom myotoxins (Ownby et al., 1983; Lomonte et al., 1991). However, even in situations where antivenoms have been shown to contain high levels of antibodies to myotoxins, their neutralizing efficiency in animal studies still remains very limited, due to the rapid action of the toxins (Rucavado and Lomonte, 1996). One approach to cope with this problem has been the search of alternative neutralizing molecules, other than antibodies, aimed at inhibiting venom myotoxins if administered immediately at the site of venom entry or parenterally. An increasing number of natural and synthetic compounds have been reported to have inhibitory effects upon snake venom myotoxins, such as heparin (Melo and Suarez-Kurtz, 1988; Lomonte et al., 1994a), suramin (de Oliveira et al., 2003; Murakami et al., 2005), fucoidan (Angulo and Lomonte, 2003), animal serum factors (Fortes-Dias, 2002; Lizano et al., 2003), and a wide variety of plant components (Soares et al., 2005).

Fucoidan is a sulfated polysaccharide extracted from the brown seaweed *Fucus vesiculosus*, which is composed primarily of a branched polymer of α 1→ 3-linked fucose, with sulfate groups substituted at position 4 on some of the fucose residues (Patankar et al., 1993). Earlier work described the ability of this natural compound to neutralize the cytolytic and myotoxic effects of a number of group II PLA_2s and PLA_2 homologs (Lys49 PLA_2s) present in the venoms of crotalid snakes. The mechanism underlying this inhibition was shown to involve the direct interaction of the polyanionic fucoidan chains with the cationic C-terminal region 115–129 of the myotoxins, forming complexes (Angulo and Lomonte, 2003). This region has been identified as the key structural determinant responsible for the membrane-damaging and myotoxic actions of the Lys49 PLA_2 homologs (Lomonte et al., 1994b; Núñez et al., 2001; Chioato et al., 2002; Ambrosio et al., 2005).

Experiments using a mouse model of myonecrosis induced by the venom of *Bothrops asper*, the most relevant snake species in Central America from a medical point of view, evidenced that the local administration of fucoidan immediately after intramuscular venom injection can reduce muscle damage by approximately 50%, in comparison to untreated controls (Angulo and Lomonte, 2003). Since crude fucoidan is heterogeneous in terms of polysaccharide chain lengths, with a relatively large average molecular weight, it was of interest to investigate if smaller fragments of this molecule would perform more efficiently in the neutralization of myotoxins, due to their higher diffusibility to tissues. The purpose of the present study was to obtain high- and low-molecular weight fractions of fucoidan, and to compare their neutralizing ability in a mouse model of myonecrosis induced by a purified myotoxin or the whole venom of *B. asper*.

2. Materials and methods

2.1. Fucoidan fractionation and hydrolysis

In order to purify a high-molecular weight fraction of fucoidan, a commercial preparation (Sigma-Aldrich) was fractionated by gel filtration chromatography on Sephadex G-100 (Pharmacia), using phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) as eluent, at 0.15 ml/min. Crude fucoidan aliquots of 100 mg were applied to a column (45 × 2.5 cm) and monitored at 280 nm. The presence of protein (which would interfere at this wavelength) in the initial crude fucoidan was ruled out by staining overloaded gels with Coomassie R-250 after electrophoresis (SDS-PAGE). The elution peak corresponding to the exclusion volume was collected and desalted by ultrafiltration using a membrane of 10 kDa nominal cut-off limit (Amicon). This final preparation of high-molecular weight fucoidan (F_H) was lyophilized and stored at −20 °C.

In order to generate low-molecular weight fragments, fucoidan was subjected to acid hydrolysis. The method described by Colliec et al. (1994) was followed, using 2 N sulfuric acid at 60 °C, at different incubation times (0, 1, 3, 6, and 24 h). Hydrolysis was stopped by addition of NaOH until pH was neutral. The extent of digestion at each time was evaluated by comparing the corresponding Sephadex G-100 chromatography profile with that of crude fucoidan, as described above. After selecting a hydrolysis time of 24 h, the low-molecular weight fucoidan preparation (F_L) was desalted by ultrafiltration using a membrane of