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Inhibitory effect of fucoidan on the activities of crotaline snake venom myotoxic phospholipases A_2

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Abstract

Myotoxic phospholipases A_2 account for most of the muscle necrosis that results from envenenomation by crotaline snakes. In this study, we investigated the protective effect of fucoidan, a natural sulfated polysaccharide obtained from the brown seaweed *Fucus vesiculosus*, against the cytotoxic and myotoxic activities of a group of phospholipase A_2 myotoxins from crotaline snake venoms: *Bothrops asper* myotoxins I, II, III, and IV, *Cerrophidion godmani* myotoxins I and II, *Atropoides nummifer* myotoxins I and II, and *Bothriechis schlegelii* myotoxin I. All of the toxins tested were efficiently inhibited by fucoidan, in both their cytotoxic and myotoxic effects. The basis for this inhibition appears to be the rapid formation of complexes between fucoidan and myotoxins, as evidenced by turbidimetric analysis. The possible binding site of fucoidan on the myotoxins was investigated using short synthetic peptides that represent the membrane-damaging region (residues 115–129) for three of these toxins. Fucoidan clearly inhibited the cytolytic activity of the peptides, indicating its ability to interact with the C-terminal myotoxic region of these phospholipases A_2. Fucoidan significantly inhibited muscle damage in mice, when administered locally, immediately after experimental envenomation with crude venom from *B. asper*. These results encourage further studies of sulfated fucans as compounds of potential use to improve the treatment of envenomations by crotaline snakes.

Keywords: Fucoidan; Myotoxin; Phospholipase A_2; Snake venom; Inhibition; Polysaccharide

1. Introduction

Phospholipases A_2 (PLA_2; EC 3.1.1.4) are ubiquitous enzymes that catalyze the hydrolysis of the *sn*-2 position of glycerophospholipids, leading to production of free fatty acids and lysophospholipids [1]. Although all PLA_2s catalyze essentially the same reaction, their biologic activities vary considerably. In snake venoms, PLA_2s have evolved into potent toxins with diverse specific activities such as neurotoxicity, myotoxicity, stimulation or inhibition of platelet aggregation, anticoagulant, hypotensive, cardiotoxic, edema-inducing [2], and bactericidal [3] effects.

Crotaline snakes (family Viperidae, subfamily Crotalinae) are widely distributed in America [4], comprising a large number of species which are responsible for the majority of snakebite envenomations in this continent [5]. Myotoxic PLA_2s have been described in the venoms of crotaline species from the genera *Bothrops*, *Agkistrodon*, *Porthidium*, *Trimeresurus*, *Atropoides*, *Crotalus*, *Bothriechis*, *Calloselasma*, and *Cerrophidion* [6–10], as components that have a prominent role in the induction of skeletal muscle necrosis. These PLA_2s are structurally classified into group IIA [11], belonging either to the enzymatically-active (Asp49-type) or enzymatically-inactive (Lys49-type) subgroups.

The clinical relevance of myonecrosis in snakebite envenomations has motivated the search for nonimmune neutralizing molecules against myotoxic PLA_2s, including natural inhibitors of animal and plant origins [12–16], that could complement conventional antivenom therapy. Among the different types of inhibitors studied, some anionic polysaccharides such as heparin have been shown to neutralize myotoxic PLA_2s isolated from *Bothrops jararacussu* [17] and *B. asper* [18,19] venoms. On this basis, we decided to investigate if fucoidan, a complex sulfated polysaccharide extracted from the brown seaweed *Fucus vesiculosus*, could inhibit the toxic activities of a group of myotoxic PLA_2s from crotaline venoms.

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Abbreviations: PLA_2, phospholipase A_2; CK, creatine kinase; LDH, lactic dehydrogenase.

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The structure of fucoidan has been characterized in detail. The core region of the fucan is composed primarily of a polymer of α1→3-linked fucose, with sulfate groups substituted at the 4 position on some of the fucose residues. Fucose is also attached to this polymer to form branch points, one for every 2–3 fucose residues within the chain [20,21]. This polysaccharide mediates a variety of biological effects in mammals, including leukocyte recruitment inhibition [22], revascularization of ischemic tissue [23], platelet aggregation [24], collagen contraction [25], and inhibition of smooth muscle cell proliferation [26]. Fucoidan interacts with several components of the coagulation and fibrinolysis systems, such as heparin cofactor II, antithrombin III, thrombin, glutamic plasminogen, tissue plasminogen activator and low molecular weight-urokinase [27,28], and exerts antitumor [29], as well as antimicrobial activities [30].

In this study, we report and characterize the inhibitory effect of fucoidan on the cytotoxic and myotoxic activities of different myotoxic PLA_2s isolated from crotaline snake venoms, and evaluate its potential to reduce myonecrosis when rapidly administered after the injection of *Bothrops asper* venom in a mouse model.

2. Materials and methods

2.1. Isolation of myotoxic PLA_2s

Each venom was a pool obtained from specimens collected in Costa Rica and kept at the serpentarium of the Instituto Clodomiro Picado. Myotoxic PLA_2s were purified by cation-exchange chromatography on carboxymethyl-Sephadex C-25 (Pharmacia, Sweden), as described: *B. asper* myotoxins I [31], II [32], III [33] and IV [34]; *C. godmani* myotoxins I and II [35]; *A. nummifer* myotoxins I [36] and II [37]; and *B. schlegelii* myotoxin I [38]. Toxin homogeneity was assessed by urea-PAGE for basic proteins [39], and reverse-phase high performance liquid chromatography (RP-HPLC) on a C4 column (25 mm × 4.6 mm; Vydac), eluted at 1.0 mL/min with a gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid (v/v), using an Agilent model 1100 HPLC system.

2.2. Inhibition of cytotoxic activity in vitro

Cytotoxic activity of the purified PLA_2s and its inhibition was assayed on murine C2C12 skeletal muscle myoblasts (ATCC CRL-1772) grown in 96-well plates, as described [40]. Toxins alone, or mixed with fucoidan (molecular mass 135 kDa; Sigma) at different molar ratios, were incubated for 30 min at room temperature. Then, aliquots of 150 μL (containing 30 μg of toxin in DME medium) were applied to the cultures, after aspirating their growth medium (DME with 15%, v/v fetal calf serum). After 3 hr of incubation at 37°, lactate dehydrogenase (LDH; EC 1.1.1.27) released to supernatants was determined with a colorimetric procedure (Sigma 500C). Reference controls for 0 and 100% cytotoxicity consisted of medium alone and medium containing 0.1% (v/v) Triton X-100, respectively. Additional controls consisted of cells incubated with fucoidan in the absence of toxins. Assays were carried out in triplicate.

2.3. Time-course of the inhibition of *B. asper* myotoxin II

In order to determine the time required for the inhibition of cytotoxicity by fucoidan, *B. asper* myotoxin II alone, or mixed with fucoidan at a 1:1 molar ratio, was incubated for 5, 10, 15, or 30 min, at room temperature. Then, cytotoxicity was quantified on the C2C12 cultures, as described above.

2.4. Inhibition of the cytotoxic activity of synthetic peptides of Lys49-PLA_2s

Three synthetic peptides corresponding to the C-terminal regions (sequence 115–129) of *B. asper* myotoxin II (KKYRYYLKPFCKK), *Agkistrodon piscivorus piscivorus* Lys49-PLA_2 (KKYKAYFKLKCKK), and *A. contortrix laticinctus* myotoxin (KKYKAYFKFKCKK), respectively, have been shown to exert direct cytotoxic activity upon C2C12 cultures, therefore mimicking the effects of their parent toxins [41,42]. These peptides, either alone (100 μg/150 μL) or mixed with fucoidan at a molar ratio of 0.25 (fucoidan/peptide), were incubated for 30 min at room temperature, and then applied to the C2C12 cultures to assay for cytotoxicity, as described above.

2.5. Inhibition of myotoxic activity in vivo

Myotoxic activity of the PLA_2s was estimated by determining the plasma levels of creatine kinase (CK; EC 2.7.3.2) in groups of four CD-1 mice (18–20 g body weight), after an intramuscular injection (in the gastrocnemius) of 75 μg of each toxin, either alone, or preincubated with fucoidan at a 1:1 molar ratio for 30 min at room temperature. Control groups received an identical injection (100 μL) of PBS, pH 7.2 alone, or fucoidan alone. In addition, inhibition of the myotoxic activity of whole *B. asper* venom by fucoidan was tested similarly, using a venom challenge dose of 50 μg. After 3 hr, blood samples were collected from the tail into heparinized capillary tubes, and the plasma CK activity was determined by a kinetic assay (Sigma CK-10). Activity was expressed as U/L (1 unit defined as the amount of enzyme which produces 1 μmol of NADH/min at 30°).

2.6. Inhibition of myotoxicity in vivo by locally administered fucoidan

Three groups of four mice received an intramuscular injection of crude *B. asper* venom (50 μg) in the gastrocnemius. In two of the groups, this was followed immediately by

an injection of fucoidan (90 or 270 μg) at the same site. Assuming that the venom contains 20% of myotoxins by weight, these fucoidan amounts would represent approximate molar ratios of 1:1 and 3:1 (fucoidan/myotoxin). Control animals received PBS alone, or fucoidan alone. Myonecrosis was estimated as described, 3 hr after venom injection. All *in vivo* experiments were approved by the University Committee on Animal Use and Care.

2.7. Phospholipase A_2 activity

PLA_2 activity of two Asp49-type myotoxins (*B. asper* myotoxins I and III) was determined using micellar egg yolk phospholipids (suspended in 0.1 M Tris–HCl, 0.01 M $CaCl_2$, pH 8.5) as substrate, in the presence of 1% (v/v) Triton X-100. Toxins (10 μg), either alone or preincubated with fucoidan at different molar ratios for 30 min at room temperature, were added to 1 mL of substrate. After an incubation of 15 min at 37°, the free fatty acids were extracted and titrated with 0.018 M NaOH, as described by Dole [43]. Controls consisted of substrate with PBS, or substrate with fucoidan. Assays were carried out in triplicate.

2.8. Formation of macromolecular complexes between fucoidan and B. asper myotoxins

Complex formation between fucoidan and *B. asper* myotoxins I–IV was assessed by turbidimetry, as previously described [19]. Two-hundred μg of each toxin were added to 2 mL of Tris–KCl 0.01 M, pH 7.0, followed by consecutive additions of 1 μL of fucoidan (20 mg/mL) to give different final ratios of fucoidan/myotoxin. After each addition, the mixtures were incubated for 1 min before reading the absorbance at 340 nm.

2.9. Competition binding enzyme-immunoassay

Competition between fucoidan and rabbit antibodies against the C-terminal region 115–129 of myotoxic PLA_2s [44], for simultaneous binding to *B. asper* myotoxin II, was evaluated using an enzyme-immunoassay. Microplates (Dynatech Laboratories) were coated with *B. asper* myotoxin II at 0.4 μg per well, by overnight incubation in 0.1 M Tris 0.15 M NaCl, pH 9.0 buffer. After five washing with solution A (0.05 M Tris, 0.15 M NaCl, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4), varying dilutions of the rabbit serum to region 115–129 were added to triplicate wells, diluted in solution A containing 2% (w/v) BSA, and incubated at 4° for 24 hr. A parallel set of rabbit serum dilutions was incubated in the presence of 500 μg per well of fucoidan. After five washings with solution A, bound antibodies were detected with an antirabbit inmunoglobulin–alkaline phosphatase conjugate (Sigma), diluted 1:2000 in solution A-BSA, and incubated for 1 hr. After washing as described, color was developed with *p*-nitrophenylphosphate, and absorbances were recorded on a microplate reader (Labsystems Multiskan RC) at 405 nm. Normal rabbit serum was utilized as a negative control.

3. Results

Fucoidan inhibited the cytotoxic activity of all the myotoxic PLA_2s tested, although with some quantitative variations among them. *B. asper* myotoxins II and IV, *A. nummifer* myotoxins I and II, *C. godmani* myotoxins I and II, and *B. schlegelii* myotoxin I were completely inhibited by preincubation with fucoidan, whereas the activity of *B. asper* myotoxins I and III was reduced by 50–65%, at a 1:1 molar ratio (Fig. 1). Toxin inhibition, as evaluated by LDH release, was consistent with microscopic observations of cell culture morphology. Fucoidan alone, at the maximal concentrations utilized in inhibition experiments, did not alter cell morphology, and did not cause LDH release.

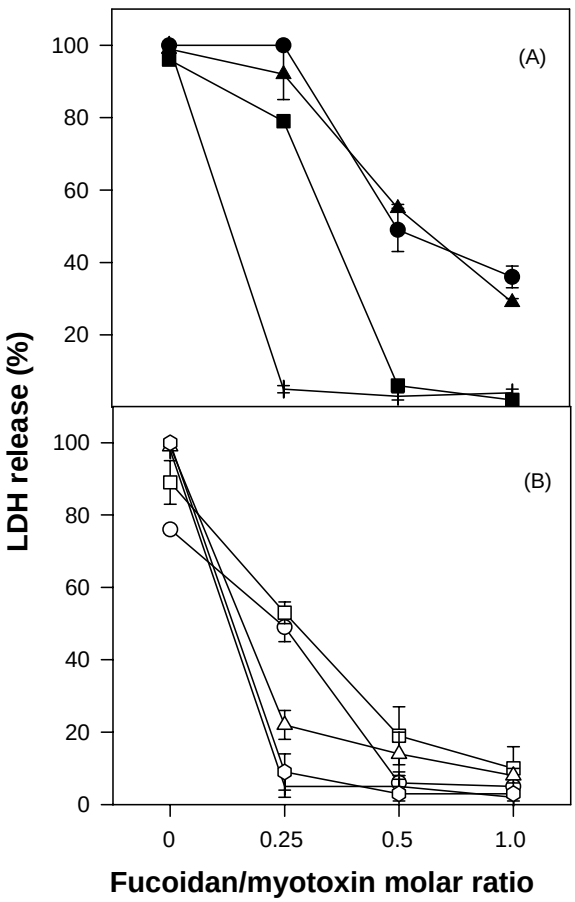

Fig. 1. Inhibition of the *in vitro* cytotoxic activity of myotoxic phospholipases A_2 by fucoidan. Cytotoxicity was determined by the release of lactate dehydrogenase (LDH) from C2C12 skeletal muscle myoblasts, 3 hr after exposure to myotoxins (30 μg), either alone or preincubated with fucoidan at the indicated molar ratios, as described in Section 2. (A) *B. asper* myotoxins I (●), II (■), III (▲), and IV (◆). (B) *C. godmani* myotoxins I (○) and II (□), *A. nummifer* myotoxins I (Δ) and II (◇), and *B. schlegelii* myotoxin I (⬡). Each point represents the mean ± SD of triplicate cultures.

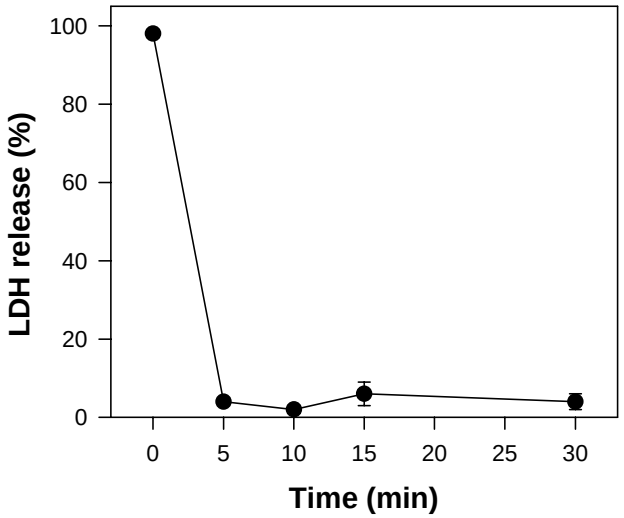

Fig. 2. Time-course of the inhibition of the *in vitro* cytotoxic activity of *B. asper* myotoxin II by fucoidan. Cytotoxicity was determined by the release of lactate dehydrogenase (LDH) from C2C12 cells, 3 hr after exposure to myotoxin II (30 μg) alone (time 0) or preincubated with fucoidan at a 1:1 molar ratio, for the indicated periods of time. Each point represents mean ± SD of triplicate cultures.

Fucoidan pretreatment of the cells, followed by washing with medium, and subsequent exposure to the toxins, did not inhibit their cytolytic activity (data not shown).

B. asper myotoxin II was selected to study the time-course of the inhibitory effect of fucoidan upon its *in vitro* cytotoxic activity. As shown in Fig. 2, the action of fucoidan was very rapid, with a complete toxin inhibition achieved within only 5 min of incubation at room temperature, before the application of the mixture to cell cultures. These results are in agreement with the rapid formation of macromolecular complexes between fucoidan and *B. asper* myotoxins I, II, III, and IV, as observed by turbidimetry assays. Addition of fucoidan to these toxins in solution resulted in a rapid increase of turbidity at 340 nm, indicating the formation of insoluble complexes (Fig. 3). These complexes caused maximal turbidity at a molar ratio of approximately 0.1:1 (fucoidan/myotoxin), and subsequently redissolved by the gradual addition of a polysaccharide excess (Fig. 3).

The possible interaction site of fucoidan on the myotoxic PLA_2s was investigated by a solid-phase competition binding assay, and by the use of cytolytic synthetic peptides. In the former test, the presence of fucoidan caused a significant reduction in the binding of rabbit antibodies towards the C-terminal region 115–129 of *B. asper* myotoxin II, to immobilized myotoxin II (Fig. 4). In addition, cytotoxicity experiments showed that the activity of three cytolytic synthetic peptides, corresponding to the region 115–129 of different myotoxic PLA_2s, was completely inhibited by their preincubation with fucoidan (Fig. 5).

Using the two Asp49-type PLA_2s from *B. asper* (myotoxins I and III), the possible inhibition of their catalytic activity by fucoidan was investigated. When fucoidan was

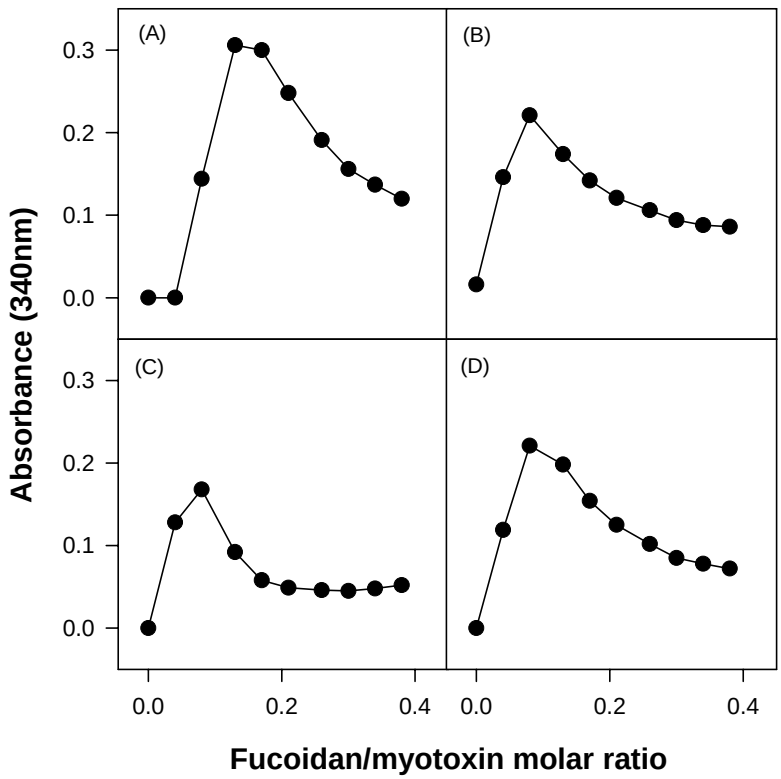

Fig. 3. Formation of macromolecular complexes between fucoidan and *B. asper* myotoxins. Two-hundred μg of *B. asper* myotoxins I (A), II (B), III (C) or IV (D) were added to 2 mL of Tris–KCl 0.01 M, pH 7.0, followed by consecutive additions of 1 μL of fucoidan (20 mg/mL) to give the indicated final ratios of fucoidan/myotoxin. After each addition the mixtures were incubated for 1 min before reading the absorbance at 340 nm.

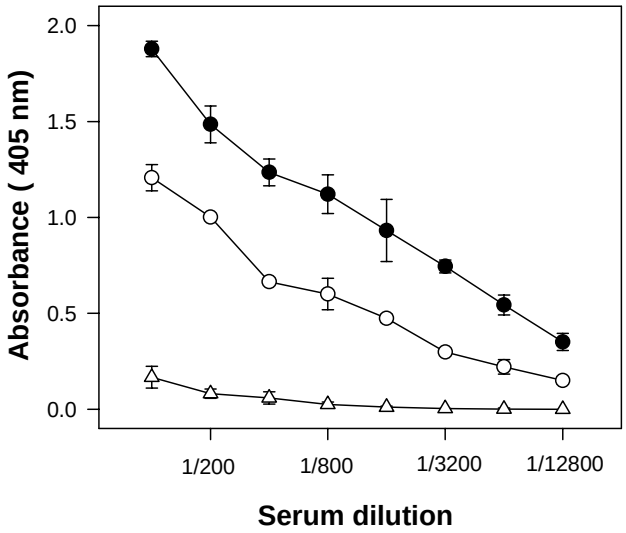

Fig. 4. Competition between antibodies to peptide 115–129 and fucoidan for binding to immobilized *B. asper* myotoxin II. Binding of rabbit antibodies raised against the C-terminal region of *B. asper* myotoxin II (115–129) was tested by enzyme-immunoassay, using microplates coated with myotoxin II, as described in Section 2. Varying dilutions of the rabbit immune serum were tested either alone (●) or in the presence of 500 μg per well of fucoidan (○). Normal rabbit serum (Δ) was included as a negative control. Each point represents the mean ± SD of triplicate wells.

incubated with these toxins, up to a molar ratio of 2:1 (fucoidan/myotoxin), their PLA_2 activity was identical to that of the control toxins incubated without fucoidan, evidencing that this enzymatic activity was not inhibited by fucoidan (data not shown).

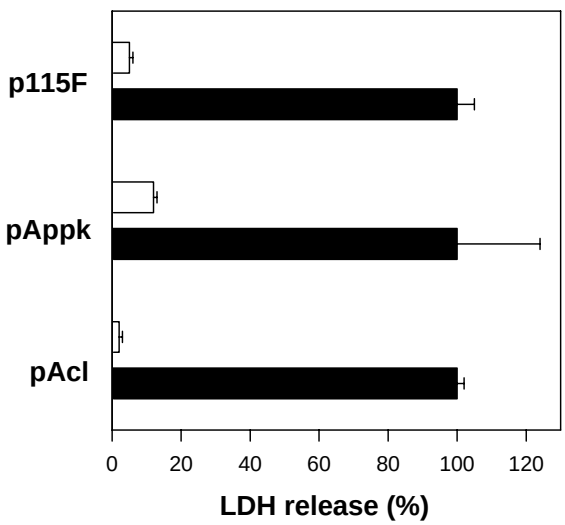

Fig. 5. Inhibition of the *in vitro* cytotoxic activity of synthetic peptides 115–129 of myotoxic phospholipases A_2 by fucoidan. Cytotoxicity was determined by the release of lactate dehydrogenase (LDH) from C2C12 cells, 3 hr after exposure to synthetic peptides (100 μg) representing the C-terminal region 115–129 of *B. asper* myotoxin II (p115F), *Agkistrodon piscivorus piscivorus* Lys49 phospholipase A_2 (pAppk), and *A. contortrix laticinctus* myotoxin (pAcl), either alone (filled bars) or preincubated with fucoidan (empty bars) at a 0.25 molar ratio (fucoidan/peptide). Peptide sequences are described in Section 2. Each bar represents the mean ± SD of triplicate cultures.

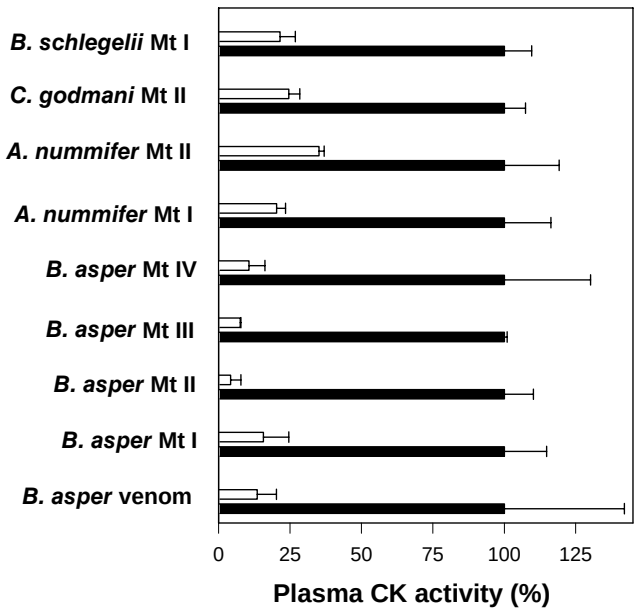

Fig. 6. Inhibition of the *in vivo* myotoxic activity of phospholipases A_2 and crude *B. asper* venom by fucoidan. Myotoxins (75 μg) or crude *B. asper* venom (50 μg) were injected intramuscularly, either alone (filled bars) or preincubated with fucoidan (empty bars) at a 1:1 molar ratio, as described in Section 2. After 3 hr, plasma creatine kinase (CK) levels were determined. Values are expressed as a percentage of the CK activity resulting from the injection of each toxin (or venom) alone. One-hundred percent values varied from 2105 to 4223 U/L. The plasma CK values of mice receiving a PBS injection alone were subtracted in all cases. Each bar represents the mean ± SD of four animals.

The inhibition of myotoxic PLA_2s by fucoidan was subsequently examined *in vivo*, by estimating skeletal muscle damage through the increase of plasma CK levels in mice. Data summarized in Fig. 6 show a clear reduction

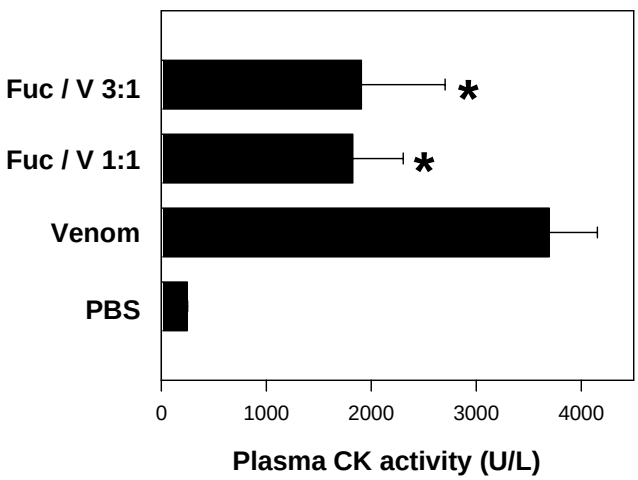

Fig. 7. Inhibition of the *in vivo* myotoxic activity of crude *B. asper* venom by independent local injection of fucoidan. Venom (50 μg) was injected intramuscularly into three groups of mice, two of which received immediately an injection of fucoidan (90 μg, Fuc/V 1:1; or 270 μg, Fuc/V 3:1) at the same site. A control group received an injection of PBS, pH 7.2 alone. After 3 hr, plasma creatine kinase (CK) levels were determined. Bars represent the mean ± SD of four animals. The values corresponding to Fuc/V 1:1 and Fuc/V 3:1 are significantly lower ($P < 0.05$) than those of the venom group.

in the plasma CK activity induced by all toxins tested, after their incubation with fucoidan at a 1:1 molar ratio. Although there are minor quantitative differences between toxins, inhibitions varied between 70 and 95% (Fig. 6). Accordingly, fucoidan also inhibited the myotoxic action of crude *B. asper* venom, in these preincubation experiments (Fig. 6). Injection of fucoidan alone did not increase plasma CK values above those of control animals receiving a PBS injection (data not shown).

Finally, the protective effect of fucoidan was evaluated in independent administration tests, in which the polysaccharide was injected locally, immediately after an intramuscular challenge with *B. asper* venom in mice. Fucoidan administration significantly reduced the extent of myonecrosis, resulting in CK values that corresponded to approximately 50% of those of the control group receiving venom alone (Fig. 7). A 3-fold increase in the dose of fucoidan administered *in situ* (from 90 to 270 μg) did not improve the level of protection (Fig. 7).

4. Discussion

Within the last decade, a growing number of inhibitors of snake venom myotoxic PLA_2s have been reported, ranging from animal plasma proteins [12–14] and polysaccharides [17–19], to plant components [15,45]. Together with the gradual advances in our understanding of the molecular determinants and mechanisms of action involved in the toxic effects of myotoxins, the search for efficient inhibitors might lead to a significant improvement of snakebite treatment, in addition to conventional antivenom therapy, in the future.

The discovery of enzymatically-inactive Lys49 PLA_2 variants in the venoms of crotalines [46,47] prompted the search for a toxic site in these proteins, not necessarily related to the known catalytic site of PLA_2s. Current evidence indicates that the C-terminal region of Lys49 variants, combining cationic and hydrophobic amino acids, constitutes a key determinant for their myotoxic activity [19,41,44,48,49]. Thus, polyanionic molecules with the ability to bind to this toxic site constitute rational candidates for evaluation of myotoxin inhibitory activity.

The present study focused on fucoidan, a complex sulfated polysaccharide extracted from marine algae. Although sharing some biological properties with heparin, sulfated fucans constitute alternative polyanionic molecules with a range of different pharmacological profiles [50]. Using a group of myotoxic PLA_2s purified from different crotaline venoms, it was demonstrated that fucoidan efficiently inhibits both their cytotoxic (*in vitro*) and myotoxic (*in vivo*) effects. Moreover, in the case of two catalytically-active (Asp49) PLA_2s, fucoidan inhibited their toxic actions without affecting their ability to hydrolyze phospholipids. This result further exemplifies the dissociation between toxicity and catalysis in the family of class II Asp49-type myotoxins, reported in a number of previous studies [7,18,51,52].

The inhibition mechanism of fucoidan against myotoxic PLA_2s appears to be the rapid formation of complexes, probably mediated through multivalent, electrostatic interactions between the anionic sulfates of the polysaccharide and the numerous cationic residues of the toxins, which have highly basic isoelectric points. Reversible complex formation was evidenced by turbidimetry measurements, where the light scattering properties of the fucoidan/myotoxin mixtures varied according to the relative proportions of these two interacting components, resulting in typical bell-shaped absorbance curves.

The possible binding site of fucoidan on the myotoxins was investigated using short synthetic peptides that represent the membrane-damaging region (residues 115–129) for three of toxins [42]. Fucoidan clearly inhibited the cytolytic activity of the three synthetic peptides, demonstrating its ability to interact with the C-terminal myotoxic region of these phospholipases A_2. Further support to this conclusion was provided by competition binding data using antibodies raised against the synthetic peptide 115–129 of *B. asper* myotoxin II [44]. Binding of these antibodies to the immobilized toxin was reduced in the presence of fucoidan. The region 115–129 of Lys49 myotoxic PLA_2s, such as *B. asper* myotoxin II, was previously shown to constitute a heparin-binding site, and to be involved in its cytolytic action *in vitro* [19,41]. In this regard, fucoidan appears to act in a similar mode as heparin [19], in agreement with their common polyanionic nature, provided by a high density of sulfate substituents. However, it should be noted that the density of negative charges on a polysaccharide backbone is not the only factor determining its ability to interact with the myotoxic PLA_2s. For example, the binding of chondroitin sulfate and dermatan sulfate to *B. asper* myotoxin II is weaker than the binding of heparan sulfate, although the latter is less sulfated [19], highlighting the contribution of the type of polysaccharide backbone upon an optimal interaction with proteins. Therefore, it was of interest to characterize and evaluate the inhibitory activities of fucoidan against myotoxic PLA_2s.

Using the crude venom of *B. asper*, the ability of fucoidan to inhibit muscle damage when administered locally, immediately after envenomation, was evaluated in mice. Under these conditions, fucoidan injection partially decreased the myonecrosis induced by the venom, to a level of approximately 50% of that of untreated animals. Thus, its efficiency is clearly limited in experiments that resemble the actual situation of a snakebite, although a 50% reduction in muscle damage is still a considerable benefit. A major limitation for the development of any clinically effective inhibitor for myotoxins is the dramatic rapidity by which these proteins affect skeletal muscle, as recorded by intravital miscroscopy techniques [53]. The high molecular weight of the fucoidan (135 kDa) may also

be a factor that could limit its distribution and diffusion in the tissue. Further characterization of fucoidan fragments of lower mass, or of different types of sulfated fucans obtained from a variety of natural sources [50] might be of interest in the search for a clinically useful, optimal inhibitor of myotoxic PLA_2s from snake venoms.

Acknowledgments

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