Myonecrosis induced in rat by a myotoxin isolated from the venom of *Bothrops neuwiedi* from Alfenas, MG

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ABSTRACT: *Bothrops* venoms are composed by several protein fractions. Among these fractions there are myotoxins which induce an important muscle lesion. The purification of this component involves some steps, although providing a pure material, is time consuming. In the present study, we describe a quick method for myotoxin fraction isolation from the venom of *Bothrops neuwiedi* using one-step high performance liquid chromatography (HPLC). The complete procedure took 30 min. The pur6+y of the two myotoxic fractions isolated was assessed by SDS-PAGE. Upon i.m. injection into rat tibialis anterior, both toxins induced early morphological changes, indicating that the plasma membrane was the first cellular structure affected. Afterwards, the lesion was typically myonecrotic and inflammatory infiltrate was present.

Key words: *Bothrops neuwiedi*, HPLC, myonecrosis, myotoxin

RESUMO: Os venenos de *Bothrops* são compostos por várias frações protéicas. Dentre elas, as miotoxinas induzem lesões musculares importantes. A purificação deste componente envolve várias etapas e, embora forneça frações puras, é muito trabalhosa. Neste trabalho foi descrito um método rápido para o isolamento da fração miotóxica do veneno de *Bothrops neuwiedi* (jararaca pintada), utilizando-se uma etapa única de purificação por Cromatografia Líquida de Alta Eficiência (CLAE). O procedimento completo demorou apenas 30 min. Foram isoladas duas frações com atividade miotóxica, cuja pureza foi verificada por eletroforese em gel de poliacrilamida-SDS. A injeção intramuscular no músculo tibial anterior de ratos induziu alterações morfológicas precoces, indicando que a membrana plasmática foi a primeira estrutura celular afetada pela fração miotóxica. A lesão muscular obtida foi tipicamente mionecrótica e foi observado infiltrado inflamatório.

Unitermos: *Bothrops neuwiedi*, CLAE, mionecrose, miotoxina

INTRODUCTION

Snake bite envenomation produces a wide range of local and systemic symptoms in animals and humans. Muscle-damaging toxins (myotoxins) are present in a variety of *Bothrops* venoms, producing prominent local tissue damage which may lead to tissue loss, disability and amputation (Nishioka and Silveira, 1992). During the last past years there has been a growing interest in the study of myotoxins, as they could induce muscle necrosis and show other pharmacological properties, such as neurotoxic, cardiotoxic, anticoagulant and produce inflammatory effects (Diaz et al., 1991).

Several myotoxins have been isolated from *Bothrops* venoms. Some of them have basic phospholipase A_2 activity but there is a group of *Bothrops* myotoxins which lack or has extremely

low enzymatic activity because they are a lysine-49 phospholipase A_2 (Diaz et al., 1991, 1992).

Myotoxicity does not apparently depend on enzymatic phospholipid degradation. Instead a membrane-disorganizing activity, Ca^{+2} independent and does not involving a catalytic activity, has been suggested (De Azevedo et al., 1999). On the other hand the anticoagulant effect of some toxins seems to depend on their phospholipase A_2 activity (Calderon and Lomonte, 1999).

Almost all *Bothrops* toxins with a skeletal muscle damaging activity are basic proteins (Homsi-Brandengurgo et al., 1988; Moura-da-Silva et al., 1990; Cintra et al., 1993; Mancuso et al., 1995) and their purification procedures include gel filtration followed by cation-exchange chromatography in order to separate myotoxins from acidic venom components. These methods yield pure fractions although they are time consuming. Some attempts have been focused on less time consuming procedures for myotoxins isolation (Toyama et al., 1995; Spencer et al., 1998). In this work we report a quick procedure for *Bothrops neuwiedi* myotoxin isolation as well as some data on the pathogenesis of skeletal muscle damage after injection of this toxin in rat.

MATERIALS AND METHODS

Venom. A pool of venoms obtained from more than 10 specimens of *Bothrops neuwiedi* from the University of Alfenas serpentarium was used in this work. The venom was desiccated and kept at 4 °C.

Isolation of myotoxins. Myotoxic proteins were purified from the *Bothrops neuwiedi* whole venom by reverse-phase high performance liquid chromatography (HPLC) according to Toyama et al. (1995) with few modifications. Samples (2 mg) of the desiccated whole venom were dissolved in 500 mL of 0.1% (w/V) trichloroacetic acid (TCA) and centrifuged at 1,000 g at 4 °C for 10 min. The supernatant was collected and loaded on a Bondapack C-18 column equilibrated with TCA. Elution was carried out with a linear acetonitrile gradient from 0% to 60% V/V in 0.1% (w/V) TCA, at a flow rate of 2.0 mL/min. Absorbances were monitored at 280 nm. Active fractions were dialyzed against distilled water, desiccated and stored at 4 °C.

Phospholipase A_2 activity. Isolated fractions were tested for phospholipase A_2 activity by potenciometric tritation, using egg yolk as substrate diluted (1:5) with 0.005 M Tris, 10 mM $CaCl_2$, pH 8.5, containing 1% (V/V) Triton X-100. One enzymatic unit of phospholipase A_2 is defined as the amount (mg) of enzyme that releases 1 mmol of fatty acid per minute at 37 °C.

Protein electrophoresis. Crude venom and myotoxic fractions were analysed by SDS-PAGE (Laemmli, 1970). Samples were diluted in sample buffer, heated at 100 °C for 2 min and run at 30 mA for 1 h in a 12% (w/V) acrylamide slab gel. Gels were silver stained.

Myotoxic activity. Groups of four adult male Wistar rats (270 ± 30 g body weight) from Universidade de Alfenas were injected intramuscularly in the right tibialis anterior with 50 mg of protein purified by HPLC dissolved in 100 mL of physiologic saline solution. Rats were sacrificed 3 or 24 h after injection. Injected muscles were removed and processed for paraffin wax embedding. Paraffin sections (5 mm thick) were mounted on slides and stained with hematoxilin-eosin. Controls received 100 mL of physiologic saline solution under the same conditions as myotoxin-injected animals.

RESULTS

Isolation and properties of myotoxin. The chromatographic separation of *B. neuwiedi* venom showed several protein fractions (Figure 1). The fractions with retention time corresponding to 29% and 37% of acetonitrile, respectively peaks 1 and 2, showed myotoxic activity. SDS-PAGE (Figure 2) showed that the whole venom of *B. neuwiedi* presented several protein bands. The

HPLC-purified protein fractions applied to the gel resulted in a single band with a molecular mass of 14,000 Da for the peak 1-protein and 15,750 Da for the protein corresponding to HPLC-peak 2, so the appearance of a single protein band on SDS-PAGE demonstrated its purity. Both myotoxic protein fractions were devoid of phospholipolitic activity, at least on the conventional egg-yolk substrate.

Myotoxic activity. Macroscopically, few minutes after myotoxin injection, rats had difficulty in moving their right hind leg. Histologically, muscle that received physiologic saline solution had the typical morphologic pattern of skeletal muscle. Muscles analysed 24 h after the injection of myotoxin showed a widespread myonecrosis, including cells in a different stage of damage, with areas with dense clumps of myofibrils and spaces apparently devoid of myofibrilar material (Figure 3). The myofibrillar material in necrotic cells was more hyaline and its distribution within the cellular space was more homogenous, instead of being in dense, clumped masses. An abundant inflammatory infiltrate was also observed.

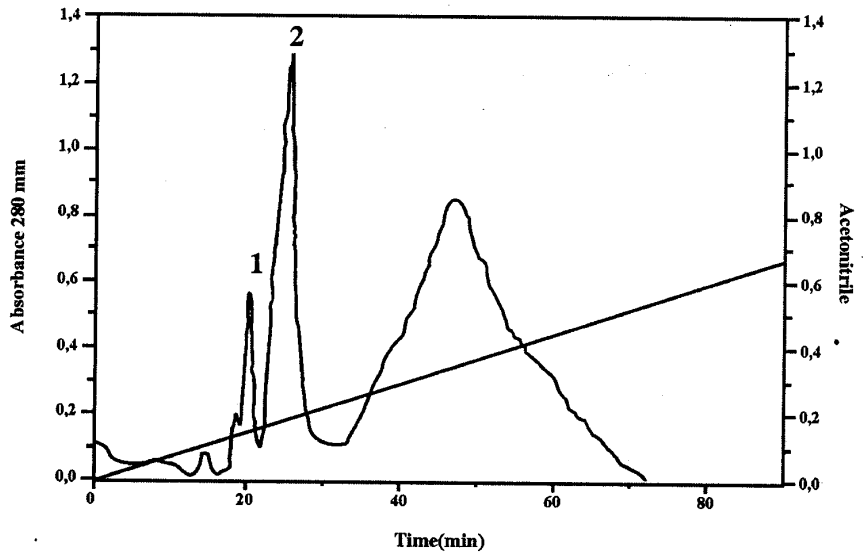

FIGURE 1. Chromatographic profile of *Bothrops neuwiedi* snake whole venom from reverse-phase HPLC. Proteins corresponding to peaks 1 and 2 showed the presence of myotoxic activity.

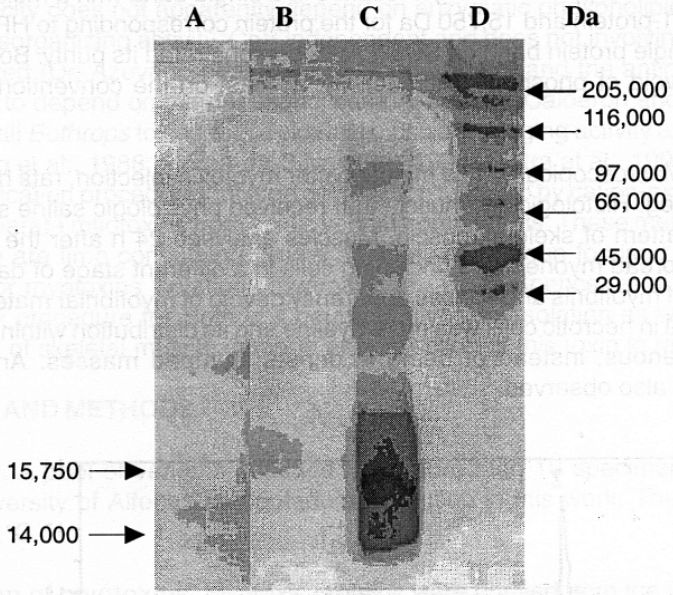

FIGURE 2. Electrophoretic pattern (SDS-PAGE) of the purified myotoxin under reducing conditions. Ten ng of protein were applied on a 12% (w/V) acrylamide slab gel and the running was performed at 30 mA, for 1 h. Lane A: myotoxic fraction purified by reverse-phase HPLC, corresponding to peak 1; Lane B: myotoxic fraction purified by reverse-phase HPLC, corresponding to peak 2; Lane C: *Bothrops neuwiedi* whole venom; Lane D: molecular mass markers (Da).

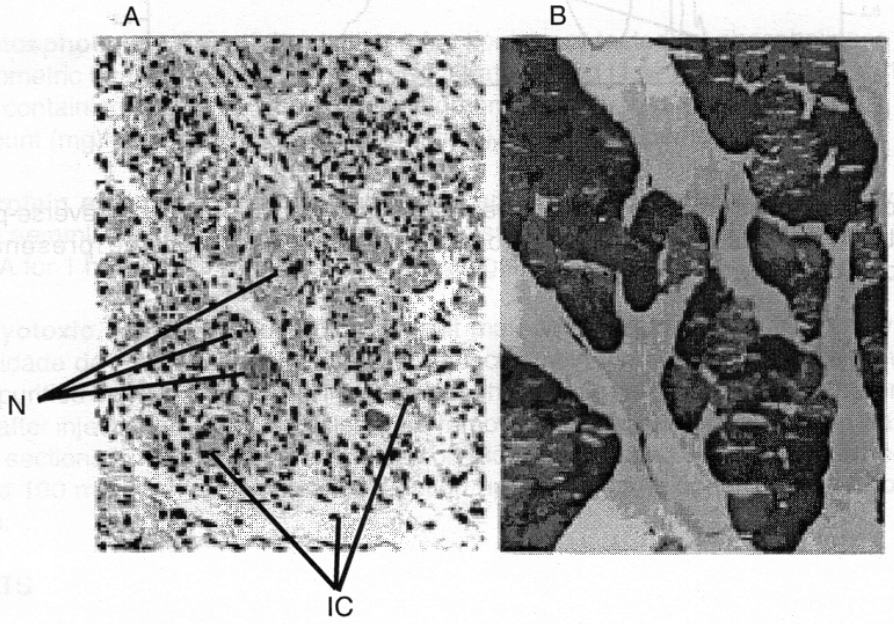

FIGURE 3. Cross-section of right rat tibialis anterior muscle 24 h after injection of 50 mg *Bothrops neuwiedi* myotoxin in 100 mL saline (A), or of 100 mL physiologic saline solution (B). Notice in (A) different stages of muscle fiber necrosis (N) and inflammatory cells (IC).

DISCUSSION

Myotoxins or myonecrotic toxins are a group of venom proteins that damage specifically skeletal muscle, affecting muscle fiber, without causing connective tissue and nerve lesion. These proteins are closely related structurally to phospholipase A_2 (PLA2), although they not necessarily present PLA2 activity (Fletcher et al., 1997). These proteins usually have 120-140 amino acid residues and 6-8 disulfide bridges and are typical components of South-American bothropic venoms (Homsi-Brandenburgo et al., 1988).

The myotoxic activity of the isolated HPLC-fractions was tested histologically. The myonecrosis pattern observed after the myotoxin injection was similar to the pattern previously described for close related species (Mancuso et al., 1995; Gutiérrez et al., 1984, 1991) or not, such as *Agkistrodon contortrix laticinctus* (Morini et al., 1998; Salvini et al., 1999). The muscle fiber damage, 24 h after the myotoxin injection, was characterized by clumps of myofibrils, fiber disorganization and disintegration and inflammatory cells, specially leukocytes. The inflammatory response demonstrated in this study is similar to the response described for *Bothrops asper* crude venom injection in muscle (Lomonte et al., 1994).

Myonecrosis induced by the isolated fractions is apparently independent of PLA2 activity, since in the present work, we could detect no PLA2 activity for both myotoxic protein fractions isolated from *B. neuwiedi* venom. This was not surprising, since there are some reports on the bothropstoxin lack of PLA2 activity (Cintra et al., 1993; Toyama et al., 1995; Diaz et al., 1995; Fletcher and Jiang, 1998). Myotoxic proteins of low molecular mass such as crotamine (Gonçalves and Polson, 1974; Giglio, 1975), myotoxin A (Cameron and Tu, 1978), toxins isolated from *Crotalus viridis concolor* venom (Engle et al., 1983) and bothropstoxin-1 (Homsi-Brandenburgo et al., 1988) also lack PLA2 activity.

The chromatographic profile of *B. neuwiedi* venom was compatible with the profile previously described for *Bothrops* species (Mancuso et al., 1995; Toyama et al., 1995). Using HPLC procedure we isolated two myotoxic fractions in a single-step purification and the complete procedure took 30 min. The proposed method reduced drastically the purification time when compared to the method described by Homsi-Brandeburgo et al. (1988). Their purification protocol involved gel filtration on Sephadex G-75 followed by cation exchange chromatography using SP Sephadex C-25. Although this method yielded a pure product, it was quite time consuming. Similar methods (Cintra et al., 1993; Mancuso et al., 1995) have been described to purify bothropstoxin, but although the high amount of purified protein obtained, these methods are quite complex. Spencer et al. (1998) described a single-step method for purification of bothropstoxin-1 using Resource-S cation exchange column connected to a FPLC system. Their procedure was shorter than the previously reported works, but the amount of protein purified was low and dialysis or desalting was required after purification. In the present work myotoxic fraction proteins purified, although low in amount, were pure, and the entire procedure took only 30 min. We believe that this method may be useful for small-scale purifications, but it could be readly scaled up for larger amounts of material. As we started with a small amount of whole venom, a relatively small amount of purified protein could be obtained. An improvement of this method could be done using larger amounts of whole venom.

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