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A high molecular weight protein Bengalin from the Indian black scorpion (*Heterometrus bengalensis* C.L. Koch) venom having antiosteoporosis activity in female albino rats

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ABSTRACT

This study reports the presence of a high molecular weight protein (Bengalin) from the Indian black scorpion (*Heterometrus bengalensis*) venom having antiosteoporosis activity in experimental osteoporosis developed in female albino Wister rats. Bengalin was purified through DEAE-cellulose ion exchange chromatography and high performance liquid chromatography. The molecular weight of the Bengalin was found to be 72 kDa and the first 20 amino acid sequence was found to be G-P-L-T-I-L-H-I-N-D-V-H-A-A/R-F-E-Q/G-F/G-N-T. Bengalin exhibited significant antiosteoporosis activity in experimental female rats, which was confirmed through analysis of urine Ca^{2+}, PO_4^{3-}, CRE & OH-P. Bengalin (3 μg and 5 μg/100 g rat/i.p.) antagonized osteoporosis by restoring urinary Ca^{2+}, PO_4^{3-}, CRE and OH-P, serum/plasma Ca^{2+}, PO_4^{3-}, ALP, TRAP, PTH, T_3, TSH, Osteocalcin, IL1, IL6 and TNF α and bone minerals Ca^{2+}, P, Mg^{2+}, Zn^{2+}, Na^{+}, as compared with the sham operated control rats. Bone minerals density of osteoporosis female rats was improved due to Bengalin, observed through DEXA scan. Subacute toxicity studies in male albino mice, Bengalin showed cardiotoxicity. In vivo experiments, Bengalin showed cardiotoxicity on isolated guinea pig heart, guinea pig auricle, and neurotoxicity on isolated rat phrenic nerve diaphragm preparation. Further detail studies on the toxicity, antiosteoporosis and structural identity of Bengalin are warranted.

1. Introduction

Osteoporosis is a worldwide socio medical problem, with a high prevalence not only in the western countries but also in Asia and Latin America (Delmas, 2002). In United States, forty four million men and women aged fifty years and older have a low bone mass and osteoporosis (Follin and Hansen, 2003). Conventional therapy recommended for the treatment of osteoporosis includes supplementation with estrogen, progesterone, calcitonin, bisphosphonates etc. that possesses several shortcomings including cancer in breast or uterus. Selective estrogen receptor modulators such as raloxifene, bisphosphonates are used for beneficial effects on bone mineral density

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; Condyle L, condyle left; Condyle R, condyle right; CRE, creatinine; DEXA, dual energy X-ray absorptiometry; EDTA, ethylene diamine tetraacetic acid; EST, estrogen; FSH, follicle stimulating hormone; L1, lumber-1; L2, lumber-2; L3, lumber-3; L4, Lumber-4; Neck L, neck-left; Neck R, neck right; OH-P, hydroxy proline; OPG, osteoprotegerin; OST, osteoporosis; PTH, parathyroid hormone; RANK, receptor activator of nuclear factor kappa β; RANKL, receptor activator of nuclear factor kappa β ligand; SV, scorpion venom; T12, thoracic-12; TNF α, tumor necrosis factor alpha; TRAP, tartrate-resistant acid phosphatase; Troch L, touchier left; TRIS, tri hydroxy amino methane.

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(Goldstein et al., 2000) but serious side effects were reported (Gorman et al., 2002). The current treatment follows with recombinant PTH (teriparatide), strontium ranelate therapy.

Scorpion envenomation, alter several hormone level including estrogen, prostaglandin etc in clinical (Ben Nasr et al., 2007) and in experimental condition (Nassar et al., 1990). Scorpion venom also alters the serum mineral constituents like calcium, phosphate, potassium (Omran and Abdel-Rahman, 1992). Such alteration in blood mineral may affect bone mineral composition. American dental association reported that a compound (kaliotoxin) found in the venom of the scorpion *Androctonus mauretanicus*, could significantly inhibit the bone loss resulting from advanced periodontal disease (Valverde et al., 2004). Mercer et al. (1998) reported that contortrostatin, a homodimeric snake venom disintegrin (a small disulfide-rich protein containing an Arg–Gly–Asp sequence near their carboxyl terminus), is a potent inhibitor of β3 integrin-mediated osteoclast attachment. Sato et al. (1990) worked out with the snake venom protein Echistrin derived from saw scale viper and was found to be a potent inhibitor of bone resorption on isolated osteoclast. Oursler and Spelsberg (1993) made an extensive review on the use of Echistatin as a potential drug for osteoporosis.

Attempts have been taken to identify newer therapeutic agent active against osteoporosis from natural resources. Gomes et al. (2009) reported for the first time that the venom of the Indian black scorpion (*Heterometrus bengalensis*) possessed antiosteoporosis activity in experimental female albino rats. The present communication identified the presence of a high molecular weight protein (Bengalin) from the *H. bengalensis* venom and established its antiosteoporosis activity in female albino rats.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents used were of analytical grade unless otherwise stated. And following kits were used calcium, phosphorous, magnesium, creatinine (Merck, India), osteocalcin (Biosource, Belgium), PTH (Biomerica, Germany), rat IL1, rat IL6 and rat TNF α kit (R & D, USA).

2.2. Purification and characterization of antiosteoporosis factor (Bengalin)

2.2.1. Collection of scorpion and scorpion venom

Adult live scorpions (*H. bengalensis*) of both sexes were collected and scorpion venom (SV) was extracted once in a month by applying square wave electrical stimulation (25 V, 1 ms) to the telson. The venom was pooled, lyophilized and stored at 4 °C in amber colour bottle until further use. Before use, SV was weighed, dissolved in phosphate buffer saline 0.01 M, pH 7.2 and expressed in terms of dry weight.

2.2.2. DEAE-cellulose ion exchange chromatography

Lyophilized scorpion (*H. bengalensis*) venom (200 mg) was dissolved in phosphate buffer (pH 7.2) over night and centrifuged (15 min × 1800 g). The supernatant was subjected to DEAE-cellulose column (20 × 80 mm). The flow rate was adjusted at 25 ml/h. Phosphate buffer (0.02 M, pH 7.2) containing increasing molarities of NaCl (0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 M) was used to elute the proteins from the column. Fractions (5 ml) were collected at room temperature (24 ± 2 °C) and protein levels were estimated (Lowry et al., 1951). The antiosteoporosis activity of the fractions were examined in female albino osteoporosis rats.

2.2.3. High performance liquid chromatography

DEAE-cellulose purified venom protein (P3) was further purified through HPLC (WATERS 600, USA). Protein-Pak 300 SW column (7.5 × 300 mm) and 2487 γ Absorbance detector, using 10 mM sodium phosphate buffer containing 0.1 M sodium chloride (pH 7.5) with a flow rate 1 ml/min. Detection of protein peak was done at 280 nm and the retention time of the protein was calculated.

2.2.4. Homogeneity testing

Slab gel (7.5%) was performed according to Davis (1964) with HPLC purified venom fraction (20 μg protein) that possessed the antiosteoporosis activity. Tris–glycine buffer (pH 8.8) and 15 mA current was used for 3 h at 4 °C. The gel was stained with 0.01% coomassie blue, destained with 7% acetic acid and 10% methanol. Protein bands were visualized and photographed.

2.2.5. Determination of molecular weight

Molecular weight of the purified protein was determined by SDS-PAGE (10% acrylamide containing 1% SDS), using standard molecular weight marker proteins (50–160 kDa) after the method of Laemmli (1970).

2.2.6. Determination of amino acid sequence

The HPLC purified fraction were concentrated and ≈15 μg protein (following SDS-PAGE) was electro-transferred to PVDF membrane (0.4 μm pore size) using 10 mM CAPS transferring buffer (pH 11) containing 10% (v/v) methanol at 100 mA for 6 h. The PVDF membrane was stained with 0.2% (w/v) Ponceau S dried and used for N-terminal amino acid sequence using Applied Biosystem precise protein sequencer.

2.2.7. Development and confirmation of osteoporosis

Female Wister albino rats (28–30 week old, 150–160 g) were collected from M/S B N Ghosh & Company, Calcutta, India. Bilateral ovariectomy was done and osteoporosis was confirmed through urine markers (Gomes et al., 2009).

2.2.8. Evaluation of antiosteoporosis activity

After confirmation of osteoporosis, all the osteoporosis rats were divided into three groups ($n = 12$ in each group). Sham operated control Gr I was kept different from the osteoporosis group. Osteoporosis Gr II received vehicle (0.9% saline), osteoporosis Gr III received Bengalin (3 μg/100 g rat) × 15 i.p. dose alternate days, osteoporosis Gr IV received Bengalin (5 μg/100 g rat) and osteoporosis group V received standard drug vitamin D3, arachitol (Vieth, 2005), 200 mg/kg and calcium, 1500 mg/kg (Flynn, 2003) × 15 i.p. dose alternate days. At day 60, urine was collected and subjected to urine analysis. The rats were anesthetized,