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Toad Toxin from the Skin of *Bufo regularis* has Bimodal Effects on Neuronal Electrical Properties of Cray Fish Stretch Receptor and Rat Subfornical Organ (SFO)

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Abstract

Background: The skin of various amphibians contains potentially diverse bioactive principles. Some of these are speculated to have pharmaceutical, medicinal, toxicological or other chemical importance. Moreover, such active principles have the potential to be used as chemical tool in biomedical research in quest for other discoveries. **Objectives:** The present study aims at isolating and purifying bioactive principles from the skin of *Bufo regularis,* and studying their effect on the neurons of Cray fish stretch receptors and rat subfornical organ (SFO). High performance liquid chromatography (HPLC) was used to isolate the toad toxin. The HPLC fractions were initially tested on guinea pig ileum for bioactivity and potency using organ bath method. The effects of potent semi-purified and purified extracts were then tested on neurons of Cray fish stretch receptors and hypothalamic SFO using two-electrode voltage clamp and patch clamp methods. **Results:** The HPLC purification resulted in potent bioactive components with a λ_{max} UV absorbance pattern at around 295 nm. From the methanol preparative run, the 79 min elute had potent bioactivity. This was further purified using acetonitrile run, where the Fraction 40 min showed the maximum bioactivity of 57% of the weighed response on guinea pig ileum. When this was tested on cray fish stretch receptor neurons it had persistent inhibitory effect on action potentials generation to current steps under voltage clamp. Action potential generation was completely abolished (100% inhibitory response) in this neuron in response to the toxin. When the SFO neurons were studied under patch clamp, the neurons exhibited sustained depolarizing inward current (5.2 ± 3.4 pA) with complete inhibition of action potential generation. **Conclusion:** The HPLC eluate at 79th

min in methanol preparative run corresponding to the eluate at 40th min in the acetonitrile run has complete inhibitory effect on action potential generation and a depolarizing effect on the resting membrane potential of neurons, which is deduced as a bimodal response.

Keywords

Bufo regularis, Toad Toxin, Stretch Receptor Neurons, SFO, Membrane Potential

1. Introduction

Amphibians are class of anurans fauna that are abundantly diversified in Ethiopia. Currently, in the Afro-tropical zone, Ethiopia has about 63 species of amphibians, of which a remarkable proportion (25 species) are not known to exist elsewhere [1].

It is reported that the secretions from the amphibian skins contain diverse chemicals that could be of interest from taxonomical, zoological, medical and toxicological point of view [2]. Although there are a number of reports concerning the anatomical and taxonomical characteristics of amphibians, little or no investigations have been made on the chemical, medicinal and toxicological or other properties of their secretions. Nevertheless, the medicinal and toxicological importance of amphibian skin secretions have been known for many millennia [3].

In many countries of the western world, the dried skin extracts of toads were used to treat people with dropsy [4], and also for cardiac ailments as diuretics [5], as well as for many other inflammatory conditions [6] [7].

When fresh skin secretion of some toads comes in contact with human conjunctiva, it produces congestion and blurring of vision [3]. It immobilizes animals if they are applied to blow darts during game hunting [8]. Different investigators have documented similar toxic and lethal effect of toad toxins. For instance, it was reported that, dogs biting toads have their mouth swollen, and frog-eating snakes were seen rejecting them from their ingested meal and some developed dyskinesia [9] [10]. It is found out that the toxicity of the secretions from poisonous darts of Latin America is primarily due to the presence of neurotoxic batrachotoxins [10]-[12].

Certain skin secretions are particularly prevalent in distinctly different species of frogs and toads residing in specific locations [8]. Each frog or toad may contain an array of bioactive principles. The isolation of novel peptides, alkaloids, steroidal bufodienolides and amines shows that amphibians are potentially a good source of new natural products that will have huge impact on the progress of research and pharmaceutical industry [13]. Since, the type and abundance of bioactive principle vary between species and geography, the different and diversified species of toads and frogs can be the source of new hitherto unknown principles.

Some of the amphibian skin secretions were reported to have antinociceptive

[14] and antimicrobial activity [15]. *Rana ridibunda* is one of those toads whose skin secretion has been studied to have broad antimicrobial activity [15]. Another reported antimicrobial chemical from *Bombina variegata* skin secretions was named bombinin [16].

The current assumption is that, novel amphibian skin products could exist that may serve as a chemical tool in physiological, biochemical or pharmacological research. Their effects on physiological variables can help investigate the existence of as yet unknown substances or to identify a physiological mechanism behind a given cell or organ functions or dysfunction. Hence study of the nature and effects of amphibian secretions, particularly in neurons is imperative.

In the current study, the species of *Bufo regularis* which is relatively common in Ethiopia in habitats that include moist savannah, montane grassland and forest margins [1] was selected. They live in the valley of permanent water source at higher elevations. For the purpose of this study, they were collected from moist grassland areas around the town of Robe, Bale Zone, Oromia Administrative Region, 500 km Southeast of Addis Ababa, Ethiopia.

2. Material and Methods

2.1. Collections of Toads

The toad *Bufo regularis* (n = 16) were collected from the nearby permanent water source, Togona river, found in Bale Administrative Zone (Soth-East Ethiopia, 550 Km from Addis Ababa) at an elevation of about 2700 - 3000 m. The species was identified using Catalogue of the Ethiopian Amphibian [1] and assistance from national museum in Addis Ababa. In the process, the animal was photographed, scanned and sent online to expert, Professor Malcolm Largen (UK), who kindly assisted the author in the identification of the species.

After handling the collected toads humanely, they were carefully sacrificed by stunning and decortications 20 min after collection. The skin of each toad was then removed and pooled into a jar containing 120 ml of 70% ethanol and 1% acetic acid. The storage fluid, covered the skin (n = 16) and the jar was wrapped in an aluminum foil to prevent possible decomposition. The jar was then kept in a refrigerator at 4˚C until use. The storage fluid was later used as the raw extract since it was found to display bioactivity.

2.2. Purification of Toxin by HPLC

The following three-step purification scheme was conducted using a method described earlier by T. Tolessa. Briefly,

1) Initial concentration and treatment of raw the extract by solid-phase extraction on Sep-Pak C18 columns. This procedure yielded three crude fractions labeled I to III (**Figure 1**).

2) Crude fraction III was further purified by an HPLC separation procedure, using a linear methanol gradient system or methanol preparative run (**Figure 1** and **Figure 2**).

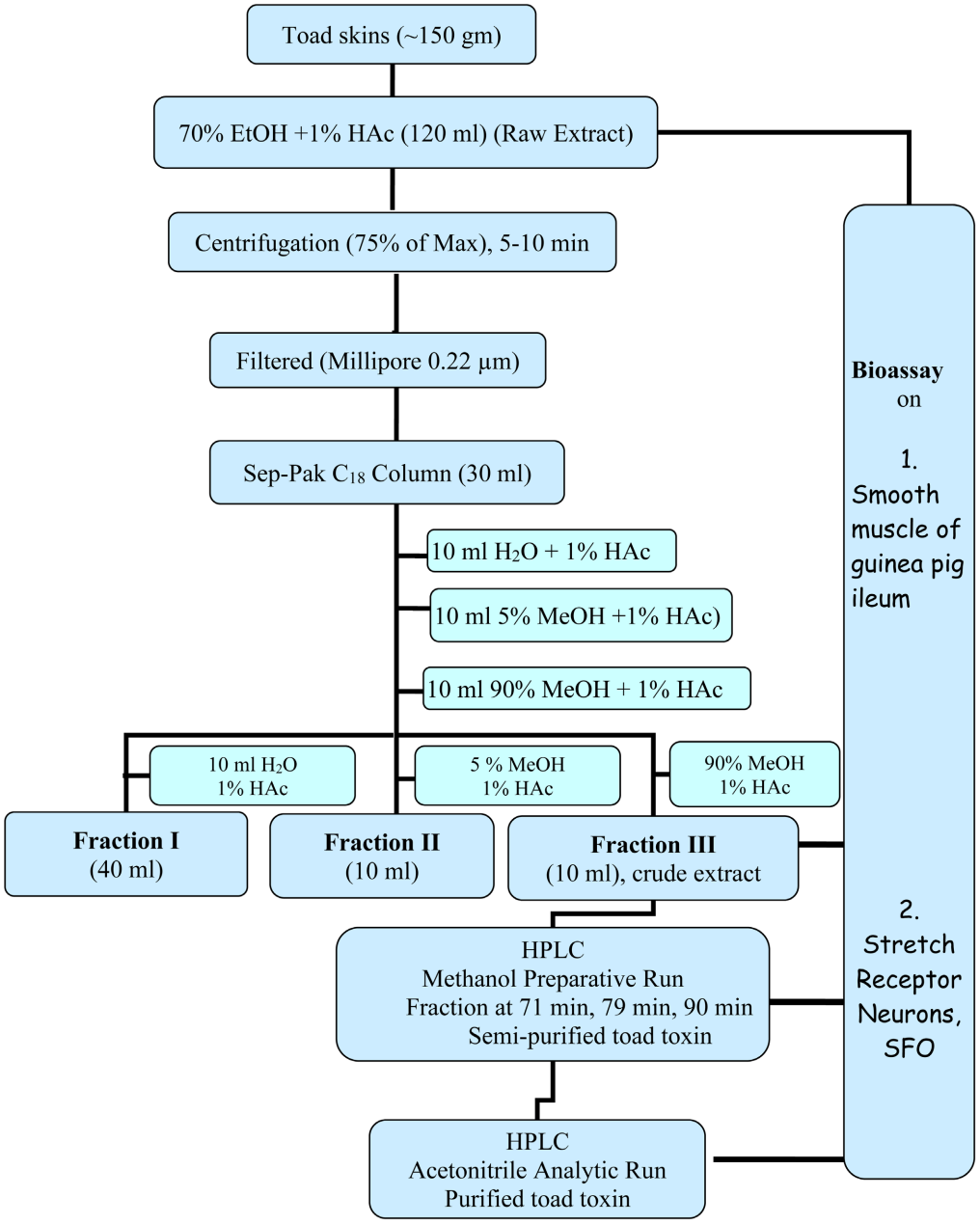

Figure 1. Flow chart indicating extraction and purification scheme of toad toxin from *Bufo regularis*. HPLC = high performance liquid chromatography, Hac = acetic acid, MeOH = methanol, SFO = Sub-fornical organ.

3) Bioactive fractions from the second step were further purified by a second HPLC separation procedure, using non-linear gradient (acetonitrile run) (**Figure 1** and **Figure 3**).

Then, 120 ml of the raw extract obtained as the 70% ethanol and 1% acetic acid preservative was divided into 10 test tubes and centrifuged. The supernatants from all test tubes were pooled and evaporated in a rotary evaporator to remove the ethanol. After evaporation, the remaining supernatant (30 ml) was filtered using Millipore Millex GV 0.22 µm filters (Millipore S.A., Moulsheim, France).

The mobile phase consisted of mixture of solvents, an aqueous phase (MilliQ water) and an organic phase (methanol and acetonitrile) in automatic linear and no linear gradient. The filtrate was loaded on five Sep-Pak C18 cartridge and rinsed with distilled water containing 1% acetic acid (Fraction I). The second rinse was made with 10 ml of 5% methanol in distilled water containing 1% acetic acid (Fraction II). The third rinse was made with 90% methanol in distilled water with 1% acetic acid (Fraction III). Fraction II and III constituting the crude extract were lyophilized separately, and fraction I was discarded. The residues were dissolved in 6 ml of distilled water and stored at 4˚C for HPLC methanol preparative run.

2.3. The Methanol Preparative Run

A preparative HPLC (detection wave length of 190-350 nm) columns (Waters radial compression PrepPak 25 × 100 mm and 6 µm NovaPak-C_{18}; Waters Inc., Millford, MA, USA) were preconditioned with blank runs of 0 - 100% methanol in 0.1% acetic acid using a 100 min linear gradient with a flow rate of 5 ml·min^{-1}. The chromatography system consisted of two LDC Constameric-II HPLC pumps (Milton Roy Co., Riviera Beach, FL, USA), a solvent mixer, a Rheodyne 7125 injector (Rheodyne L.P., Rohnert Park, CA, USA) with a 2.2 ml loop, and Waters Radial Pak Column (60Å, 4 µm, 8 mm × 100 mm).

UV absorbance was measured at 256 nm by an LDC UV Monitor-III (Milton Roy C., Riviera Beach, FL., USA) and was displayed on a BBC-120 pen-writer (ABB, Zurich, Switzerland). A Pharmacia/LKB 2140 Rapid Spectral Detector (Amershan Pharmacia Biotech, Uppsala, Sweden), controlled by Pharmacia/LKB Wavescan software on an IBM PC, was coupled directly with the fixed wavelength detector, and provided scans of UV absorbance from 190 to 370 nm at 4 s intervals. The chromatography run was set to a linear gradient operation starting with 99.9% water with 0.1% acetic acid and finishing at 100 min with 99.9% methanol with 0.1% acetic acid. The flow rate was set to 5 ml·min^{-1}.

Aliquots of 1 ml of fraction III were injected into the preparative column and fractions were obtained every 1.5 min by an automatic collection device, Pharmacia/LKB Redifrac. The preparative fractions were lyophilized in a vacuum centrifuge, and the residues were dissolved in 1 ml distilled water when bioassays were performed.

2.4. The Acetonitrile Analytic Run

HPLC fractions, with absorbance peaks around 295 nm, from the methanol preparative run corresponding to the 76 - 83 min (fractions 51 - 55) and 87 - 90 min (fractions 59 and 60) retention time were separately pooled. These fractions, now labeled 51 - 55 and 59 - 60 respectively, were separately lyophilized. The analytical columns (Waters Radial Pak 8 × 100 mm and NovaPak-C_{18}, 4 µm) are preconditioned with linear gradients of 0 - 100% of acetonitrile containing 0.1% trifluoroacetic acid. The 51 - 55 fractions were dissolved in 1 ml MilliQ water each, which was analyzed in two similar HPLC-runs. The HPLC-runs were performed with a

nonlinear gradient using MilliQ water containing 0.1% trifluoroacetic acid (pooled in bottle labeled "A"), and acetonitrile containing 0.1% trifluoroacetic acid (pooled in bottle labeled 'B'). The gradient started with a linear gradient throughout 10 min to reach 80% for bottle "A" and 20% for bottle "B". Later the gradient was changed to another linear steps during 100 min reaching 30% for bottle "A" and 70% for bottle "B" at 110 min and then altered to a third linear gradient step going to 100% for bottle "B" at 115 min. The HPLC flow rate was set to 1 $ml \cdot min^{-1}$. The absorbances of the bioactive principles were followed using computerized Pharmacia/LKB 2140 Rapid Spectral Detector (Amershan Pharmacia Biotech, Uppsala, Sweden) and SpectroMonitor III (Milton Roy C., Riviera Beach, FL., USA) connected to a BBC 120 pen-writer (ABB, Zurich). Fractions of 1 ml were collected, lyophilized and the residues were dissolved in 0.5 ml MilliQ water for bioassay on guinea pig longitudinal muscle of the ileum.

2.5. Dissociation of Neurons and the Cell Culture

All dissecting tools and dishes were sterilized in 70% ethanol. Ten days old Wistar rat pups were anesthetized with isoflurane (Abbott Scandinavia, Sverige), inhalation, and sacrificed by decapitation. The head was immersed in 70% ethanol. Then, for 3 min the brain was rinsed with sterile rodent Ringer with glucose solution (146 mM NaCl, 5KCl, $2CaCl_2$, 1 $MgCl_2$, 10 HEPES, 11 glucose, pH = 7.4). A sagittal section of the brain was made on ice plate; cortex identified, rapidly removed and put in rodent Ringer's solution. Further dissection was made by removing parts of the hemispheres leaving a thin tissue block containing the SFO. The lateral ventricle and thick vasculatures were used as a landmark to identify SFO. With the aid of a dissecting microscope, the SFO containing tissue were dissected away from all surrounding CNS tissue, thus isolating tissues composed of SFO. These were kept in Ringer's solution and continuously aerated by blowing with 95% O_2 and 5% CO_2 over the solution. The tissue was cut into chunks of less than one mm^3 and were rinsed once with 2 ml PIPES buffered saline (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MaCl_2$, 25 mM D-glucose, 20 mM piperazine-N, N'-bis [2-ethanesulfonic acid], pH 7.0). The tissue was immediately transferred to a small scintillation vial (~5.5 ml) and incubated at room temperature in 1 ml of 0.5% trypsin XI (Sigma) and 0.01% DNase-I (Sigma) in PIPES buffered saline for 30 min with 95% O_2 and 5% CO_2 blown over the mixture. This was followed by incubation for 60 min at 35˚C with gentle shaking and continuous aeration with 95% O_2 and 5% CO_2. The tissue was subsequently rinsed briefly with 1 ml of rodent Ringer containing 1 $mg \cdot ml^{-1}$ BSA and 1 $mg \cdot ml^{-1}$ trypsin inhibitor type II-O (Sigma) and rinsed again in 1 ml of growth medium (Neurobasal medium with B27 supplement, 0.5 mM glutamine, 0.02 $mg \cdot ml^{-1}$ gentamicin, pH 7.4; Gibco BRL, Life Technologies, Grand Island, NY). The tissue was lightly triturated with Pasteur pipette followed by triturating with a fire polished Pasteur pipette in fresh media; and then plated onto the center of a poly-L-lysine (Biochrome AG, Berlin, Germany) coated dish. After the cells settled (~5 min) additional growth

media was added to the dish to bring the volume up to 2 ml per dish. The preparation was incubated at 37˚C in cell culture incubator (LabRum Klimat Revco Ultima, Solna, Stockholm, Sweden) for experiments.

3. Results

3.1. Isolation and Purification of Toxin

The methanol preparative run

The HPLC/UV chromatogram profile of the methanol extract from skin of *B. regularis* showed distinctly discernible peaks that had elution time between 30 to 95 min (Figure 2(a)). The peaks had a variable bioactivity on longitudinal muscles of the guinea pigs ranging from a relative weighted response of zero to 55. The peak that corresponds to the potent toad-toxin eluted at about 65% methanol gradients (Figure 2(a)). This principle with maximum contractile bioactivity (Figure 2(b)) was eluted at a retention time of 79 min corresponding to a range of 76 - 83 min (Figure 2(a)). This had a UV absorbance of 295 nm λ_{max}. The chromatogram

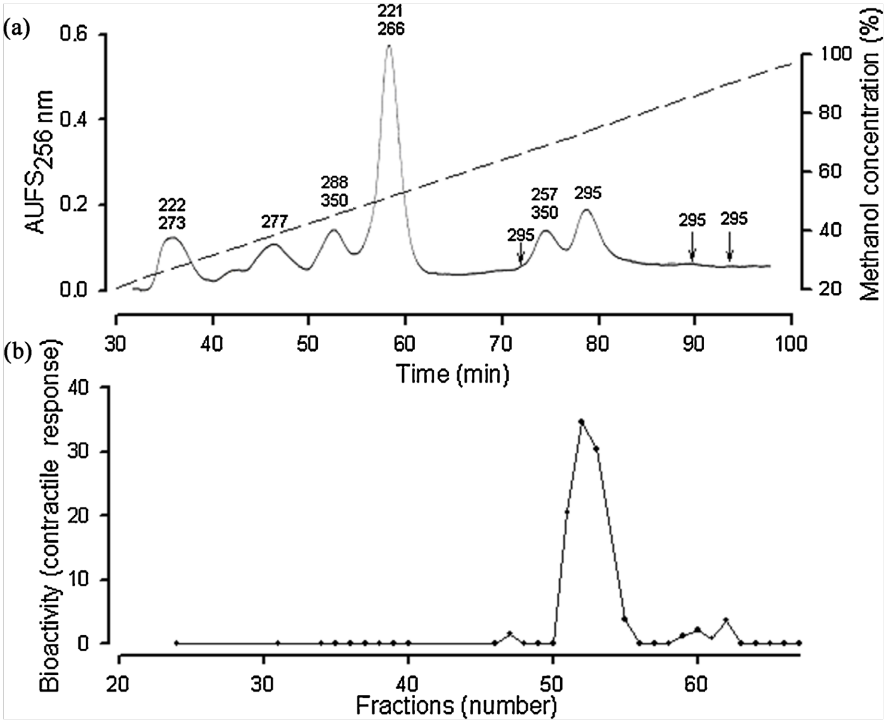

Figure 2. HPLC chromatogram of crude toad toxin, Fraction III, on a Waters Radial Pak 25 × 100 mm and NovaPak-C_{18}, 6 µm. Sample (1 ml) was injected at time zero (not shown) when a linear gradient of 0 - 100% methanol (a) was started with a flow rate of 1.5 ml·min^{-1}. Acetic acid (0.1%) was present throughout. The relative bioactivity of elutes during bioassay on longitudinal muscle containing myenteric plexus (contraction response) of guinea pig ileum is indicated as weighted response (b). Numbers above chromatogram peaks denotes UV absorbance maximum (λ_{max}), a computerized resolution, in the chromatography (190 - 350 nm). The diagonal broken-line in the top tracing indicates the linear gradient (0 - 100%) of methanol concentration in the HPLC run, as published by T. Tolessa [17]. AUFS = Absorbance Units Full Scale.

had other peaks also with lower bioactivities at retention times of 71 min and 88 - 93 min with similar UV absorbance of 295 nm λ_{max}.

3.2. The Acetonitrile Analytic Run

The 76 - 83 min from the preparative run

As in methanol preparative run, there were distinct peaks in the acetonitrile chromatogram (Figure 3(a)). When acetonitrile analytic run was conducted on the methanol preparative chromatogram from the 76 - 83 min elutes, it showed multiple different peaks. The picks had elution time between 17 to 65 min. In the organ bath bioassay, these peaks had a variable bioactivity ranging from a relative weighted response of zero to 60 (Figure 3(b)). The peak that showed a potent bioactivity on guinea pig ileum was eluted at 40 min; and eluted at about 36% acetonitrile gradients. The UV absorbance had λ_{max} of 295 nm; other peaks with moderate to minor bioactivities were eluted at 47 min, 30 min and 58 min. The corresponding UV absorbances were 296 nm, 296/252 nm and 297 nm respectively.

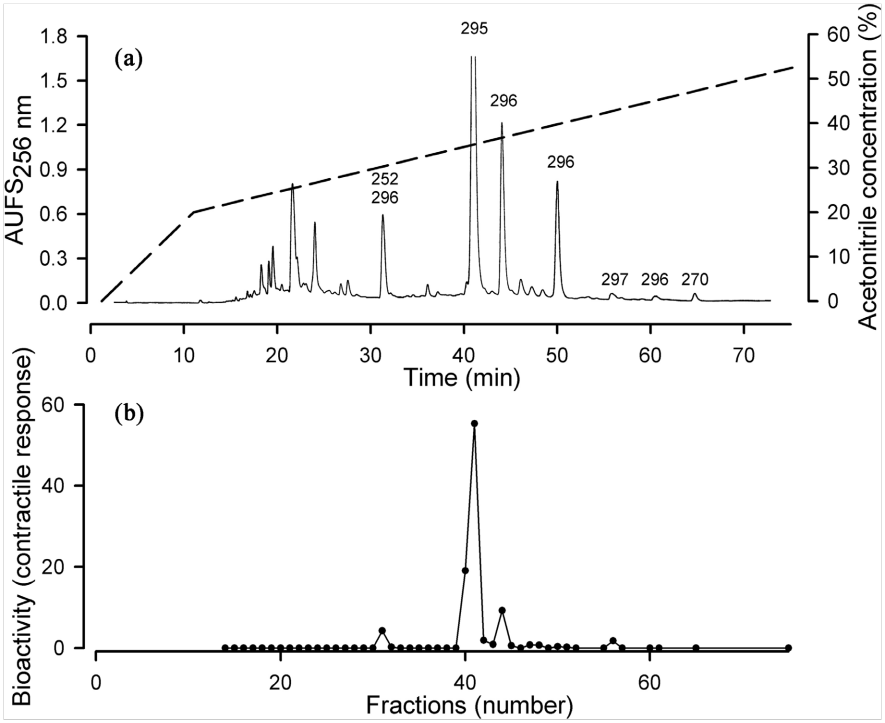

Figure 3. Elution time of the acetonitrile run with 0.1% TFA on 8 × 100 mm NovaPak-C_{18}, 4 µm column conducted on the 76 - 83 min fraction from the methanol chromatography (a). Fractions were collected at 1 min intervals. The nonlinear methanol gradient is indicated as a broken line (A). Relative bioactivity of the guinea pig ileum in organ bath, calculated as weighted response to elutes during bioassay on plexus containing longitudinal muscle of guinea pig's ileum (b) as published previously by T. Tolessa. The 40th min elute had the highest potency on smooth muscles in organ bath.

3.3. The 88 - 93 min Elutes from the Methanol Preparative Run

From the 88-93 min elutes of methanol preparative run, the acetonitrile chroma-

togram showed two major peaks (Figure 4(a)). The peak with maximum bioactivity eluted at 56 min (Figure 4(b)). This had a UV absorbance of 295 nm λ_{max}. There was a peak at 64 min with low bioactivity.

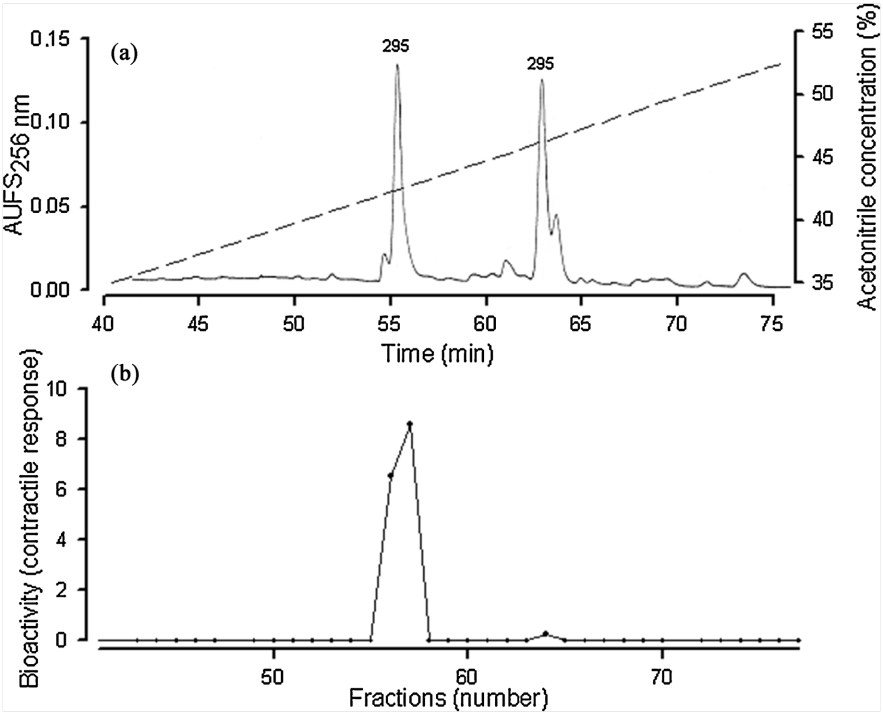

Figure 4. Elution time of the 88 - 93 min fraction from the methanol chromatography by acetonitrile run (a). Fraction with strong bioactivity was eluted at 56 min (b). The strength of its bioactivity 6× less when compared to eluate at 40 min in Figure 3(a). Numbers above peaks indicate λ_{max} UV absorbance at 190 - 350 nm.

3.4. Organ Bath

Purified toad toxin on smooth muscle of guinea pig ileum

In the acetonitrile run, the maximum effect of toxin on the isolated muscle preparation was observed from peaks eluted at 40 min. The dilution ratio of the toxin in organ bath was 1:250. 77% in increase in tone of smooth muscle and 100% decrease in the amplitude of the nerve-induced contractions contraction was observed (Figure 5). The inhibitory effect was persistent and it was reversed one hour following repeated washing. It was this toxin component that is used to investigate electrophysiological effect on neurons.

3.5. Effect of Fraction F-III of *B. regularis* Toxin on Stretch Receptor Neurons

Two Electrode Voltage Clamp: Cray fish stretch receptor neuron was hyperpolarized to holding potential of −80 mV before applying the stimulating rectangular current pulses, whose amplitude varied from −6 nA to +12 nA. The current protocol elicited depolarization in the control recordings at the given holding potential of −80 mV. When the amplitude of the injected depolarizing currents into the

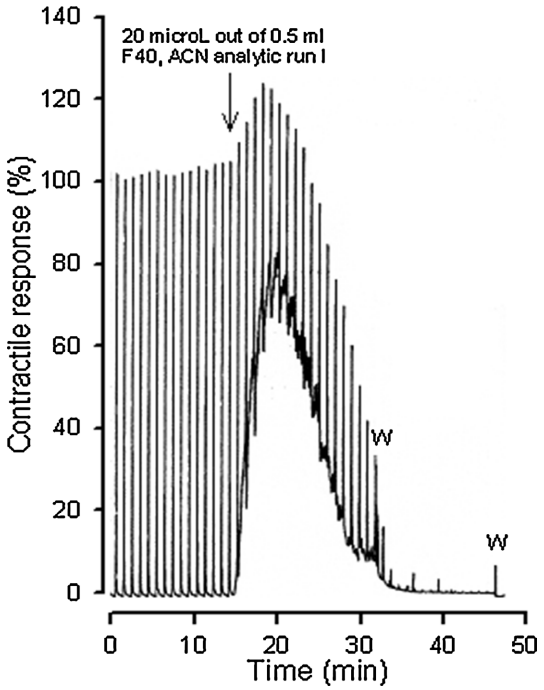

Figure 5. The effect of eluate at 40 min from first acetonitrile run on isolated longitudinal muscle of guinea pig ileum. 20 μL of toxin taken from a solution of 0.5 ml MilliQ water was used. Transmural nerve stimulation was applied at 3 Hz, 0.2 ms, 15 pulses at 1 min. W = wash.

neuron was increased, the resulting depolarization generated action potentials (Figure 6(a)). The amplitudes of the action potentials were reduced by more than 30 mV after 4 minutes of exposure (Figure 6(b)) and completely abolished 10 min after exposure of the neurons to 20 μL of Fraction III (Figure 6(c)). The neuron action potentials were able to recover only after repeated washing.

3.6. Recording of Current from Hypothalamic SFO Cell in Whole-Cell Voltage Clamp Mode

The successive voltage clamp step (−120 to +60 in a 20 mV) steps from a holding potential of −80 mV produced membrane currents (Figure 7(a)). The membrane current consists of early inward and the late outward currents in cultured hypothalamic SFO cells in whole-cell recording mode. The early component of the response corresponds to inward sodium current (b) whereas the later response corresponds to slow outward potassium current (c).

3.7. Effect of F 40 from the Acetonitrile Runs on SFO Inward Current and *I-V* Curve

On the same neuron described above, when a stimulating voltage was linearly changed from −120 to +80 in 2 ms intervals over 2 sec (instead of holding at a potential of −80 mV), a resultant current and *I-V* curve were recorded from a slowly adapting stretch receptor neuron (Figure 8). Before treatment with toxin, the ionic current varied with the potential of the test pulse, showing leak current

(slope), regenerative inward currents (negative deflection) and outward currents (positive deflection) (Figure 8(a)). The regenerative inward current appeared in the *I-V* curve only in the voltage sensitive region of the curve and its magnitude changed with increasing stimulating voltage changes. However, after treatment with F40 toxin, the regenerative inward and outward currents were completely abolished (Figure 8(b)). In addition, the *I-V* curve and its slope (indicator of the leak current), shifted upward and to the left beginning at a holding potential of

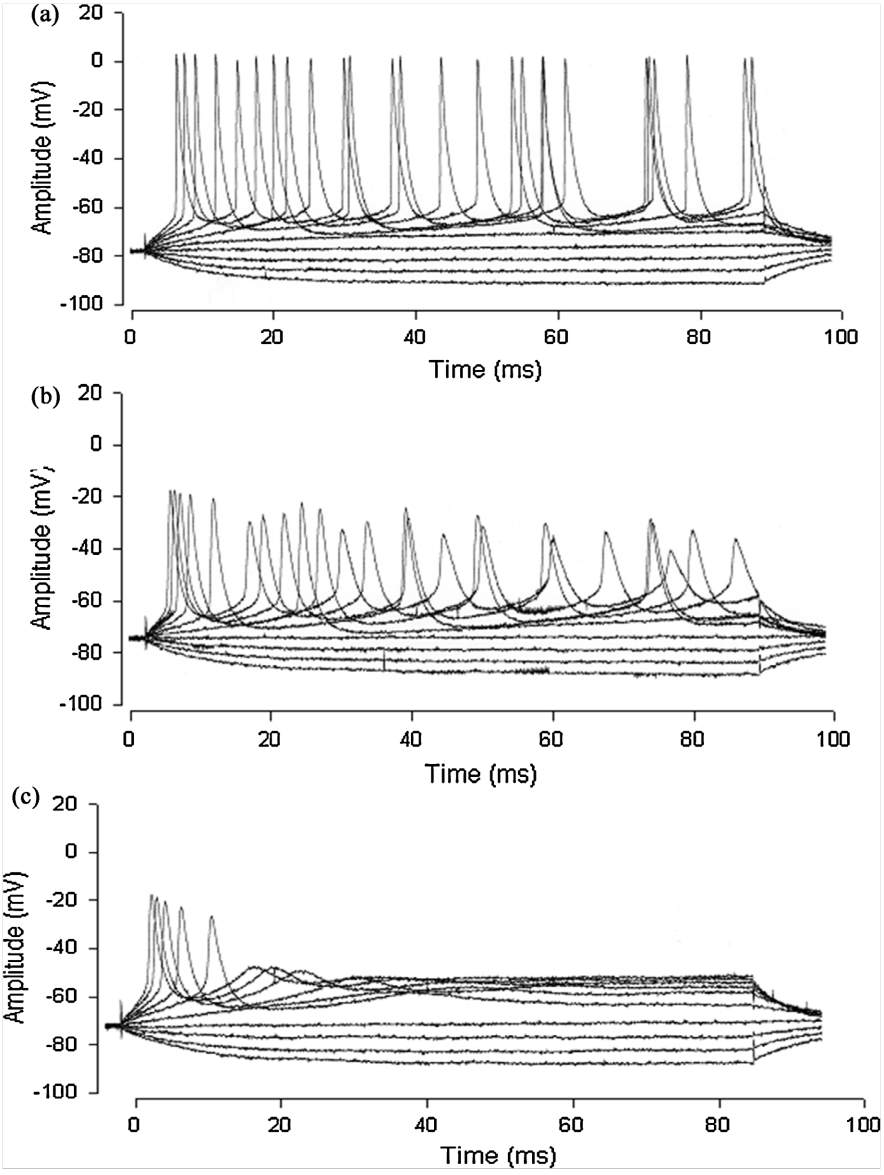

Figure 6. The effect of Fraction III (20 µL of stock solution in 200µL of solution in the chamber) on action potentials of slowly adapting cray fish stretch receptor neuron. The cell was hyperpolarized to holding potential of –80 mV before being stimulated. The stimulating rectangular current pulses (–6nA to +12nA) elicited depolarization and actions potentials in the control recordings (a). The amplitude of these action potentials was reduced to –30 mV 4 minutes after exposure to extract (b), and completely abolished the actions potentials 10 min later (c); n = 8.

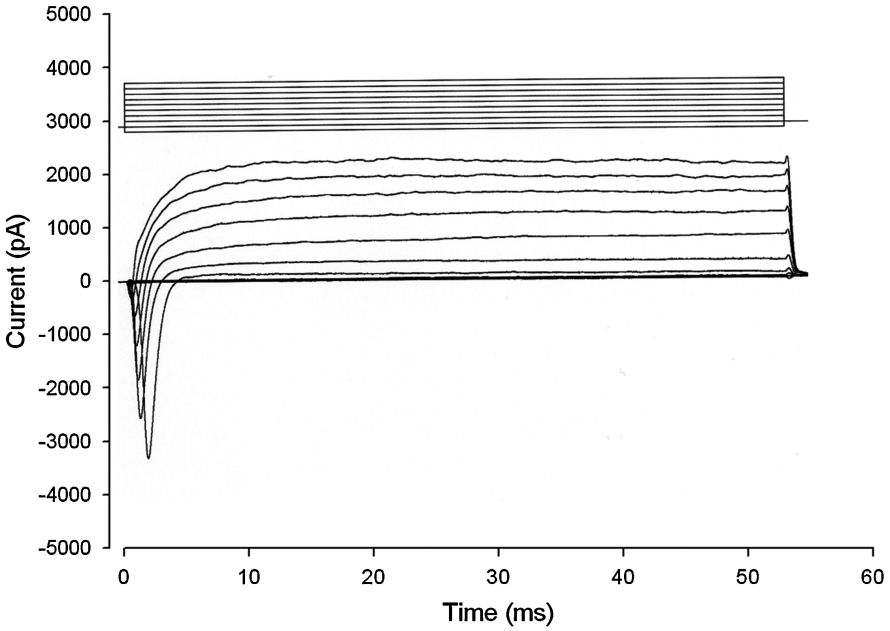

Figure 7. Control recording of the effect of successive voltage clamp step from a holding potential of −80 mV on SFO cell using patch clamp. The potentials were stepped from −120 mV to +60 mV in 20 mV steps and then the membrane current was recorded. The direction and time course of ionic current varied with the potential of the test pulse, showing that these cells are neurons; different from the glial cells found intermixed with the neurons. E_{hold} = −80 mV; Erest = −80 mV. Electrode resistance = 1 - 10 MΩ; n = 8.

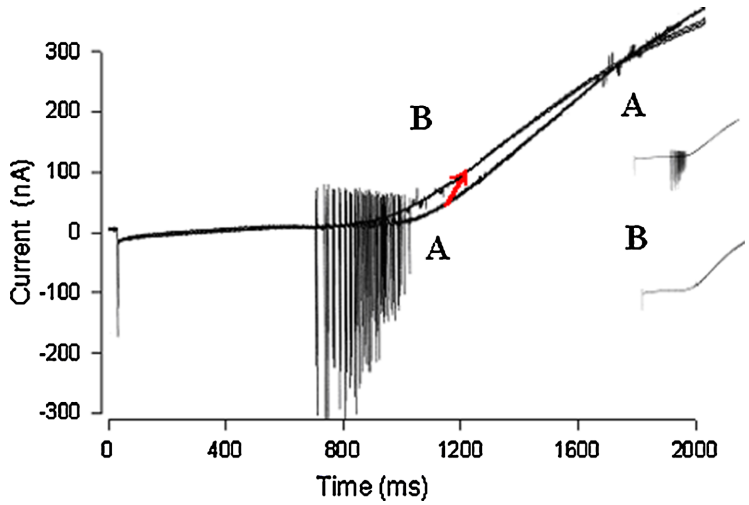

Figure 8. Current-voltage (*I-V*) curve showing the effect of F40 on crayfish stretch receptor neuron. The voltage was changed linearly from −120 to +80 in 2 s intervals and then the resultant current was recorded. The ionic current varied with the potential of the test pulse, showing that the regenerative inward currents (a), disappeared following toad toxin application (b). When A and B curves are overlapped during the recording, it shows that the *I-V* curve shifted upward and to the left (arrow) at the start from the holding potential of E_{hold} = −60 mV (n = 6).

−60 mV. At more negative potentials which is at voltage step before appearance of the regenerative currents, no leakage conductance was observed.

3.8. Effect of F 40 (ACN Analytic Run) on Stretch Receptor Neuron Action Potentials

The stretch receptor neuron was again hyperpolarized to holding potential of −80 mV before applying the stimulating rectangular current pulses which was varying from −6 nA to +12 nA. When the amplitude of the depolarizing rectangular current pulse was increased, the resulting depolarization generated action potentials in the initial control recording of stretch receptor neuron (Figure 9(a)). Exposure of this neuron to F40 of the toad extract reduced the number and amplitude of the action potentials within 5 ms and followed by the disappearance of the action potentials after 10 min, during the 100 ms recording (Figure 9(b)). Recording of membrane potential changes in current clamp showed that the neuron responded to the toxin by membrane depolarization of about 3 mV (not indicated).

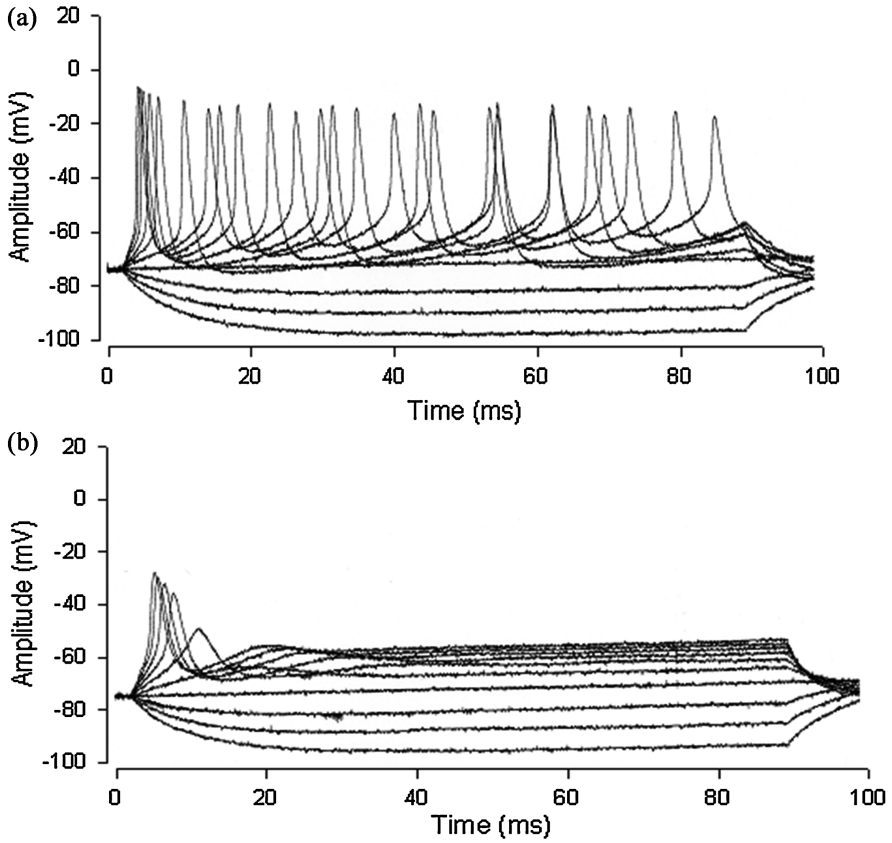

Figure 9. The effect of F 40 (50 µL) on action potentials of a stretch receptor neuron from a crayfish. The cell was hyperpolarized to a holding potential of −75 mV before being stimulated. The stimulating rectangular current pulses, whose amplitude varied from −6 nA to +12 nA elicited current-dependent action potentials in the control recordings (a). The number and amplitude of the action potentials were reduced in 10 minutes during the application of F40 (b). Electrode resistance = 1 - 10 MΩ; n = 8.

3.9. Effect of Ouabain on Cray Fish Stretch Receptor Neuron Action Potentials

Increasing the amplitude of the depolarizing currents resulted in depolarization

followed by generation of action potentials (Figure 10(a)). Ten minutes after exposure of the neuron to ouabain (5 mmol), the amplitude and number of the action potential was reduced by about 10 mV (Figure 10(b))

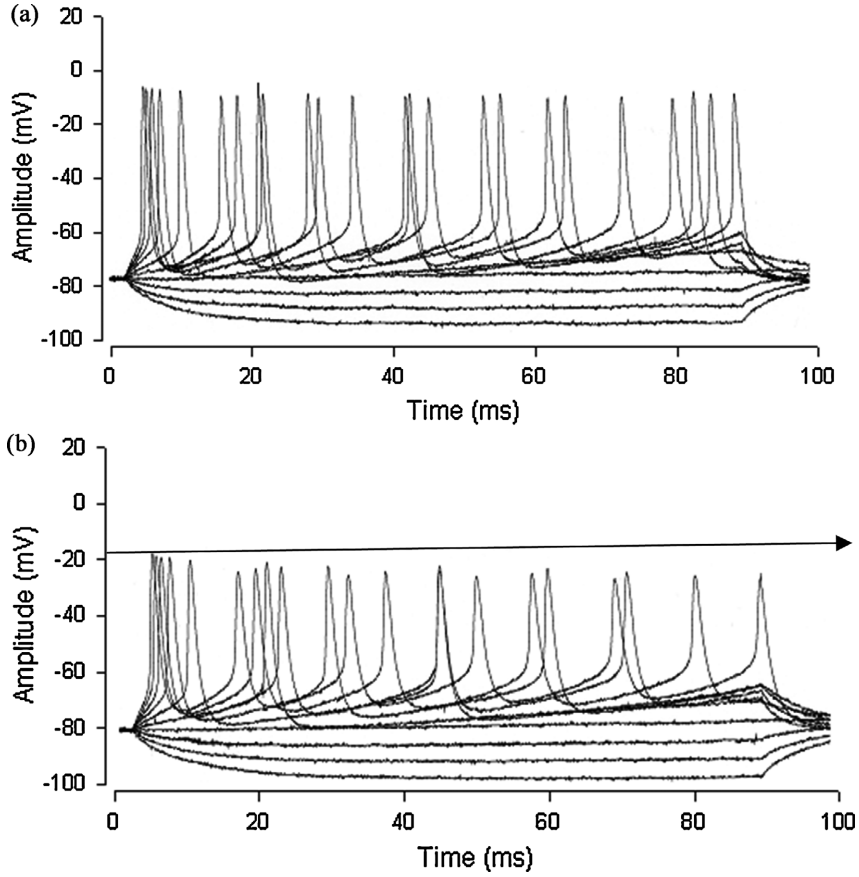

Figure 10. Effect of ouabain (5 mmol) on action potential of crayfish stretch receptor neuron (a) Control, (b) 4 min after ouabain (1 mM). Holding potential was −80 mV. The duration of the pulse protocol was 100 ms in the X-axis. Y-axis repents the voltage magnitude in mV. The horizontal arrow indicates and overtime (from 1 ms to 100 ms) shift in action potential magnitude after exposure to toxin.

3.10. Effect of Fraction III on Membrane Current of SFO Cells

Single Electrode Patch Clamp: A typical whole-cell voltage clamp recording using the patch clamp technique on SFO cells (Figure 11). The cell was clamped at a holding potential of −60 mV and the change in membrane current was recorded before and after application of the raw extract. In response to the toxins, inward depolarizing membrane current of −8 pA (Figure 11) was measured over a period of three minutes. The current response in SFO neuron in response to Fraction III was 5.2 ± 3.4 (Figure 12).

4. Discussion

The distribution of the various bioactive chemicals in skins of toads and frogs varies with the geographical locations. Allopatric and yet different amphibians

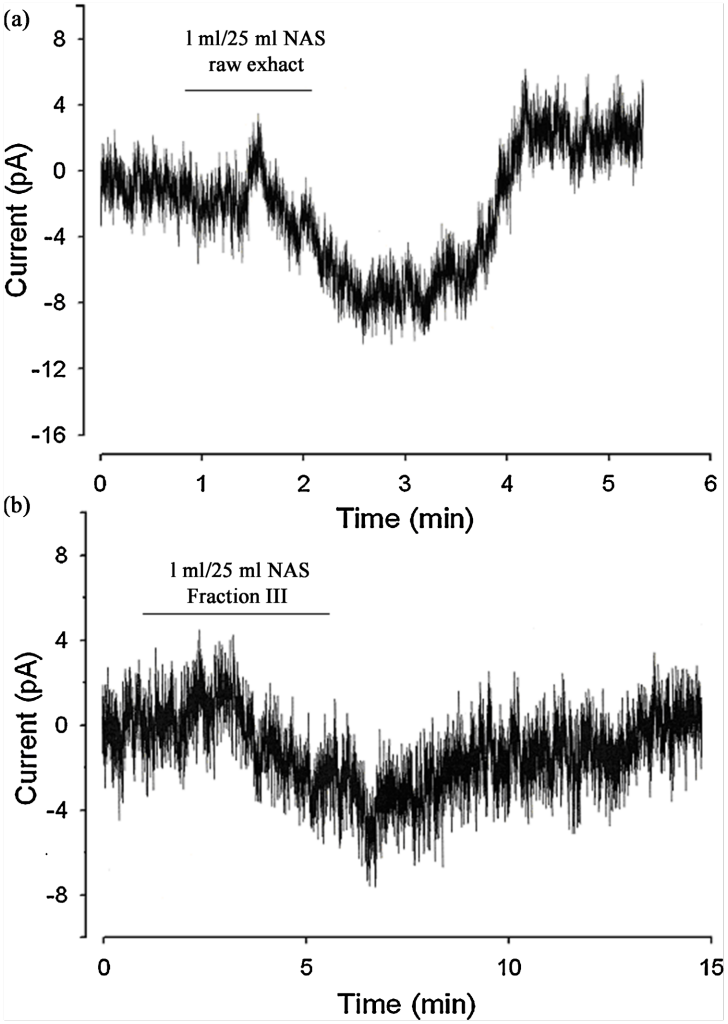

Figure 11. Whole-cell voltage clamp recording of current changes from SFO cells in response to toxin in Fraction III. The toxin produced an inward current of −8 pA (a) and −5 pA (b); holding potential −60 mV (n = 8).

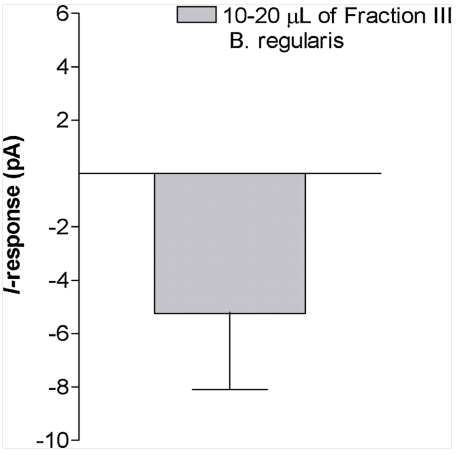

Figure 12. Mean of the maximal current responses of SFO neurons after treatment of the cells with 10 - 20 µL Fraction III prepared from the skin of *B. regularis*. Values are mean ± SEM (n = 8).

may contain similar principles, and sympatric and similar frogs may synthesize different principles, and the abundance of toxin varies within and between species [18] [19]. The various toads, specially the bufonids are richly endowed with and bufodienolides (ouabain-like compounds) and indole alkylamines. The ouabain-like compounds are evenly distributed in most bufonids and other genera, as well as in some species of the Ethiopian toads such as the *Bufo garmani* [20].

Buodienolides are steroidal compounds, if present in large quantities in animals or plants can provide protection against natural enemies, such as pathogens and predators. There are strong relationships between toad toxin presence and bacterial community structure of the aquatic habitat [21]. Such relationships may have arisen due to adaptation to local bacterial communities, phenotypic plasticity and differential biotransformation of toxin compounds by different bacterial communities. Bacterial groups that contribute to variation in toad toxin content remain to be investigated.

Ouabain-like compounds from *Bufo marinus* and other species having a retention time between 6 - 15 min have been identified by Flier and associates [20]. Shimoni *et al*. [22] also isolated a bufodienolide from the skin and plasma of *Bufo viridis*, and the bioactive compound was eluted at the retention time between 26 - 30 min on an amino acid column; and in a reverse column it was between 7 - 10 min. In serial HPLC runs, using a reverse phase preparative column, the active fraction was eluted at a retention time between 25 and 50 min [23]. When this fraction was re-chromatographed on semi-preparative and reverse-phase analytical columns, its elution time indicated that the active principle was a steroid derivative [23]. In a similar HPLC separation performed on skin toxin from Ethiopian *B. garmani*, the active fraction was eluted at 47 and 75 min [24]. In the current study, the retention time values (between 30 to 95 min) for the extract from *B. regularis* seems to be different from the values for resinobufagenin, a bufodienolide from *B. viridis* and *B. marinus* but appears to be similar to the ouabain-like compound from *B. garmani*.

The λ_{max} of other bufodienolides, which were extracted from *Bufo marinus*, was reported to be at 300 nm, preceded by a minimum at about 254 nm [20]. The ouabain-like compound from *B. viridis* was reported to have a UV λ_{max} at 278 nm [4]. In the present study, the methanol preparative run had discernible UV spectrum with absorbance pattern ranging from 295 - 297 nm, but peaks with maximum bioactivity had UV absorbance spectrum at λ_{max} of 295 nm indicating a difference in the retention time with the others finding. The acetonitrile run revealed a UV absorbance range between 230 to 297 nm for the possible toxin retention time.

The peak with maximum bioactivity in organ bath showed two λ_{max}, a smaller one at 296 nm and a larger one at 295 nm. The semi-purified fraction eluted at the 79th min in the methanol preparative run; and the purified fraction eluted at the 40th min in the acetonitrile run had a similar UV absorbance at λ_{max} at 295 nm. Both the preparative and analytical runs have thus yielded the same princi-

ple. The main difference between the previous and the current finding might be due to time and sensitivity, which depends on the nature of the different columns used and on the concentration of the organic solvent. Alternatively, the toxins from *B. regularis* skin could be different steroid chemical with difference in bioactivity and HPLC profile from that isolated from other species of toads. In this study, the earliest bioactive peak isolated from the analytical run was at the 30th min and this had two absorbance values, 252 and 296 nm. The time and peak absorbance in this study was different from others experimental finding on bufonid species, indicating the existence of different toxins in the same genus of toads from Ethiopia.

The most potent purified principle from both the methanol and acetonitrile run had persistent and potent inhibitory effect on neurogenic-induced contractile response of the longitudinal muscle of guinea pig ileum. The slowly-induced inhibitory effect was not immediately reversed even after repeated washing. The amplitude of contraction in response to transmural nerve stimulation was gradually abolished, and this effect was comparable to that of ouabain [17].

The electrophysiological approach to study neuronal effect using two-electrode voltage clamp technique on the cray fish stretch receptor sensory neurons showed that the semi-purified toxin had potent inhibitory effect on action potential generation. Exposure of the receptor neuron to semi-purified eluate at 79th min of the preparative resulted in complete ablation of action potential generation, this being the second phase of a bimodal response of a neuron to the toxin. This neuronal response was similar to the effect of tetrodotoxins on sensory neurons [25]. Contrary to membrane depolarization observed, the amplitude of the action potential of the sensory neurons was initially reduced until it was completely inhibited. Therefore, the action potential response of neurons to this toxin indicates the presence of chemical component in the extract that blocks voltage-gated channel. Voltage-gated channels are transmembrane proteins which are important for generating and propagating action potentials in neurons and muscle cells. They open in response to changes in voltage, allowing ions such as sodium to flow into the neurons, which leads to depolarization, generation and propagate action potentials [26].

The electrophysiological approach using single electrode whole-cell patch clamp technique on the hypothalamic SFO cells showed that the extract had slow and prolonged effect on the membrane currents. When these cells where exposed to the toxin, a sustained inward current of about -8 pA was observed. For SFO cells with a whole-cell resistance of 2 - 3 MΩ, the membrane potential change was 3 mV. This shows that the toad toxin had a compound that has slow depolarizing effects on the membrane potential, this being the first components of the bimodal response of neuron to the toxin. For comparison, when ouabain was studied at dose of 5 mmol, it produced changes in membrane current that was similar but smaller (2 mV) in potency and duration.

In excitable cell membranes, where an electrochemical gradient is necessary

for normal function, the steady-state resting membrane potential is maintained or restored by an electrogenic Na^+-K^+ pump. The resting membrane potential is determined by the intracellular and extracellular concentration of Na^+ and K^+ and cell membrane's permeability to K^+. To a minor degree the permeability to Na^+ and other ions also contribute to the membrane potential. Ouabain and many other glycosides block the Na^+-K^+ pump, thus increasing the intracellular concentration of Na^+ and decreasing the intracellular K^+ [27]. This results not only in depolarization of neurons, but also affect ion channel-mediated membrane currents by changing the chemical driving force. In addition, in the cray fish stretch receptor neurons, the Na^+-K^+ pump is electrogenic similar to what was found in the lobster stretch receptor neuron and blocking the pump can depolarize the cell by 5 - 10 mV. This was similar to what has been observed in the crayfish stretch receptor neuron and rat hypothalamic neurons when they were treated with the raw and crude toad toxin. However, it is not conclusive whether the depolarizing effect is due to the blockage of an electrogenic pump or non-electrogenic component.

5. Conclusion

The skin of the toad *B. regularis* contains neurotoxin that elutes at 79 min in methanol preparative run and at 40 min in acetonitrile run in HPLC. This toxin has bimodal effect on smooth muscles of guinea pig ileum, that is, increase in the basal tone of the muscle followed by decrease in electrical induced contractions. Similarly, the neurotoxin has bimodal effect on cray fish stretch receptor and subfornical organ neurons. It increases membrane depolarization, decreases and later completely inhibits current-induced action potential generation.

Ethical Considerations

This study was approved by Faculty Research and Publication Committee (FRPC) of the Faculty of Medicine, Addis Ababa University, Ethiopia, and the local Ethics Committee for Animal Experimentation in northern Stockholm, Sweden.

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Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

The author declares that AI-assisted technologies were not used in any way in conducting or in the writing process of this manuscript.

Author's Contribution

Tesfaye Tolessa Dugul designed, conducted and wrote the final report.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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