Shendi University
Faculty of Graduate Studies and Scientific Research

Characterization of protein in the secretions of paratoid gland of toads (Amietophrynus *spp)* collected from shendi area

A thesis submitted for fulfillment of the requirements the degree of M.Sc. in zoology

By

Weam Yassin Ahmed Ali

Supervisor:

Assistant Professor. Husam Eldin Elhag Abugabr Elhag

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Dedication

To my parents who taught me that the means to ends is patience.

To my Husband Hassan Elamin

TO all who stood beside mi and pushed me ahead particularly my

Brothers and sisters.

Acknowledgements

First I deeply thank my God upon completion of this work successfully. I wish to express my sincere appreciation and much thanks to my supervisor Dr: **Husam Eldin Elhag Abugabr Elhag**, faculty of sciences and technology, university of Shendi *for his sustained encouragement* .Thanks to my family for their continual support .Also my thanks extend to zoology laboratory staff In Shendi University, faculty of sciences and technology, *For their fine help in laboratory investigation***.**

Abstract

The parotoid glands secretions of two species, namely *Amietophrynus regularis* and *Amietophrynus xeros*, were investigated by Bradford estimation test for determining the concentration of proteinaceous compound in the secretions. The results revealed a wide range of proteins that differed between the two species. The average of protein content in *A. regularis* secretions was estimated as 26.95± 8.89% while the *A. xeros* was 23.86± 8.94 %. Statistical test by F test and t student test illustrated significant difference between the two groups of protein concentrations.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) illustrated two different protein profiles for the two species that indicated the evolutionary and taxonomical value of the paratoid secretions. Furthermore it revealed different protein profiles within each species, indicating the existence of subspecies or the existence of another species that was extremely similar to the species, especially in the case of *Amietophrynus regularis*. The Retention factor (*Rf*) and molecular weights of the protein

الملخص

تمت دراسة تركيز المركبات البروتينيه فى إفرازات الغده النكفيه لنوعين من الضفادع هما A.regularis و A. Xeros بإستخدام إختبار برادفورد وقد أوجدت النتائج كميه مقدره من البروتين بإختلاف طفيف بين النوعين ووجد إن متوسط محتوى البروتين للنوع الاول8.89±26.95وللنوع الآخر8.94±23.86.

كما أوضحت الإختبارات الإحصائيه وجود فرق واضح في شكل المجموعات البروتينيه وأوضح إختبار الرحلان الكهربي Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS- PAGE) نوعين مختلفين من البروتينات في أنواع الضفادع تحت الدراسه مما وضح انه يمكن وجود قيمه تصنيفيه لافرازات الغده النكفيه وأيضا أوضح الفرق بين البروتين والأخر مما دل علي إمكانية وجود تحت انواع او انواع جديده تتشابه لحد كبير مع بعضها والي حد كبير مع نوعA. regularis

كما تمت دراسة معامل المكوث (Rf) والوزن الجزيئ للبروتين الموجود فى الافراز

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CHAPTER ONE

1.Introduction and literature review

1.1 Background of amphibians and their glands

1.1.1 Amphibians and their glands

The amphibians first appeared on earth about 360 million years ago during late Devonian period from the common ancestry of Sarcopterygian bony fish (Fritzsch, 1990). Modern amphibians (Lissamphibians), appeared during the Triassic period and they have survived till today (Bolt, 1991; Marjanović and Laurin, 2007). Triado batrachus massinoti was considered the earliest Lissamphibian fossil available from Madagascar. Among the Modern amphibians, Anura and Gymnophiona appeared during the early Jurassic period and Caudata during middle Jurassic (Jia and Gao, 2016).

Amphibians were the first vertebrates to leave ancestral aquatic life style to venture terrestrial mode. Among the vertebrates, amphibians are the only class to have free-living tadpole stage and adult stage. All the living amphibians throughout the world have been grouped in 3 orders, namely *Apoda* (Caecilians), *Salientia* (Frogs and Toads) and *Caudata* (Salamander and Newts) (Clarke, 1997). Amphibians were characterized by their ability to exploit both aquatic and terrestrial habitats. The name amphibian, derived from the Greek word "amphibias" meaning "living a double life," reflects this dual life strategy—though some species are permanent land dwellers, while other species have a completely aquatic mode of survival (Conant, et al 1999; Harding and Holman 1992). They

were able to spend longer periods in terrestrial environment through acquisition of sets of adaptations; one of these adaptation sets is the possession of exosecretion granular gland that are highly specialized skin structures which appear during metamorphosis as epithelial derivatives that develop to gland alveolus formed by a secretary layer covered externally by an epithelial layers (Rollins-Smith et al., 2002; Toledo and Jared, 1995).

The skin also helps in oxygen uptake and release of carbon dioxide (permeable to gases) from the surrounding environment. It is also permeable to water. Due to this permeable nature of skin, water readily evaporates from the skin and dehydrates the amphibians easily (Wood, 1991). Color of the skin is produced by xanthopores, iridophores and melanophores (color bearing cell organelles) and they can change their skin color according the surrounding environment (Rohrlich and Rubin, 1975).

Amietophrynus is a large genus of true toads native to Africa. Originally, all Amietophrynus species were included in the genus *Bufo*. Recently the *Bufo* genus was split due to large enough taxonomic divergence that *Sclerophrys capensis* Tschudi, 1838 is the same species as *Bufo regularis rangeri* Hewitt1935 (Ohler and Dubois, 2012; Ohler and Dubois, 2016).

Toad of the genus *Amietophrynus* are typically large and compact with a skin and a dark olive brown basic color at the dorsal, often

turning lighter towards the venter. they have compact parotoid glands situated on the neck , on sides of the head or shoulder regions which accumulate a milky secretion , these parotoid glands have large prominent kidney or parallel rod shape , with a relatively smooth appearance because the warts are quite flat in this region (Perry, 2000 : Rodel 2000).

1.1.2. Classification of the investigated anura species

Kingdom: Animalia

Phylum: Chordate

Sub phylum : vertebrata

Class: Amphibia

Family: Bufonidae

Genus: *Amietophrynus*

Scientific name: 1- *Amietophrynus regularis*

2- *Amietophrynus xeros*

1.1.3. The African common toad (*Amietophrynus regularis*)

The African common toad is a large sturdy toad with a warty skin. Males grow to a snout-to-vent length of 62 to 91 mm (2.4 to 3.6 in) and females reach 70 to 130 mm (2.8 to 5.1 in) (Rödel, 2000). The paratoid glands are large and either parallel or kidney-shaped and the male has a single vocal sac under the chin. The dorsal surface is dark olive-brown

with dark patches on the back, often arranged fairly symmetrically, and in younger animals, there is a paler band along the spine (Rödel, 2000).

1.1.4. Amietophrynus xeros

Amietophrynus xeros is a species of toad in the family *Bufonidae*. It is a sub-desert toad is a medium-sized species with a broad head and blunt snout. The dorsal surface bears conical warts tipped with black spines. This toad varies in colour from cream or pale grey to dark brown and has three pairs of symmetrical dark-edged markings and various other dark blotches. The underparts are cream with variable amounts of mottling. Females have pale throats while those of males are darker, and males also have vocal sacs on the throat and some irregular red markings on the outer thighs (Rödel, 2000).

1.1.5. Amphibian glands and their secretions

Amphibians were able to spend longer periods in the terrestrial environment through gradual acquisition of a set of adaptions both morphofunctional and behavioral, on such adaptation was the multicellular exocrine glands of the skin. Two fundamental types of cutaneous glands are to be observed in the amphibians: mucous and granular (serous or venom) glands. The mucous glands produce a mucous which plays a part in a variety of functions: cutaneous respiration, reproduction, thermoregulation and defense. The granular glands produce a toxic or repellent secretion with an effect on varous

vertebrate species (Toledo and Jared, 1995) and also produce antimicrobial peptides (Rollins et al., 2002).

The amphibian dermatous glands or granular glands are highly specialized skin structures which appear during metamorphosis as epithelial derivatives and generally made up of a gland alveolus formed by a secretory layer covered externally by a myoepithelial layer. This contractile layer is related with the extrusion of glandular products. They are responsible for the production of noxious or toxic substances with a variety of pharmacological effects (Lazarus and Attila, 1993).

Since the discovery of bombinin in the skin of the frog *Bombina variegata* by Csordás and Michl in 1969 (Csordás and Michl, 1970), amphibian skin had become an important source of new antimicrobial agents and several novel molecules of antimicrobial peptides that showed unprecedented structural features (Barra and Simmaco, 1995). Amphibians release the secretions in response to stress, injury and predator attack, the secretions are a complex plethora of biologically active components including alkaloids, biogenic amines, steroids, peptides and proteins (Lazarus and Attila, 1993; Perry, 2000).

In the Anura, the granular glands have a syncytial secretory layer; the syncytium vary in type and concentration. In general the venoms contain peptides, guandidine derivatives, biogenic amines, steroids, and alkaloids. In terms of pharmacological effects, these substances are cardiotoxic, haemotoxic, neurotoxic,

myotoxic, hypotensive, hypertensive and anesthetic. Clusters of granular glands are to be observed in certain regions of the body. These are known as macroglands, and may be divided into parotoid, paracnemid, lumber, hedonic and pectoral. Parotoid macroglands consist principally of a large concentration of granular alveoli, which contain the venomous secretion. In toads, the secretion is basically steroids and biogenic amines (Toledo and Jared, 1995)

1.1.6. *Amietophrynus spp*. glands and their glands secretions

Toads of the genus *Amietophrynus* are typically large and compact with a warty skin and a dark olive brown basic color at the dorsal often turning lighter towards the venter .they have compact parotoid gland situated on the neck, The parotoid gland (alternatively, paratoid gland) is an external skin gland on the back, neck, and shoulder of toads and some frogs and salamanders (Abugabr Elhag,et al 2009). It can secrete a number of milky alkaloid substances (depending on the species) known collectively as bufotoxins, which act as neurotoxins to deter predation (Chen & Kovaříková, 1967). These cutaneous glands are called parotoid as they are somewhat similarly positioned to mammalian parotid gland, although these have a different function, excreting saliva within the mouth rather than externally excreted defensive chemicals (Jared et al., 2009).

Parotoid glands of toads (*Amietophrynus*) consist of large aggregations of granular glands located between the otic region of the skull and the scapular region (Rödel, 2000). The circulatory patterns of the paratoid glands were determined by prefusing the vascular systems of *Bufo alvarius*, *B. marinus*, *B. terrestris*, and *B. valliceps* with either India ink or Microfil then studied by gross dissection and microscopic investigations in comparison with the arrangement of the vessels of *Rana sphenocephala* which lacked paratoid glands (Hutchinson and Savitzky, 2004).

In genus *Amietophrynus*, these gland are histologically parotid macroglands (Toledo and Jared, 1995), which are compact glands situated on the neck or sides of the head or shoulder regions (Rödel, 2000) and accumulate a milky secretion which is known to be rich in low molecular weight constituents of varied molecular types such as steroids, amines and peptides; much of the interest in these secretions is a consequence of their bioactivities, which may have partly evolved as a mode of anti-predator defense (Clarke, 1997). While examining secretion from two *Amietophrynus* species for the presence of such low relative molecular mass peptides by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE) , it was observed that there were a number of proteins present of much higher relative molecular mass than might have been expected (Perry,2000). A study based on using thin layer chromatography revealed differences in

peptides content in the paratoid secretions of *A. regularis* and *A. xeros* (Abugabr et al., 2008).

1.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most qualitative protein analyzing method which is based on the separation of proteins according to their sizes (Laemmli, 1970). The polyacrylamide gels is a formation of polymerized acrylamide monomers in the presence of a crosslinking agent named *N*,*N*'-methylene-*bis*-acrylamide (*bis*-acrylamide) which consists of two acrylamide molecules linked by a methylene group. The polymerization of acrylamide was initiated by the addition of ammonium persulfate and the base *N*,*N*,*N*',*N*'-tetramethylenediamine (TEMED) as the TEMED catalyzed the decomposition of the persulfate ion to give a free radical. Eventually a well-defined crosslinked matrix gel is formed.

A stacking gel over the separating gel was achieved to increase the protein concentration that assisted the sharpening of the separated proteins into bands that differ in the protein size. The band-sharpening effect (isotachophoresis) relied on the negatively charged glycinate ions. Protein samples are runned on SDS- PAGE in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduced the presence of disulfide bridges. Sodium dodecyl sulfate (SDS) is an anionic detergent and bonded strongly to the proteins and caused their

denaturation which converts the total denaturated protein to a rod shape structure accompanied with the negative charge of SDS. Protein samples pass through the stacking gel and then been separated by the separating gel as they move to the anode under the applied electric field. While passing through the porous gel, segregation caused retention of larger proteins. The experiment would reach the end when the unretarded dye reaches the bottom of the gel. Then the gel was washed by destain solutions overnight to remove the background dye from the gel leaving the stained proteins to be visible.

Precautions should be considered while applying SDS-PAGE which included that acrylamide is a potential neurotoxin particularly when weighing out acrylamide. Proteins, pure or in simple mixtures, should be dissolved at 1–0.5 mg/mL with the avoidance of low pH levels of buffers, this called to treat the proteins by dialysis. β-mercaptoethanol is susceptible to oxygen which any exposure reduces its power.

1.3. Bradford assay

Bradford assay is a rapid and accurate method for the estimation of protein concentration (Bradford, 1976) and more sensitive than the Lowry method and is subjected less interference by common reagents and non-protein components of biological samples (Lowry et al 1951). Bradford assay relied on the binding of the dye Coomassie Blue G250 to

protein, mostly most readily to arginyl and lysyl residues of proteins, and the protein concentration was estimated by determining the amount of dye in the blue ionic form at 595 nm.

1.4. Objectives

1.4.1. Main Objective

The study aimed to evaluate the taxonomical value of peptides and proteins in the parotoid glands secretions of *A. regularis* and *A. xeros* collected from Shendi area

1.4.2. Specific Objectives

To Estimation of total protein concentration between two *Amietophrynus* species in Shendi area

Characterization of protein of the parotoid gland secretions of *A. regularis* and *A. xeros* using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE)

CHAPTER TWO

2. MATERIALS AND METHODS

2.1. Collection of Toad individuals

Amietophrynus species were collected from stagnant pools in Shendi state and kept in wet glass and plastic aquaria for this study, the individuals were classified by examining their morphological features according to (Abugabr Elhag 2007)(Abugabr Elhag et al 2009)(Rödel, 2000) and (Abugabr et al., 2008). Individuals of *A. regularis* and *A. xeros* were separated in different wet aquaria

2.2. Parotoid glands secretion of Amietophrynus spp.

Within each species, parotoid glands secretions were obtained manually from each one, separately, by manual compression and massaging the parotoid glands; the secretion dissolved in 5 ml deionized water.

2.3. Total protein concentration of parotoid gland secretions

For estimating the total protein the study adopted Bradford essay of total protein estimation (Bradford, 1976; Kruger, 2002). Coomassie Blue G250 (100 mg) was dissolved in 50 mL of 95% ethanol and then mixed with 100 mL of 85% phosphoric acid and been completed 1 L with distilled water and then filtered by Whatman no. 1 filter paper and then stored in a dark bottle at (25^{o}C).

The standard protein used to determinate the total concentration of protein was bovine serum albumin (BSA). The standard solution was

prepared by solving 0.1gram of albumin in 100 ml distilled water (1\1000, w\w). Different concentration were obtained by using dilution method. All concentration were tested by Bradford test to detect the correspondent absorbance. Results were used to obtain a standard formula for this study.

The experiment was conducted by inserting 100 µg of protein in a test tube. each experiment a 5 mL of protein reagent was added to each tube and mixed by gentle vortex mixing to avoid the phenomena of foaming which could lead to poor reproducibility. The mixtures were measured at 595 A^{o} beside the standard solutions and the blank reagent that contained all the ingredients except the protein sample which was substituted by distilled water.

2.4. Statistical analysis

Statistical analyses were applied on the experimental values of the protein concentration in the paratoid gland secretions by employing t – test and F- test in excel 2016.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS -PAGE)

2.5.1. Experimental steps of SDS-PAGE

The molecular weights of the peptides and the proteins were established by SDS PAGE (Judd, 2009; Laemmli, 1970; Walker, 1996). The electrophoresis buffer was consisted of a mixture of Tris (12 g), glycine (57.6 g), and SDS (2.0 g); the volume was completed up to 2 L

with distilled water. The sample buffer was made by mixing 0.6 *M* Tris-HCl, pH 6.8 (5.0 mL), SDS (0.5 g), Sucrose (5.0 g), β-Mercaptoethanol (0.25 mL), 0.5% Bromophenol blue (5.0 mL) and the mixture was completed to 50 mL with distilled water. The protein stain consisted of 0.1% Coomassie brilliant blue R250 in 50% methanol and 10% glacial acetic acid by dissolving the dye in the methanol and water followed by the addition of acetic acid. The solution was filtered by Whatman No. 1 filter paper. The destain solution consisted of 10% methanol and 7% glacial acetic acid. Samples and dyes were loaded by micropipettes with fine types.

The internal surfaces of the plates were cleaned and dried then joint together forming a cassette then clamped in a vertical position. After insuring the position of the plates the separating gel was prepared to be inserted in the cassette. The selected separating gel was 15% that was made by the mix of 1.875 *M* Tris-HCl, pH 8.8 (8.0 mL), water (11.4 mL), Stock acrylamide (20.0 mL), 10% SDS (0.4 mL) and Ammonium persulfate (10%) (0.2 mL). The mix was degassed for 30 seconds. Then 14 μL of TEMED was added gently with swirling the flask for 15 minutes to ensure mixing to initiate the polymerization reaction.

The gel was transferred to the gel cassette by a pipet by pouring it carefully down one edge between the glass plates until it was nearly 1 cm below the comb. Distilled water was added carefully done one edge into the cassette to ensure a creation of a smooth surface. After the gel

was left to set, the distilled water was removed and the stacking gel was added. The stacking gel was made by mixing 0.6 *M* Tris-HCl, pH 6.8 (1.0 mL), Stock acrylamide (1.35 mL), Water (7.5 mL), 10% SDS (0.1 mL) and 10% Ammonium persulfate (0.05 mL) followed by a degassing process. Then 14 μL of TEMED was added. The stacking gel was added to the gel cassette until the solution reached the cutaway edge of the gel plant. The comb was inserted to the solution and left to set for about 20 minutes. The comb was then removed carefully from the stacking gel, followed by rinsing any nonpolymerized acrylamide solution from the wells by the electrophoresis buffer. Spacers were removed and the cassette was assembled in the electrophoresis tank that was filled after that with the electrophoresis buffer.

Samples were loaded slowly by a syringe needle just at the bottom of the wells followed by the connection of the power pack to the apparatus. The electrophoresis was exposed to a 200V and experiment was stopped when the visible bromophenol blue reached the bottom of the gel. Then the gel apparatus was dismantled, the stacking gel was discarded and the separating gel was placed in stain solution accompanied with gentle shaking for 2 hours. The gel was destained to visualize the protein bands.

2.5.2. Calculation of Rf values and molecular weight of peptides and proteins in the paratoid gland secretions of Amietophrynus spp.

Mobility of peptides was calculated by the *Rf* values and molecular weights by the following equations (1 and 2) (Rybicki and Purves, 2006; Rybicki Edward and Maud, 1996).

$\boldsymbol{Rf}_{\textbf{peptide}}$**:**	$\frac{\boldsymbol{D_p}}{\boldsymbol{D_{Bpb}}}$	**(Eq. 1)**
$\textbf{log}_{\textbf{10}}\,\boldsymbol{MW_{peptide}}$**:**	mD+b	(Eq.2)

Where *Rf* is the retention factor of the mobility of the peptide in the gel, D_p was the distance moved by the peptide, D_{Bpb} was the distance moved of the Bromophenol blue, *MW* was the molecular weight of the peptide, *m* was the slope of the standard curve, *D* was the distant moved by the peptide and b was the *Y* axis part.

CHAPTER THREE

3. RESULTS AND DISCUSSION

3.1. Preliminary observations of the paratoid gland secretions

Both *Amietophrynus* spp. Paratoid glands withintheir distinguishable morphological features (Clarke, 1997). These glands were distinguished by their protruded appearance as rod-like shapes (Abugabr Elhag et al., 2007). The two species were differentiated by the appearance of a red patch on the thighs of *A. xeros* (Rödel, 2000).

Paratoid glands showed high response to the applied extraction method to secrete the skin secretions, the textures of the collected secretions varied in their density indicating variation in the moisture content regardless to the species; this implied that the texture of the secretion is not relevant to any evolutionary or taxonomical parameter. However, all the secretions illustrated tendency to dissolve entirely in distilled water with the assistance of gentle shaking; an observation that indicated either none or very minute existence of hydrophobic compounds.

3.2. Bradford test of protein content in paratoid gland secretions

Bradford test was conducted according to paragraph (2.3.). The standard solutions of the protein (BSA) were employed to establish the standard curve (Figure. 1) for estimating the protein content in each sample. Results illustrated high variance intra and inter-species (Figure. 2). This variety indicated that further studies should be conducted for

further clarification. However, the results revealed the existence of higher amounts of proteins in the secretions of the *A. regularis* accompanied with a wider range of concentrations, indicated by the higher standard deviation that could be related to evolutionary perspectives that is distinguished to the certain species.

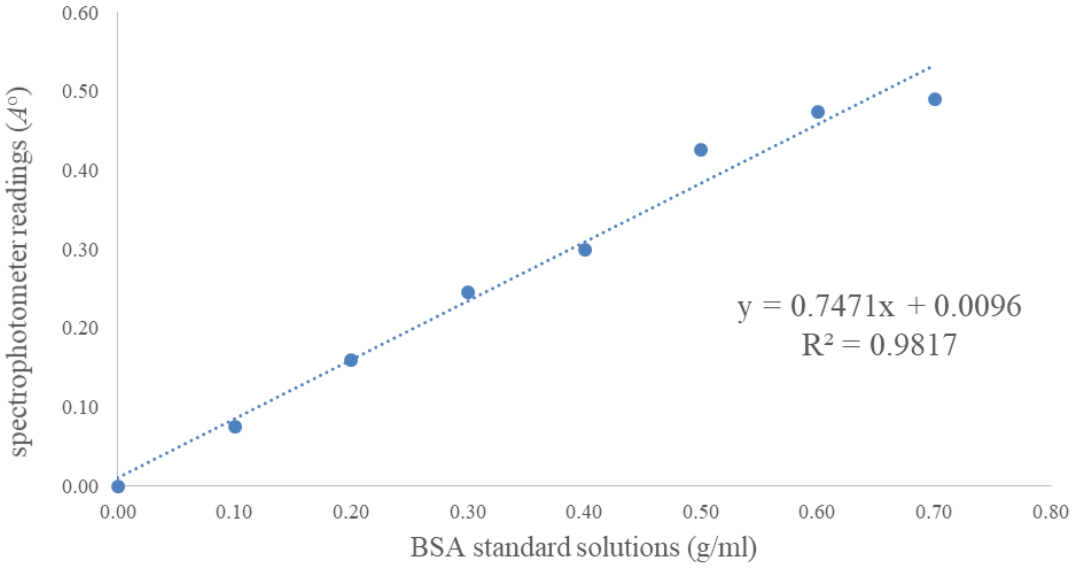

Figure 1: NM spectrophotometer readings vs. concentrations of BSA(g/mL)

Table 1: Total protein concentration (g/g) in paratoid gland secretions (A comparison between the Amietophrynus spp.

Protein concentration (g/g)		Protein concentration (%)	
A. regularis	*A. xeros*	*A. regularis*	*A. xeros*
0.103	0.110	10.293	11.024
0.104	0.116	10.379	11.562
0.107	0.116	10.710	11.624
0.110	0.122	11.031	12.151
0.137	0.125	13.679	12.530
0.149	0.128	14.931	12.812
0.153	0.129	15.307	12.912
0.161	0.131	16.068	13.124
0.166	0.131	16.593	13.135
0.174	0.132	17.365	13.212
0.200	0.141	20.031	14.135
0.225	0.145	22.507	14.451
0.227	0.169	22.693	16.895
0.228	0.171	22.753	17.135

0.228	0.184	22.768	18.430
0.228	0.209	22.807	20.862
0.229	0.213	22.893	21.257
0.229	0.217	22.931	21.662
0.230	0.217	23.031	21.691
0.231	0.224	23.065	22.395
0.231	0.226	23.079	22.591
0.231	0.226	23.110	22.612
0.271	0.228	27.053	22.824
0.280	0.228	28.031	22.835
0.285	0.229	28.493	22.851
0.290	0.229	29.010	22.857
0.292	0.229	29.165	22.862
0.293	0.229	29.253	22.866
0.294	0.229	29.431	22.895
0.295	0.229	29.468	22.930
0.300	0.240	30.010	23.957

0.301	0.245	30.079	24.466
0.307	0.257	30.710	25.735
0.309	0.261	30.879	26.062
0.322	0.267	32.193	26.657
0.323	0.272	32.293	27.191
0.333	0.272	33.253	27.212
0.337	0.296	33.665	29.566
0.339	0.299	33.907	29.930
0.344	0.314	34.353	31.366
0.354	0.320	35.393	31.991
0.355	0.335	35.507	33.495
0.360	0.367	36.031	36.724
0.369	0.371	36.893	37.066
0.394	0.378	39.431	37.751
0.397	0.392	39.731	39.191
0.409	0.403	40.868	40.257
0.411	0.405	41.068	40.495

0.417	0.414	41.693	41.430
0.418	0.415	41.765	41.451

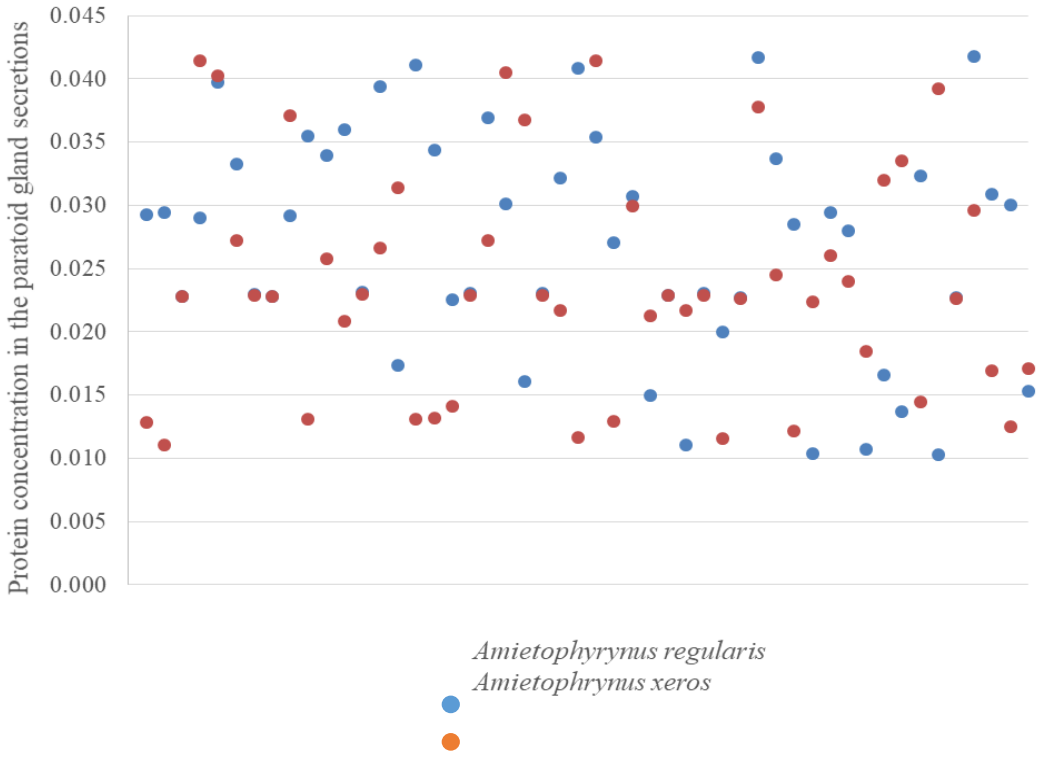

Figure 2: Dispersion of protein concentrations in the parotoid gland secretions of the Amietophrynus population in Shendi area

The average of protein content of the *A. regularis* was 0.2695± 0.0889 g/g while the *A. xeros* was 0.2386± 0.0894 g/g which corresponded to the percentages 26.95± 8.89% and 23.86± 8.94 % respectively (Figure 3). The results were in accord with the statement of Perry (2000) that the paratoid gland secretions of *Amietophrynus* spp. (formally *Amietophrynus spp*) ranged between 25-35% when *B. mauritanicus* and *B. calamita* were studied. The study also strongly agreed with previous findings that detected the range of protein concentration in the paratoid secretions of the *Amietophrynus* spp. (Formally *Bufo* spp.) was 22.6-40.8% (Abugabr Elhag et al., 2007)(Abugabr Elhag et al., 2009). These finding suggested that the total range is approximately constant for the Amietophrynus spp.; however, the *A. xeros* illustrated a less average than that of *A. regularis*, which might indicate a differentiation factor between the species that could be indicated at physiological and molecular levels.

3.3. Statistical analysis

The results of the Bradford estimation of protein concentrations in the paratoid gland secretions were evaluated by F test and t student test for estimating the statistical significance of the experimental values.The F statistic test was conducted to compare the joint effect of all the experimental variables together. Results (Table 1) showed that the F statistical value was higher than f critical which basically illustrated

significant difference between the two groups of toad secretions: furthermore the variables also showed different significancy due to the lower values of p than 0.05.The *t* student test compared the two averages of the protein concentrations in the toads' secretions indicating any differences between them and the possibility of these differences to occur by chance. On the other hand, the *t* score is a ratio between the difference between two groups and the difference within the groups. Table 2 illustrated that the values of *t* for one tail and two tails were larger than critical t values.

3.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Results of Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) illustrated a high variety of peptide bands in paratoid gland secretions of both toad groups. Based on differences in the morphology features of the toads in each group, *A. xeros* were divided to 4 groups while *A. regularis* specimens were divided to 5 groups. Therefore further explanation of this diversity was strengthened by simple mathematical models of Retention factor (*Rf*) and determination of molecular weight according to the equations (1 and 2) (Rybicki and Purves, 2006). The *Rf* was a factor that indicated the mobility of the peptide related to the mobility of the bromophenol blue

that represented the whole path that the protein crosses through the gel. Table 3 illustrated this factor to each peptide.

Further on, the molecular weights were determined by (Eq.2); first the standard curve was established by plotting the Log_{10}MW of the protein markers against the distance (Figure 4), the generated equation was employed to generate the Log_{10}MWs of the peptide samples which were then reversed to the actual molecular weights (Table 4).

Results of SDS-PAGE implied that the secretions from the two species contained mixtures of peptides in relative molecular mass range of 50-281 kDa. The results indicated a distinguishable peptide pattern for the *Amietophrynus* spp. (Figure 5). A common peptide (52kDa) was identified in all the secretions which suggested a mutual gene origin in both species so genus is identified by band size 52, *A. xeros* spp. illustrated exclusively 3 peptides (178-281 kDA) that were not found in *A. regularis secretions*. One of the *A . xeros* (X_3) specimens demonstrated a lonely peptide (60kDa), this implied that there are two sub species of *A. xeros* with the existence of a majority that doesn't possess the gene that produced the (60kDa) protein.X_2 ,X_3 andX_4 samples possessed amutual protein(106kDa).So species X is identified by band 106.

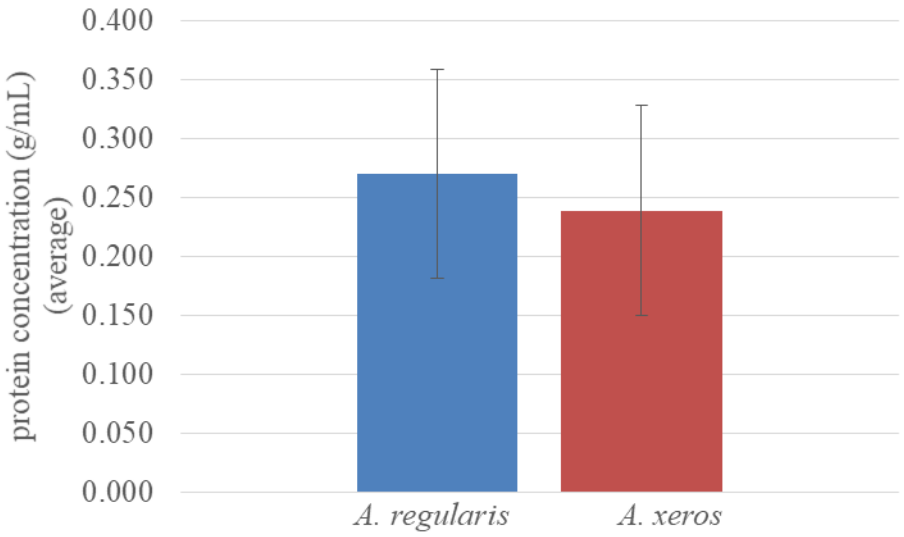

Figure 3: A comparison of the average of protein concentration (g/mL) between the paratoid gland secretions of A. *regularis* and *A. xeros*

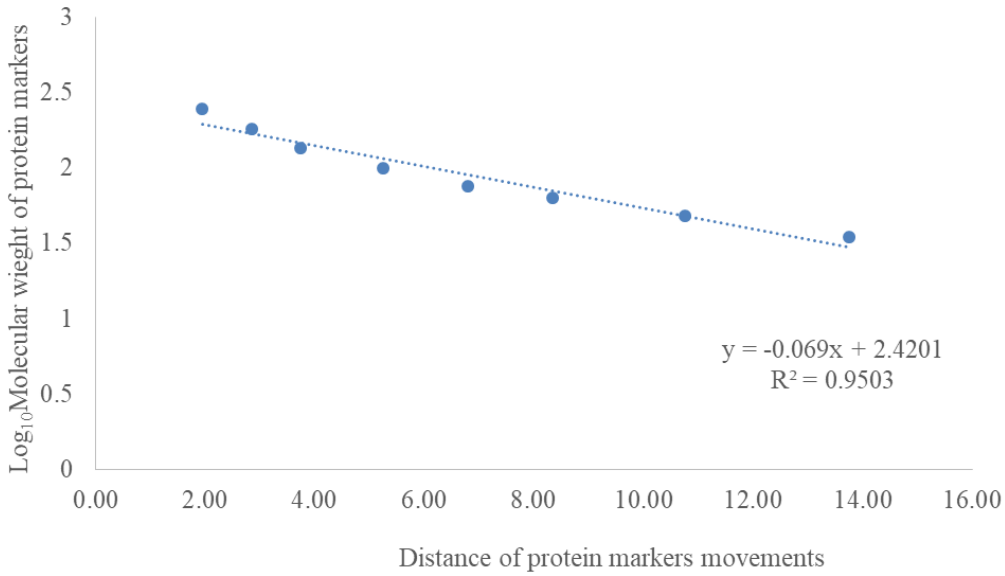

Figure 4: standard curve generated by the plotting of Log10 of the molecular weights of the protein markers vs. the distances moved in the gel

Table 2: Retention factors of protein from the paratoid gland secretions of A. xeros (X1,2,..,4) and A. regularis (R1,2,..,5).

Protein marker	X_1	X_2	X_3	X_4	R_1	R_2	R_3	R_4	R_5
0.132	0.038	0.047	0.045	0.051	0.318	0.194	0.329	0.200	0.252
0.193	0.103	0.119	0.106	0.113	0.379	0.324	0.379	0.261	0.344
0.253	0.151	0.163	0.154	0.168	0.536	0.374	0.582	0.400	0.603
0.355	0.384	0.403	0.390	0.390	0.586	0.536	0.700	0.468	0.709
0.459	0.575	0.583	0.579	0.582	0.707	0.583		0.589	
0.564	0.716	0.722	0.637	0.712		0.705		0.707	
0.726			0.712						
0.929									

Table 3: Molecular weights of protein from the paratoid gland secretions of A. xeros (X1,2,..,4) .

number of bands	molecular weight kDa	x1	x2	x3	x4
1	281	1	0	**0**	0
2	277	0	0	**1**	0
3	275	0	1	**0**	0
4	274	0	0	**0**	1
5	247	1	0	**0**	0
6	246	0	0	**1**	0
7	242	0	0	**0**	1
8	239	0	1	**0**	0
9	185	1	0	**0**	0
10	184	0	0	**1**	0
11	180	0	1	**0**	0
12	178	0	0	**0**	1
13	108	1	0	**0**	0
14	106	0	1	**1**	1
15	69	1	0	**1**	0
16	68	0	0	**0**	1
17	67	0	1	**0**	0
18	60	0	0	**1**	0
19	52	1	1	**1**	1
	no bands present	6	6	**7**	6
				the different one	n=19

Table 4: Molecular weights of protein from the paratoid gland secretions of A. regularis (R1,2,..,5).

MW	r2	r3	r5	r4	r1	
170	1	0	0	1	0	
147	0	0	1	1	0	
130	0	0	0	0	1	
129	1	1	1	0	0	
115	1	0	0	0	0	
113	0	1	0	0	1	
108	0	0	0	1	0	
93	0	0	0	1	0	
81	1	0	0	0	0	
80	0	0	0	0	1	
73	1	0	0	0	0	
72	0	1	1	0	0	
71	0	0	0	1	1	
52	1	1	1	1	1	
no bands present	6	4	4	6	5	n=14

Table 5: Molecular weights of protein from the paratoid gland secretions of A. xeros (X1,2,..,4) and A. regularis (R1,2,..,5).

Protein markers (kDa)	X_1	X_2	X_3	X_4	R_1	R_2	R_3	R_4	R_5
245	281	275	277	274	130	170	129	170	147
180	247	239	246	242	113	129	113	147	129
135	185	180	184	178	80	115	72	108	72
100	108	106	106	106	71	81	52	93	52
75	69	67	69	68	52	73		71	
63	52	52	60	52		52		52	
48			52						
35									

The *A. regularis* group illustrated more complicated structure than the A. xeros; however, all the samples possessed a mutual protein (71-72kDa). R_1 and R_2 shared all the protein with the existence of an additional one in R_2 (171 kDa). R_3 only shared two protein with R_1 and R_2 (113 and 129 kDa). R_4 shared a protein with R_2 (170 kDa) with two un-mutual proteins and R5 had a mutual protein with R_1, R_2 and R_3 (129kDa) so species R is identified by size 129 and another mutual protein with R_4 (147kDa). This illustrated the complexity of the *A. regularis* spp. and suggested the existence of sub-species within this regularis complex. Generally, the results agreed with the literature (Abugabr Elhag et al., 2007; Perry, 2000) as it stated that the secretions contained protein ranged approximately 12 - 200 kDa, and the ability of the protein profile to exhibit markedly different banding patterns from species to species.

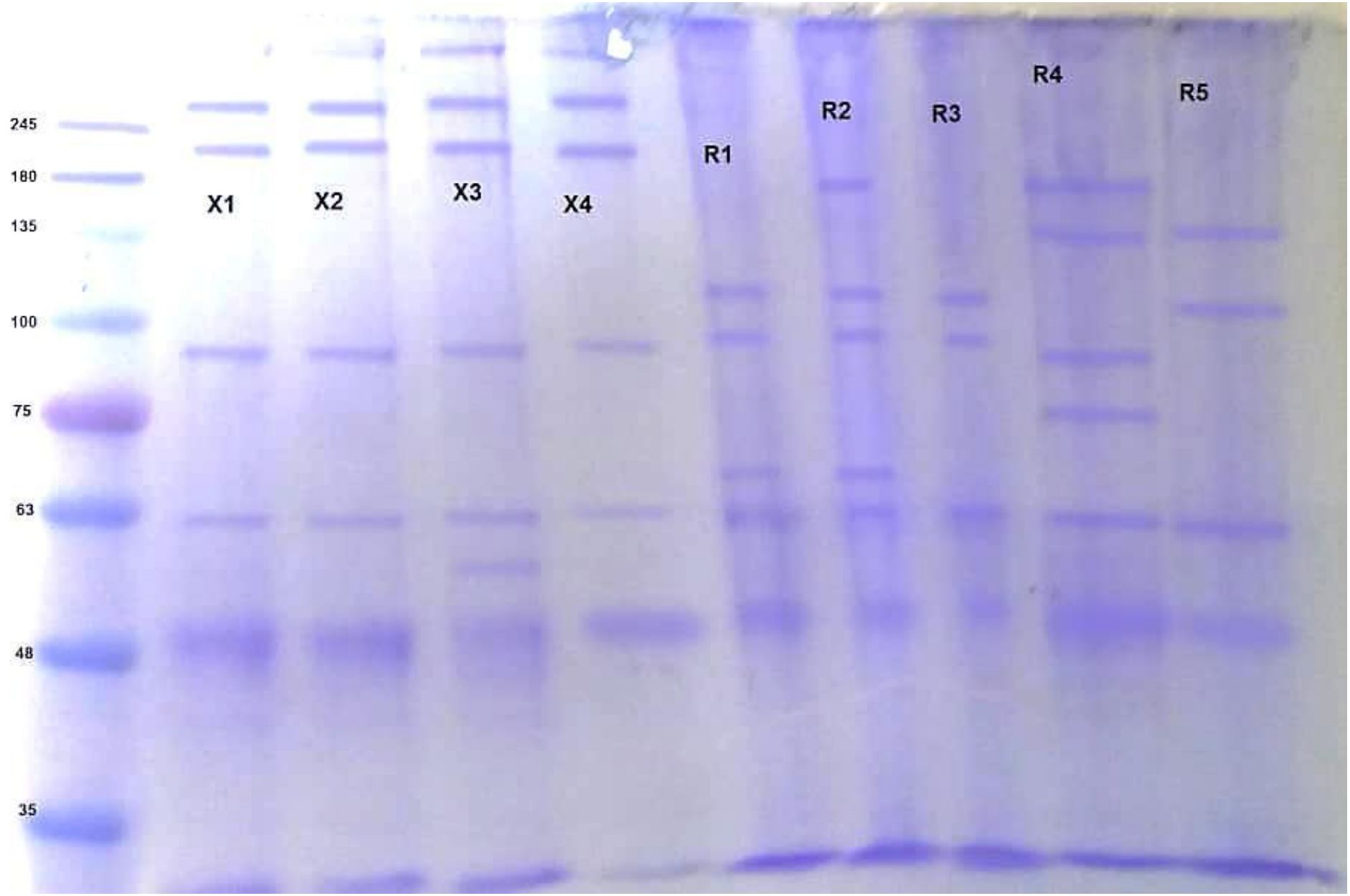

Figure 5: Peptide bands in paratoid glands obtained by SDSPAGE from A. xeros (X1,2,..,4) and A. regularis(R1,2,..,5

CHAPTER FOUR

CONCLUSIONS and RECOMMENDATIONS

CONCLUSIONS:

1-Paratoid glands showed high response to the applied extraction method which was based on physical compression

2 - Bradford test illustrated that the average of protein content was 0.2695± 0.0889 g/g for the *A. regularis* and was 0.2386± 0.0894 g/g for *A. xeros* which corresponded to the percentages 26.95± 8.89% and 23.86± 8.94 %.

3- SDS-PAGE implied that the secretions from the two species contained mixtures of peptides in relative molecular mass range of 50-281 kDa.

4- SDS-PAGE results indicated a distinguishable protein pattern for the *Amietophrynus* spp. that could be employed as a taxonomical tool

RECOMMENDATIONS:

1-Further investigation in the protein brofiles in the paratoid gland secretions of the Amietophrynus species and their potential taxonomical value

2-Further biochemical studies on the molecular structure of the protein of the paratoid gland secretions of the Amietophrynus species

3- Further investigations on the biological role of protein profiles of the paratoid gland secretions.

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Appendix:

Table 6: F test results of comparing the protein concentrations in the parotoid glands secretions of the Amietophrynus spp.

	Amietophrynus regularis	*Amietophrynus xeros*
Mean	0.269528	0.238621
Variance	0.007902	0.007993
Mean (%)	26.95278	23.86213
Variance (%)	0.7902	0.799
Observations	50	50
Df	49	49
F	0.988524	
P(F<=f) one-	0.48397	
F Critical one-	0.622165	

Table 7: t student test results of comparing the protein concentrations in the parotoid glands secretions of the Amietophrynus spp.

	Amietophrynus regularis	*Amietophrynus xeros*
Mean	0.269528	0.238621
Variance	0.007902	0.007993
Observations	50	50
Mean (%)	26.95278	23.86213
Variance (%)	.7902	.7993441
Observations	50	50
Hypothesized Mean	0	
Df	98	
t Stat	1.733415	
P(T<=t) one-tail	0.043084	
t Critical one-tail	1.660551	
P(T<=t) two-tail	0.086167	

t Critical two-tail	1.984467	