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BIOCHEMICAL CHARACTERIZATION OF INHIBIN HORMONE IN THE OVARY OF SHE CAMEL (CAMELUS DROMEDARIES)

(With One Table and 6 Figures)

By

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التعرف على هرمون الانهبين فى مبايض إناث الجمال باستخدام الطريقة الكيميائية الحديثة

أحمد عبد الفضيل رمضان ، عادل محمد أبو الفتوح البحيرى ، مصطفى عبد الفتاح مصطفى ، محمود عباس ابراهيم الجابرى

هدفت هذه الدراسة الى استخلاص وتنقية ودراسة تركيب هرمون الانهبين من مبايض الجمال المصرية باستخدام تقنيات حديثة. تم تجميع السائل الموجود داخل حويصلات التبويض الموجودة على مبايض إناث الجمال بغض النظر عن سن الحيوان أو حجم الحويصلات. تم تنقية السائل الذى تم تجميعه من هرمونى الاستروجين والبروجيستيرون باستخدام الفحم النشيط ثم تم استخدام نوعين من الكروماتوجراف السائل لفصل الأنواع المختلفة من البروتينات. النوع الأول من الكروماتوجراف (سيفاكريل س-٢٠٠) فصل ٣ بروتينات بناء على الوزن الجزيئى لها. تم استخدام النوع الثانى من الكروماتوجراف (سيفاديكس ج-١٠٠) لفصل البروتينات المكونه للبروتين الثالث المعزول من الكروماتوجراف الأول. تم الحصول على هرمون الانهبين فى صوره نقية من البروتين المستخلص من سيفاديكس ج-١٠٠. للتأكد من نقاء الهرمون المعزول من البروتين الثالث، تم استخدام تقنية فصل البروتينات باستخدام التهريب الكهربائى مرتين. فى المرة الأولى تم استخدام مواد تفكك للبروتينات لمكونات صغيره وتم صباغتها بمادة الكوماسى الأزرق اللامع حيث أسفرت عن فصل ٥ مركبات بروتينيه (تراوحت بين ٥٨,٨ -٣ , ٣٢ كيلو دالتون). فى المرة الثانية تم حذف المواد التى تفكك البروتينات وتم صباغتها بمادة نترات الفضة حيث أسفرت عن فصل مركب بروتينى واحد. تم تحليل المركب المعزول من مبايض إناث الجمال باستخدام الكروماتوجراف السائل عالى الكفائه على عامود سى-١٨ حيث تم فصل مركب بروتينى واحد عند الدقيقة ١٠١,٦٥ مما يدل على نقاء المركب. تم أيضا استخدام تقنية الكروماتوجراف ذو الألواح الرقيقة عالى الكفائه لفصل الأحماض الأمينيه المكونه للمركب المعزول، حيث تم الحصول على ٧ أحماض أمينيه مختلفة وبتركيزات مختلفة أعلاها تركيزا الهستيدين.

SUMMARY

Isolation, purification and advanced characterization of hormone inhibin in ovary of female camel are aimed in this study. Pooled follicular fluid was collected from the ovaries of she-camel irrespective to physiological status and age of the animals. Follicular fluid was subjected to 2 types of gel filtration chromatography; Sephacryl S-200 where three peaks of proteins was obtained. The suspected peak to contain inhibin (peak III) resolved from S-200 was subjected to Sephadex G-100 where three peaks were obtained, third peak was suspected to contain inhibin in pure form. To verify the purity of the isolated hormone, the lyophilized fraction containing inhibin was subjected to analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under both non reducing condition, stained with silver nitrate where a single band was resolved, and under reducing condition, where five bands resolved (ranging between 58.8 to 32.3 KDa). Using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) to fractionate the third peak, subunits of inhibin hormone eluted at different retention times. High Performance-Thin Layer Chromatography (HP-TLC) used to determine the N-terminal amino acids contents of the third peak and 7 amino acids resolved with different concentrations where histidine was the most abundant of the amino acids.

***Key words:** Inhibin hormone, ovary, she camel*

INTRODUCTION

Inhibins family is present in a wide variety of reproductive and non reproductive tissues, the main source of inhibin in both sexes appears to be the gonads since its concentration is approximately 5-8 folds higher in the ovarian vein than jugular vein and its concentration was undetectable following gonadoectomy (Roser *et al.*, 1994). The ovary is the major source of circulating inhibin and its level was rapidly disappeared from serum following ovarictomy (Robertson *et al.*, 1988). The ovarian tissues contain the mRNA and proteins of inhibin measured in granulosa cell conditioned media and the expression of the subunit mRNAs of α and β subunits of inhibin have changed with follicular development (Findlay *et al.*, 2001). Follicular fluid has shown to be a potent source of inhibin in different species: pigs (Schwartz and Channing, 1977; Channing *et al.*, 1982) cattle (Henderson and Franchimont, 1981) rats (Hermans *et al.*, 1982; Bicsak *et al.*, 1986;

Zhang *et al.*, 1987) humans (Channing *et al.*, 1984) and other primates (Noguchi *et al.*, 1987). Several studies have shown that the amount of inhibin produced by granulosa cells from large follicles was greater than that from small follicles (Channing *et al.*, 1982), and this fact is reflected in the concentrations of inhibin in the follicular fluid (Tsonis *et al.*, 1987). McLachlan *et al.* (1996) demonstrated that the circulating inhibin levels reflect the mass of active granulosa cells in the ovary, since they correlate significantly with the number of follicles detected by ultrasound. Moreover the expression of βB –subunit of inhibin was greater in the granulosa cells of antral follicles during the luteal-follicular transition, (Hayes et al. 1998), whereas the expression of α-subunit of inhibin appeared relatively constant throughout the follicular development (Drummond *et al.*, 2000 and Findlay *et al.*, 2001). The corpus luteum is also a significant source of inhibin in the luteal phase (McLachlan *et al.*, 1996). Human granulosa cells allowed to be luteinized in culture have the capacity to produce inhibin (Tsonis *et al.*, 1987). In addition the luteal cells of rat and human contained mRNA for the α-subunit of inhibin (Davis *et al.*, 1986, 1987). Indeed, the expression of the βA-subunits of inhibin was highest in the corpus luteum and the dominant follicle (Hayes *et al.*, 1998).

Inhibin was also detected in non-reproductive tissues; the mRNAs that encode inhibin β-subunits have been identified in the heart, skeletal muscle, spinal cord cerebrum, kidney, adrenal, bone marrow, and liver of mid gestational human fetus.

FSH increases inhibin secretion. In primary cultures of rat granulosa cells, crude preparations of FSH stimulated inhibin biosynthesis and its secretion (Woodruff *et al.*, 1987; Turner *et al.*, 1989; and LaPolt *et al.*, 1992). The ovarian steroids may also contribute to the regulation of inhibin subunit gene expression, such as estrogens that can directly increase inhibin mRNAs and its secretion (Turner *et al.*, 1989; Rivier and Vale 1989). The bases of molecular regulation of inhibin subunits synthesis are now clearer than ever. Gonadotropins are potent regulators of inhibin subunit gene expression. Both LH and FSH signal in target cells through G protein-coupled receptors and increase intracellular cAMP levels via activation of adenyl cyclase. Gonadotropins stimulate cAMP response element binding protein (CREB) phosphorylation in granulosa cells and stimulate transcription via CREB-mediated interaction with the CRE in the inhibin tissues specific promoter. This provides an explanation for how the gonadotropins (specifically, FSH) stimulate inhibin (A) expression

throughout the majority female reproductive cycle (Mukherjee *et al.*, 1998 and Tanimoto *et al.*, 1996).

Importance of inhibin in the reproductive cycle of many known animals is well established and understood, moreover the isolation and purification of this peptide hormone from these animals has been completed many years ago. Indeed, isolation, purification, and partial identification of this hormone from she camel is aimed in this study for many reasons: first, camel as a domestic animal in Egypt still needs great attention to fully understand its reproductive cycle with all its aspects, second, camel is a unique domestic animal in many physiological, anatomical, pathological, and reproductive aspects, third, increasing the wealth of camel in Egypt needs many efforts to gather information about its reproduction.

MATERIALS and METHODS

Collection of follicular fluid

Large follicles were collected from different abattoirs, including pregnant female camels, irrespective to the physiological status of the follicles, nor the age of the animal. The follicular fluid was aspirated from the ovarian follicles, pooled and transported on ice bags to the laboratory within 30 minutes. Suitable amount of follicular fluid (100 ml) was collected to fulfill the experiments. The follicular fluid was centrifuged at 5000 rpm in cooling centrifuge at 4° C for 15 minutes; the clear supernatant was carefully aspirated and kept at -20° C. Protein concentration was estimated using Biuret Reagent as described by (Henary *et al.*, 1968).

Refining follicular fluid from steroids

The follicular fluid was refined from all steroids according to (Welschen *et al.*, 1977) by adding 50 mg of activated charcoal to each 1 ml of follicular fluid while stirred at room temperature for 30 minutes, and centrifuged at 4° C at 5000 rpm for one hour then the supernatant was carefully aspirated and preserved at -70° C until subsequent analysis.

Extraction of ovarian peptides

The supernatant of the follicular fluid was added drop-wise to 90% (v/v) acetone; the precipitate was dissolved in 95% acetic acid and stirred overnight at room temperature. The protein was again precipitated by adding ethanol 90% (v/v); the precipitate was dissolved in water, dialyzed, and lyophilized.

Purification of ovarian peptides

1- Purification using Sephacryl S- 200 HR (2.5 x 40 cm) using ECONO System manufactured by Bio-Rad Laboratory USA. The follicular fluid was diluted in 15 ml of 0.05 mol ammonium acetate solution and slowly applied onto the top of the gel. The mobile phase was 0.05M ammonium acetate. The column effluent was monitored at 280 nm and collected in tubes (1 ml/tube). Fourteen runs were done, and the number of tubes per each run was ranged between16 to 19 tubes. Protein concentration was estimated using Bradford reagent (1976) and diagrammed to plot the resolved peaks. The tubes representing each peak in every run were pooled together.

2- Purification using Sephadex G-100 column (2.5 x 40 cm) fraction number III produced from the Sephacryl S-200 gel was applied to Sephadex G-100 column and eluted with 25% acetic acid as a mobile phase. One ml fraction size was collected per each tube and protein concentration was estimated and diagrammed to plot the resolved peaks. The tubes representing each peak in every run were pooled together.

Electrophoresis

The electrophoresis technique was deployed to find out the purity of the suspected protein peak containing inhibin. The technique was done under both non-reducing and reducing conditions using Bio-Rad USA mini gel according to (Laemmli, 1970). Under non-reducing conditions, both SDS and 2- mercaptoethanol (ME-2) were eliminated from the technique and kept under reducing conditions. The molecular weights of protein bands in the gel were estimated in comparison with that of the standard protein markers (Bio-Rad 10,000 to 100,000 Daltons containing sex proteins) using software Gel Pro Analyzer Version 3.1.

Detection of Subunits of inhibin using Reversed Phase High Performance Liquid Chromatography (RP-HPLC) (Moore et al., 1994)

Detection of inhibin subunits was carried out according to the method described by (Moore *et al.*, 1994). An HPLC CBG England supplied with UV detector and a C18 column was used (1cm x 25cm) and the mobile phase consisted of gradient of 0.01% trifluoroacetic acid (TFA) in water and 0.01% TFA in 80% acetonitrile. Total run time was 130 minutes. Flow rate was adjusted to 2 ml/min and the effluent detected at 280 nm using UV detector. The resulted chromatogram was analyzed with Winchrom 3 software.

Identification of the N- terminal amino acids contents of the separated peptides using High Performance Thin Layer Chromatography (HP-TLC)

Fraction 3 that contained inhibin was placed in a hydrolysis tube and 200 µL of hydrolysis solution (6 N hydrochloric acid containing 0.1% to 1.0% of phenol) per 500 µg of lyophilized peptide were added. The sample was hydrolyzed at 110° C for 24 hours in an inert atmosphere. The hydrolyzed sample then dissolved in 10 µl of 0.2 M sodium bicarbonate and 10 µl 1-dimethylaminonaphthalene-5-sulpfonyl chloride (Dansyl chloride, DNS-CL) and mixed well for labeling of N-terminal amino acids with fluorescent Dansyl chloride. The tube was sealed with parafilm and incubated at 37° C for 1 hour then 10µl of 50% pyridine was added. Silica plates (10X10 cm) were used to separate different amino acids of inhibin. The developing solvent was prepared by adding 5 g EDTA-disodium salt to 50 mL distilled water and the pH was adjusted to 9 using 1 N NaOH, then 10 mL of n-butanol was added and the solution was vortexed well and 35 mL diethylether was added, vortexed and the upper phase was then used as developing solvent. Densitometric evaluation of the amino acids was done by CAMAG TLC Scanner3 using Lab data System and CATS evaluation software. Scanning of the plates was made by fluorescence at 280 nm using mercury lamp, monochromator bandwidth 30 nm; slit dimensions 0.3 x 4 mm. The amino acids were detected in comparison with the standard amino acids solution.

RESULTS

Figure (1) represents the fractionation pattern of follicular fluid of she camel. The chromatogram shows 3 protein peaks (I, II, and III) resolved from Sephacryl S-200 HR gel filtration column chromatography.

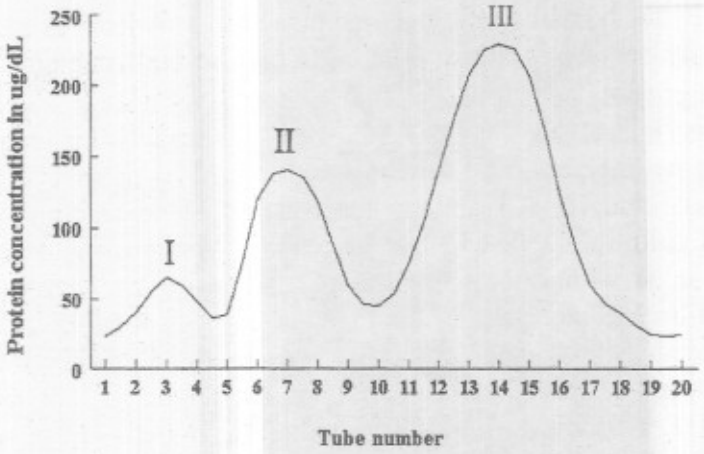

Fig. 1: Chromatogram of the eluted protein peaks from follicular fluid of she camel on Sephacryl S-200 HR gel filtration column chromatography.

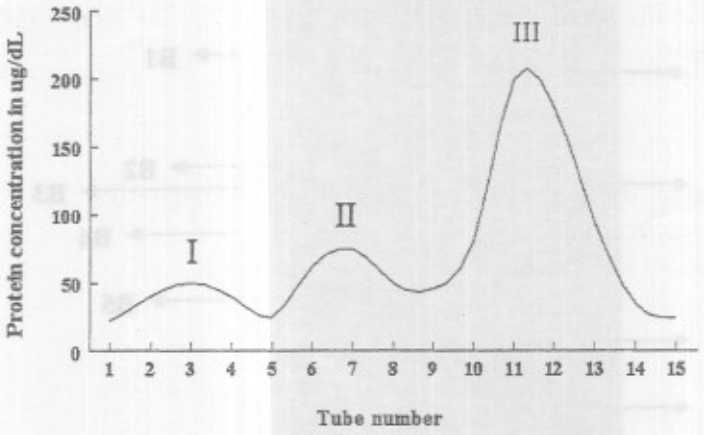

Fig. 2: Represents the fractionation pattern of peak number III resolved from S-200 gel and fractionated on G-100 gel. The chromatogram shows 3 protein peaks.

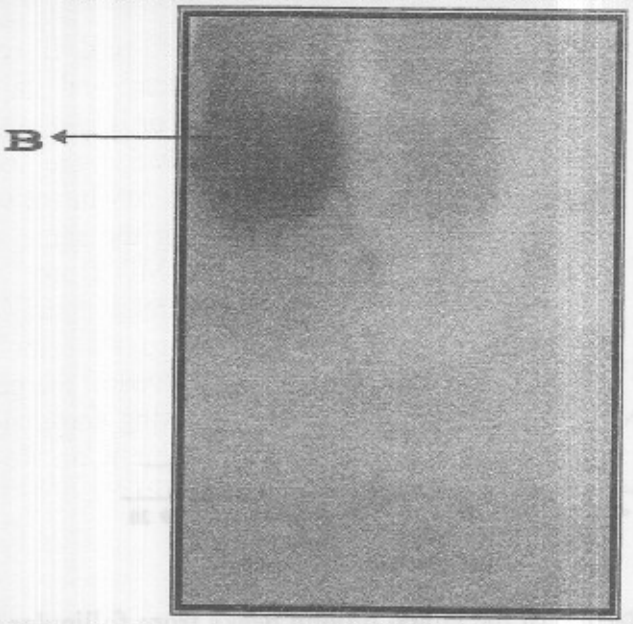

Fig. 3: Electrophoretic pattern (SDS-PAGE) of isolated and purified inhibin from ovarian follicular fluid of she camel (peak number III from G-100 gel) run under non-reducing conditions and stained using silver nitrate.

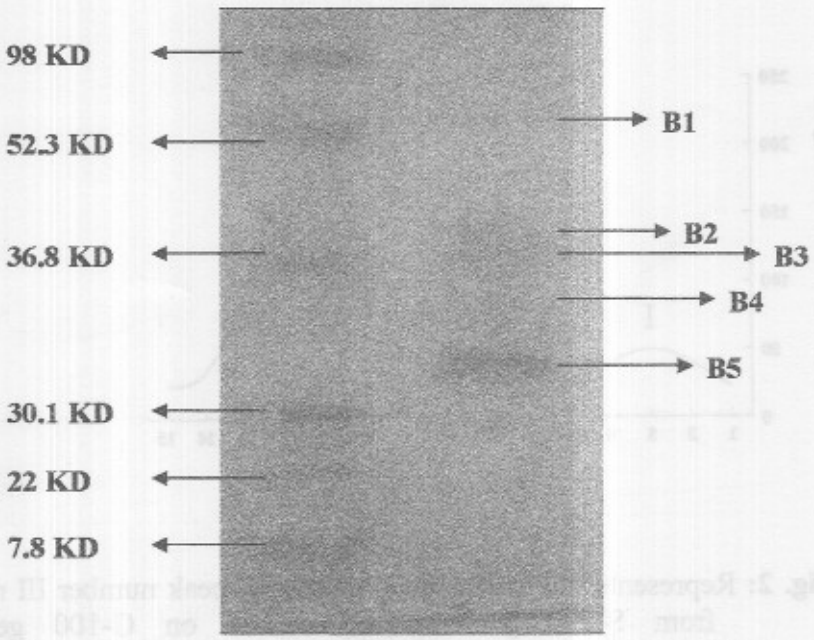

Fig. 4: Electrophoretic pattern (SDS-PAGE) of isolated and purified inhibin from ovarian follicular fluid of she camel and run under reducing conditions and stained using Coomassie brilliant blue stain

Electrophoretic pattern of isolated and purified peak III resolved from G-100 (inhibin) from follicular fluid of she-camel and stained by silver nitrate (Figure 3) clearly shows the presence of only single band of protein (B). The condition of electrophoresis excluded the reducing substances (SDS and ME-2) to allow the sample to run intact without fractionation on the gel. On the other hand running the same sample under reducing condition (in presence of SDS and ME-2) and stained with Coomassie brilliant blue R-250 allowed the fractionation of the isolated inhibin into different subunits according to their molecular weights (Figure 4). There are five protein bands resolved in the gel with different amounts and molecular weights. The following chromatogram shows the fractionation of isolated and purified inhibin on Reversed phase high performance liquid chromatography (RP-HPLC).

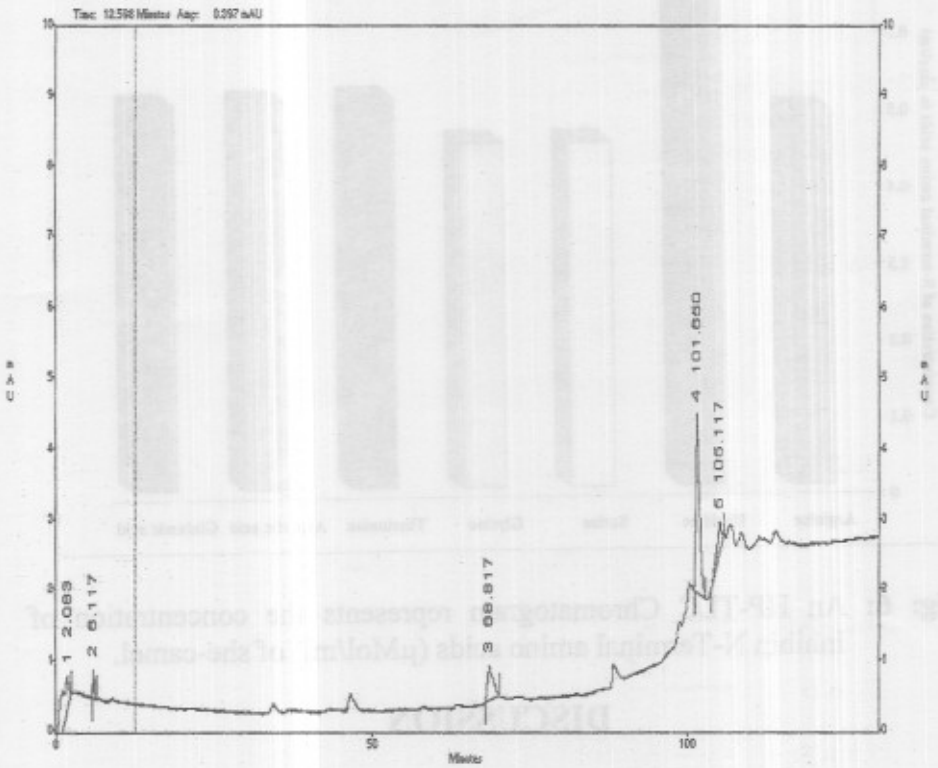

Fig. 5: Chromatogram represents different fractions of inhibin with different retention time separated on –RP-HPLC using C-18 column.

Table 1: Showing migration distance (MD), retention factor (Rf) and N terminal of amino acids concentration of she-camel inhibin on HP-TLC

Amino Acids	Migration distance	Retention factor	MW	Concentration µM/mL
Arginine	6.6	0.110	174.2	0.488
Histidine	8.9	0.148	155.16	0.684
Serine	19	0.317	105.09	0.449
Glycine	22.5	0.375	75.07	0.499
Threonine	27	0.450	119.12	0.509
Aspartic acid	33	0.550	133.1	0.506
Glutamic acid	35	0.583	147.13	0.503

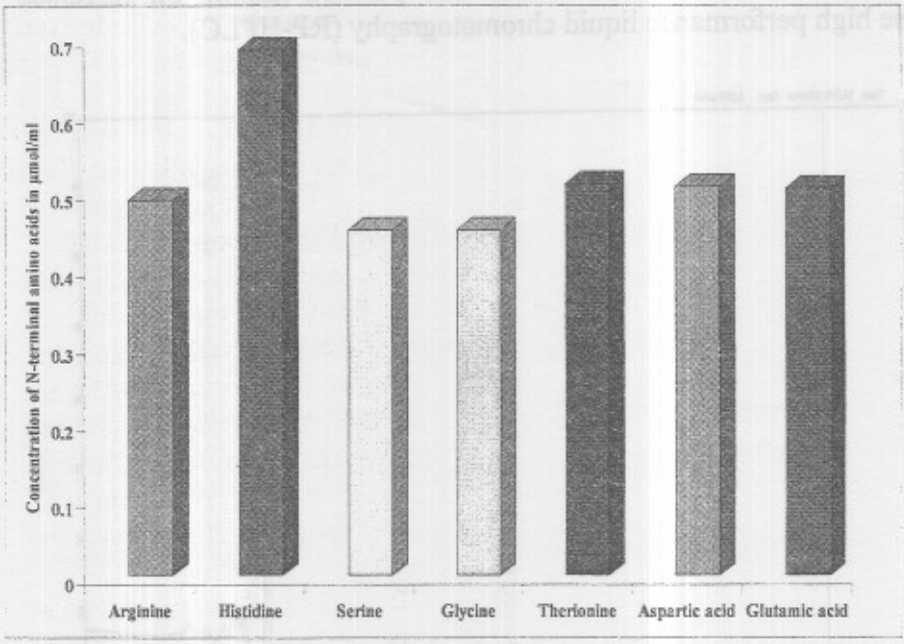

Fig: 6: An HP-TLC Chromatogram represents the concentration of inhibin N-Terminal amino acids (µMol/ml) of she-camel.

DISCUSSION

Inhibin is a peptide hormone secreted mainly from the ovary of all known domestic and experimental animals. It is a member of the transforming growth factor-β (TGF-β) superfamily that is expressed by oocytes, granulosa cells, and theca cells of the developed oocytes. Inhibin acts as an intraovarian regulatory molecule involved in follicle

recruitment, granulosa and theca cell proliferation or atresia, steroidogenesis, oocyte maturation, ovulation, and luteinization. (Knight and Glister, 2003; Kawano *et al.*, 2004; and Ocal *et al.*, 2004). The interactions of peptide and steroid hormone signaling cascades of hormonal events in the ovary are critical for follicular growth, ovulation, and luteinization. The pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play key regulatory roles and their actions are dependent on other peptides signaling pathways, including insulin-like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β) family members (e.g., inhibin, activin, growth differentiation factor-9, and bone morphogenic proteins) (JoAnne *et al.*, 2002). Inhibins family is members of TGF β superfamily of growth and differentiation factors. They were first identified as gonadal-derived regulators of pituitary FSH and were subsequently assigned of multiple actions in a wide range of tissue (Massague, 1998). Isolation and purification of this hormone has been accomplished in many species. The initial isolation of inhibin was achieved from bovine follicular fluid as a 58 kDa glycoprotein consisting of two disulphide-linked subunits of apparent molecular masses 43 kDa and 15 kDa (Robertson *et al.*, 1985). Miyamoto *et al.* (1985) reported that the isolation of a 32-kDa glycoprotein from porcine follicular fluid consisting of two subunits of 20 kDa and 13 kDa, the larger subunit has been termed α, and the smaller was β, these findings were confirmed by Ling *et al.*, 1985 who isolated two forms of inhibin, termed inhibin A and inhibin B and differentiated from each other by the differing NH2 -terminal amino acid sequences of their 13-subunits. In sheep, a 30 kDa form of inhibin with 20 and 16 kDa subunits had been isolated from follicular fluid (Leversha *et al.*, 1987).

In current study, fractionation of follicular fluid of female camel using Sephacryl S-200 gel resolved 3 protein peaks with different molecular weights and protein concentrations. Similar results obtained by Kishiko *et al.*, (1992) who obtained three fractions from bovine follicular fluid. On the other hand, Moore *et al.*, (1994) isolated four fractions from equine follicular fluid and Leversha *et al.*, (1987) found only two fractions in ovine follicular fluid. Using Sephadex G-100 to further fractionate third peak isolated from Sephacryl S-200 also resolved 3 peaks. Similar findings obtained by Robertson et al., (1992) from bovine follicular fluid, Gordon *et al.* (1986) from porcine follicular fluid and Moore *et al.* (1994) from equine follicular fluid. Checking the purity of peak 3 resolved from Sephadex G-100 using electrophoresis,

under non-reducing conditions and stained by silver nitrate, revealed only one protein band, indicating purity of the isolated peptide. Kishiko *et al.* (1992) reported similar results in bovine and Rivier *et al.* (1985) in porcine follicular fluids. Running the same protein on electrophoresis under reducing conditions resolved 5 protein bands with the different molecular weights (58, 38, 36, 34 and 32 KDa) which indicate that protein peptide isolated in peak 3 from Sephadex G-100 has multiple subunits or forms. The highest protein concentration detected in the lowest molecular weight band (32 KDa). Miyamoto *et al.* (1985) mentioned that in porcine follicular fluid inhibin is present in four molecular forms with corresponding MW of 100, 80, 55, and 32 KDa. More recently, Kishiko, *et al.* (1992) isolated three forms of inhibin from bovine follicular fluid, the highest molecular weight form (95 and 105 KDa), the intermediate form (55 and 65 KDa) and the low form (32KDa). Also Moore *et al.* (1994) stated that at least three forms of inhibin were present in equine follicular fluid with different molecular weights, 90, 56 and 32 KDa. It is obvious that the 32 KDa form of inhibin is common in the follicular fluid of most animal species studied so far. The other forms detected with different molecular weights in these animals may be attributed to 1- species differences, 2- different protocols for processing the samples and buffers with different pH values (between acidic and alkaline) used in these studies, and 3- different reproductive and physiological status of ovaries from which follicular fluids were collected. Indeed, Guthrie *et al.* (1997) reported that follicular production and/or intracellular processing of inhibin dimer and/or inhibin α subunits were changed during different phases of follicular development, supporting the notation of physiological roles for these peptides. Also Mason *et al.* (1996) concluded that the wide variation in the size of inhibins was due to incomplete cleavage of the proteolytic processing sites and the differential glycosylation of the N-linked sites.

Analyzing peak 3 resolved from Sephadex G-100 using RP-HPLC, five peaks with different retention times (2.083, 6.117, 68.817, 101.65, and 105.117 minutes) were resolved. These results are close to results recorded by Moore *et al.* (1994) who found 5 peaks of peptides in equine follicular fluid with almost same retention times.

Amino acids contents of the she camel follicular fluid inhibin peptide shown to be Arginine, Histidine, Serine, Glycine, Threonine, Aspartic acid and Glutamic acid with concentrations of (0.488, 0.684, 0.449, 0.499, 0.509, 0.506 and 0.503 μmol/ml) respectively. In 1985

Ling *et al.* found that the sequence analysis of the porcine follicular fluid inhibin (18 and 14 KDa) was Serine, Threonine, Alanine, Proline, Leucine, Tryptophan, Glycine, Glutamic, Aspartic, Asparagine and Arginine. The discrepancy in the results in four amino acids (Alanine, Proline, Leucine, Tryptophan) may be attributed to the method used (we used 6N HCL, and sample heated at 110 C^{o} for 24 hours) which may caused the destruction of Tryptophan and Leucine and the conversion of Asparagine and Glutamine to Aspartic and Glutamic acids respectively. The absence of Alanine and Proline from camel inhibin may be attributed to species difference between camel and porcine. Indeed, Rivier *et al.*, (1985) used different technique in studying N- Terminal amino acids of porcine inhibin (32 KDa) and obtained Histidine, Alanine, Serine, Glycine, Leucine, Proline, Threonine and Glutamic acid. The difference in type of inhibin studied (18 and 14 KDa vs. 32 KDa) and technique adopted in these two studies clearly revealed slight difference in amino acid residues even in the same species; porcine. Kishiko *et al.* (1992) found in bovine follicular fluid inhibin (50 KDa) N- Terminal sequence consisting of Cysteine, Histidine, Glycine, Leucine, Glutamic, Aspartic and Arginine. There is a slight difference in only two amino acids, Serine and Threonine found in camel inhibin and Cysteine and Leucine found in bovine inhibin. On the other hand Leversha *et al.* (1987) found that ovine follicular fluid inhibin N-Terminal amino acid contained the sequence of Serine, Proline, Glycine, Leucine, Glutamic, Alanine, Histidine, Valine, Aspartic and Aspargine, where the amino acids Serine, Glycine, Glutamic, Histidine and Aspartic were found in camel inhibin.

In conclusion, in current study inhibin peptide was isolated from the follicular fluid of female camel in our laboratory. Advanced biochemical analyses were adopted to study the structure of this peptide. The use of 2 types of gel filtration techniques (Sephacryl S-200 and Sephadex G-100) was necessary to obtain inhibin peptide in pure form. Also the refining follicular fluid from steroids and the extraction of ovarian peptides using chemical techniques has helped in purification process. Inhibin peptide has many forms depending on molecular weights (electrophoretic patterns) and retention times (RP-HPLC). The N-terminal amino acid residues of this peptide also vary according to its form. In our study it is clear that the protein of 32-KDa molecular weight has the greatest protein concentration and when analyzed using HP-TLC technique, it resolved to 7 amino acid residues that have some sharing residues with porcine, bovine, and ovine follicular fluids. Further

research is required to clone the genes that control inhibin peptide secretion. Moreover, we are in the process of raising antibodies against different forms of inhibin isolated and purified in our laboratory. These antibodies will help us in finding out which form has the dominant physiological effect in vivo and also can help us developing therapeutic regime for the reproductive problems originating from some pituitary hormone disturbances.

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