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A Simple Method to Purify Growth and Milk Enhancing Hormones (Growth Hormone and Prolactin) In High Yield From the Pituitary Glands of Indian Domestic Goats (*Capra hircus*)

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Abstract: Pituitary hormones GH and PRL have been used as external stimulating agents in farm animals to increase the yield of milk and meat. Here, a simple, protocol for purification of Growth Hormone (GH) and Prolactin (PRL) from the pituitary glands of Indian domestic goats (*Capra hircus*) has been described. Extraction of pituitaries with ammonium sulphate coupled with QAE-Sephadex anion exchange chromatography provided purified GH. The GH preparation was found to be pure in SDS-PAGE analysis and immuno-reactive to anti-buffalo GH antibody in ELISA. QAE-Sephadex was also useful in fractionating goat GH into different charge isoforms. PRL was purified from an alcoholic extract by gel-filtration chromatography on S-200.The PRL preparation was found to be pure in SDS-PAGE analysis. The respective yields of pure GH and PRL were 4.9 and 2.9 grams per kilogram of pituitaries. Molecular weights of major form of GH and PRL as determined SDS-PAGE was found to be 20 and 23 kDa respectively. Physico-chemical properties of goat hormones were found to be similar to the buffalo hormones. Domestic goat is an important source of farm products (milk and meat) in many developing countries. The yield of products can be improved on per animal basis by administering purified homologous GH and PRL. For this purpose, a simple yet efficient method to get pure preparations of GH and PRL, as described in the present communication may be of great importance. Also, the purified material obtained can be used as standard for various immunoassay and bioassay experiments.

Keywords: Caprine, Goat, Prolactin, Growth Hormone, Anterior pituitary, Purification, Lactogenic, Protocol

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I. INTRODUCTION

Growth hormone (GH) and Prolactin (PRL) are protein hormones which are secreted by the anterior pituitary gland [1]. These chemical messengers are responsible for general body growth and stimulation of specific tissues including mammary glands [2-7]. These share structural and physicochemical properties. Major form of both the hormones are primarily monomeric having the molecular weights of 22-20 kDa for GH and 22-25 kDa for PRL as has been found in various farm animals and humans [8-15]. In addition to the major form, several minor forms of GH and PRL, differing in size, charge and the extent of glycoslation, sulfation and phosphorylation have also been reported [4, 8,11, 13-14, 16-20]. Lower size variants are produced either naturally by alternate splicing of mRNA and proteolytic cleavage of momeric form or by artefacts of purification. Higher size variants are usually formed by aggregation of monomeric forms which are linked either via covalent bonds (disulphide linkage) or non-covalent interactions [13-14, 4, 19, 21]. It has been shown that minor forms possess different biological activities than major forms [l4, 19-20, 16].

Owing to the growth promoting ability and lactation, both GH and PRL have been used as external agent to improve yield of milk and meat in farm animals including cows, pigs and buffalos [3, 22-31]. The farm products obtained by the use of these exogenous hormones on farm animals have been shown to be safe for human consumption [22]. Hormones isolated from animal pituitaries and produced by recombinant DNA technologies, have been used for administration in farm animals [22-31]. Use of heterologous hormones (obtained from different animal species) poses a possibility of production of neutralizing anti-hormone antibodies in the recipient with subsequent reduction in the efficacy and severe anaphylactic reactions [21, 32-33].Use of homologous (of same animal species) recombinant hormones although considered safe, can also pose similar problems if not folded properly and presence of contaminating proteins belonging to organism in which

the hormone has been expressed[32-33]. Considering the above mentioned factors, it can be assumed that use of homologous hormones as stimulants, whether isolated from the tissue of animals or produced by recombinant methods, if pure, homogeneous and correctly folded may reduce the immunological complications [32-35]. A highly pure preparation is however mandatory for hormones produced by recombinant methods. Domestic goat (*Capra hircus*) is an important farm animal and globally more than 90 percent of the goat population is concentrated in low income countries of Asia and Africa, where its milk and meat are used as major source of protein and other nutrients [36-41]. In addition to milk and meat goat also provides hide and manure [42, 43]. In order to further improve the yield of farm products and to maintain a pace with ever increasing demand, yields can be improved per animal basis by the administration of exogenous stimulants such as GH and PRL. Prolactin has also been found to have a stimulating effect on the growth of secondary hair follicle of 'Cashmere goat'; an animal, which provide one of the finest wool known as '*Pashmina*' [44, 45].

In the present communication we provide a protocol to obtain pure Caprine GH (cGH) and Prolactin (cPRL) from the pituitary glands of Indian Domestic Goats (*Capra hircus*). These pure preparations can be administered in goats as homologous exogenous hormones in order to increase yield of farm products. The hormonal preparations can also be used in 'Cashmere goats'-phylogenetically a close relative of domestic goats. There is a previous report on the purification of PRL from the pituitaries of castrated male goats, however simultaneous purification of GH was not shown [46]. Production of caprine GH by recombinant DNA technology has also been shown [47]. To the best of our knowledge there is no report on simultaneous purification of GH and PRL from the same batch of pituitary glands (thus maximizing the use of resource) of domestic goats as shown in the present communication.

II. EXPERIMENTAL

Materials

Pituitary glands of domestic goat were procured from a local abattoir. Pituitary glands were from animals of mixed age and sex. Sephacryl S-200, Blue Dextran- 2000 and QAE- Sephadex were purchased from Pharmacia Bio-Tec. Ltd. Sweden. Goat anti-rabbit IgG-HRP conjugate was purchased from Bangalore-Genei Ltd. India. ELISA plates were purchased from Greiner, Greiner Bioscience Germany. SDS-PAGE was performed in an Atto make mini gel system obtained from Atto, Japan. o-phenylenediamine (O.P.D.), 4-Chloro-α-Napthol and phenyl-methyl-sulfonyl- fluoride were procured from Sigma Chemical Co., St. Louis, USA. Coomassie- brilliant blue G-250 and β-mercaptoethanol (β-M.E.) were obtained from E-Merck Ltd., Germany. Bovine Serum Albumin (BSA) was obtained from SRL, India, Ltd. All other chemicals used were of analytical grade.

Methods

Extraction of Crude GH and PRL:

Frozen pituitary glands were weighed and homogenized in 150 mM ammonium sulphate buffer containing 1mM phenyl-methyl-sulfonyl-fluoride. During the entire process temperature was maintained between 3-4°C and tissue to buffer ratio was kept to 1:4 or 1:5. After homogenization the pH of homogenate was set to 4 using 1N HCl. The resultant suspension was allowed to stir for 2.5-3 hours after which it was centrifuged at 5000g for 30 minutes. The residue obtained after centrifugation was designated as acid pellet or 'AP'. The AP was suspended in 250 mM ammonium sulfate, pH was set to 5.5 using 0.1 N NaOH and extracted for 4-5 hours in cold. Thereafter it was centrifuged at 10,000g for 30 minutes. The extract so obtained was called 'Crude GH'. The residue was re-suspended in pre-chilled 70% ethanol, (volume was kept same as homogenization buffer) pH was set to 9.5 with 0.1 N NaOH and extraction was done for 6-7 hours at subzero temperature (between - 4 to -5 °C). It was centrifuged at 5000g, -4°C, for 30 minutes. The pH of ethanolic extract was set to 5.5 using 0.1 N HCl and two volumes of pre-chilled absolute ethanol was added slowly with continuous stirring while maintaining subzero temperature. After stirring for 2 hours centrifugation was done at 5000g, 30 minutes. The pellet obtained was suspended in 0.1M ammonium bicarbonate and labelled as 'Crude PRL'.

Enrichment of GH by Chromatography on QAE-Sephadex:

Buffer of the crude GH was changed to 0.1M ammonium bicarbonate using dialysis method. The crude GH was loaded on 10 mL QAE-Sephadex column which was equilibrated with 0.1 M ammonium bicarbonate (buffer 1). 2 - 3 mg of crude GH was loaded per mL of matrix and elution of unbound/leaching proteins was done with buffer 1, till the optical density at 280 nanometre decreased to baseline value. Unbound fractions with significant optical density were pooled and called 'pure GH'. Proteins bound to the column were displaced with 0.1M ammonium bicarbonate buffer containing 1M NaCl (buffer 2). Fractions eluted with buffer 2 were pooled and called 'QAE-bound'. Fraction of 3.7 mL/tube was collected using automatic fraction collector.

Enrichment of PRL by Gel Filtration Chromatography:

Twenty milligram of crude PRL was loaded on Sephacryl S-200 column (2.42 X 145 centimetres.) which was, equilibrated with 0.1 M ammonium bicarbonate. The column was pre-calibrated with standard molecular weight markers and void volume was measured using blue dextran. Elution pattern of purified buffalo PRL [15] was also determined previously on the same column. Flow rate was maintained at 25 mL/hour and fractions were read at 280 nanometre. Fraction size was kept 5 mL/tube. Pooling of fractions was done after observing banding pattern of each tube (making peak in the chromatogram) in SDS-PAGE. Fractions having band corresponding to PRL were pooled and the material was called 'pure PRL'

SDS-PAGE:

SDS-PAGE was performed according to the method given by Laemmeli et. al. [48]. When required, β -M.E. (β-mercaptoethanol) was used as reducing agent. After completion of the run, the gel was fixed in 10% trichloro-acetic acid for 30 minutes and was stained according to the protocol given by Neuhoff et al. [49] for highly sensitive, clear background coomassie brilliant blue (G-250) staining. The staining was done for 24 hours in an air tight box. The gel was dried by vacuum drier, photographed and stored.

ELISA:

Immuno-reactivities of GH and PRL containing samples were measured using direct binding ELISA. Blocking of the nonspecific sites was done using 1% casein solution in 0.1 M phosphate buffer pH 7.5. Primary antibody developed against buffalo GH and PRL were used to probe corresponding goat hormones [13, 15]. Secondary antibody, goat-anti-rabbit IgG- HRP conjugate was used to probe primary antibody. The entire process including incubation of antibody-antigen solution was done at room temperature. Washing of the wells was done using phosphate buffer (50 mM pH 7.0) containing Tween (0.05%). Ortho-phenylenediamine (1 mg/mL) and H_2O_2 in 50 mM citrate buffer pH 5.5 solution was used as the substrate. Plates were read at 490 nanometre in an ELISA reader.

Western Blot:

Bands were electro-blotted to the nitrocellulose membrane, blocking was done using 1% casein solution in 0.1M phosphate buffer, pH 7.5. The blot was probed with primary antibody –anti buffalo PRL antibody. Goat-anti-rabbit IgG- HRP conjugate was used to probe primary antibody. Washing of the blot was done using 50 mM phosphate buffer, pH 7.0. Blot was developed with 4-chloro-α-napthol and H_2O_2 as substrate.

Estimation of Protein:

This was done according to the method given by Lowry et. al. [50]. BSA was taken as standard protein.

III. RESULTS

Homogenization and extraction of pituitaries in 150 mM ammonium sulfate at pH 4 provided residue 'AP' which was taken as starting material for isolation of goat GH and PRL. Extraction of AP with 250 mM ammonium sulfate at pH 5.5 selectively extracted GH as a turbid solution (Crude GH) leaving behind bulk of PRL in the residue (figure 1). The mean value of protein content in crude GH was found to be 13.3 gms/Kg of pituitaries (n=3, table 1). SDS-PAGE analysis of crude GH showed a prominent band corresponding to the GH and several faint bands of contaminating proteins (lane L1, figure 2). After dialyzing against buffer 1, crude GH was loaded on QAE-Sephadex anion exchanger (figure 3). In SDS-PAGE analysis, the material unbound to the anion exchanger was found to be cleared of several high molecular weight contaminating proteins (lanes L1 and L3, figure 2). Also, a prominent band was observed at the same location as in the case of buffalo GH taken as standard (lanes L2 and L3 figure 2). The QAE-Sephadex unbound material was denoted 'Pure GH'. The total protein content of pure GH was found to be 4.9 gms/Kg of pituitaries (table 1). The purity of pure goat GH was also comparable to (or purer than) the standard pure buffalo GH [15] (Lanes L2 and L3 figure 2). In the direct binding ELISA, pure GH possessed higher GH immune-reactivity as compared to the QAE bound fraction (figure 4).

The residue obtained at second step of purification (i.e. after extraction of GH, figure 1) contained bulk of pituitary PRL. It was extracted with alkaline ethanolic solution. Proteins extracted at this step were precipitated by addition of absolute ethanol at pH 5.5. The precipitate obtained (Crude PRL) had a mean protein content of 3.14 gms/Kg of pituitaries (n=3, table 1). SDS-PAGE analysis of crude PRL done under non-reducing condition showed presence of a prominent band (major form or monomeric form of PRL) at the same position as in the case of buffalo PRL taken as standard [15] and a few bands of high molecular weight (Lanes L1 and L2, figure 6). In western blot analysis, band corresponding to monomeric form of PRL as well as high molecular weight bands reacted with antibody raised against purified buffalo PRL (B, Figure 7). Crude PRL was loaded on Sephacryl S-200 gel-filtration chromatography, where pure PRL was recovered in the main peak with

Ve/Vo value of 2 (figure 5). The Ve/Vo value obtained for goat PRL coincided with the value observed for standard buffalo PRL passed through the same column previously. SDS-PAGE analysis of pure PRL showed a prominent band corresponding to the monomeric form of PRL (Lane L3 in figure 6). The yield of pure PRL was found to be 2.9 gms /Kg of pituitary glands (table 1).

IV. DISCUSSION

In the present communication we provide a scheme for purification of GH and PRL from the whole pituitary glands of Indian domestic goats.

Homogenization and extraction of goat pituitaries in 150 mM ammonium sulphate, extracts most of the pituitary LH and possibly FSH and TSH, owing to the similarity in physicochemical properties of these three hormones [51-54]. Purification of highly pure goat LH from this extract has already been shown by our laboratory [52]. The residue 'AP' obtained after extraction contained almost entire pituitary GH and PRL and it is a good material to begin with. Further extraction of AP with 250 mM ammonium sulfate almost selectively extracted GH (Crude GH), leaving behind PRL in the residue (figure 1). At this step, centrifugation of the suspension at 10,000 rpm, prevented GH from being contaminated with PRL (reducing cross-contamination of PRL in GH preparation). Crude GH contained few contaminating proteins (figure 2) which were removed by subsequent QAE-Sephadex anion exchange chromatography. In QAE-Sephadex, goat GH was found to leach out of the column (figure 3) in a manner similar to buffalo GH under identical condition [51]. This indicates closeness in the isoelectric points of Growth Hormones between the two animal species (isoelectric point of major form of buffalo GH is 7.2 and 7.7) [11].The reason for loose interaction and subsequent leaching of GH from the strong anion exchanger column could be ascribed to the proximity of isoelectric point and the pH of loading buffer (aqueous solution of 0.1M ammonium bicarbonate has pH nearly 8). A small amount of goat GH was bound tightly to the QAE column (figure 3 and 4) which was also observed in the case of buffalo GH [51]. The bound GH may represent to the more acidic isoform (isoelectric point between 5.7-7) [11].

The yield of pure goat GH was found to be 4.9 gms/Kg of pituitaries. In SDS-PAGE analysis, the molecular weight of goat GH was found to be same as that of buffalo GH i.e. 20 kDa (figure 2) [15].

PRL was extracted from the residue with ethanol. From this extract, PRL was precipitated by increasing ethanol concentration and lowering the pH (isoelectric precipitation,). Buffalo PRL (Isoelectric point of which is between 5.1-5.45) was also shown to precipitate under same conditions, indicating proximity in isoelectric point between the Prolactin of two animals [15, 20]. The yield of crude PRL was 3.41 gm /kg of pituitaries. It is clear from the SDS-PAGE and western blot analysis that the crude PRL besides having major form (monomeric) also contained several size variants of Prolactin (Figure 6 and 7). On Sephacryl S-200 gel-filtration chromatography the size variants were resolved and the major form of PRL was recovered from main peak (figure 5 and Lane L3 in figure 6). The material obtained from the main peak of gel-filtration chromatography was called pure PRL. The yield of goat PRL observed in present study was 2.9 gms/kg of pituitaries, which is comparable to the yield and purity observed by McNeilly et.al. for the same animal species (2.5gms/kg)[46]. A highly pure goat PRL can be obtained from pure PRL by re-chromatographying the latter on Sephacryl S-200 as has been done earlier for buffalo PRL [18].

Several high molecular weight bands were highlighted in western blot analysis of crude PRL, indicating that this material can be useful starting material for isolation of various size forms of Prolactin. Also, last few fractions in the descending arm of Sephacryl S-200 chromatogram (figure 5), contained several proteins having molecular weight below 20 kDa (result not shown). This material can be a good starting material for the isolation of lower size forms of PRL. In the case of buffalo, lower molecular size PRL fragments have been shown to possess anti-angiogenic properties and a possible role in tumorigenesis [20, 56].

The similarity between the PRL of goat and buffalo in SDS-PAGE banding pattern and elution profile on S-200 indicates closeness in the overall three dimensional shape and molecular weight (i.e. 23 kDa).

The protocol proposed here is simple and reproducible and pure hormones obtained in the present study can be easily converted into highly pure preparations. The process of purification can also be scaled up for bulk purification. The hormonal preparations are suitable for further physicochemical and biological studies and can be used as external agent for stimulation of growth and lactation in domestic goats, such studies however are needed to be performed.

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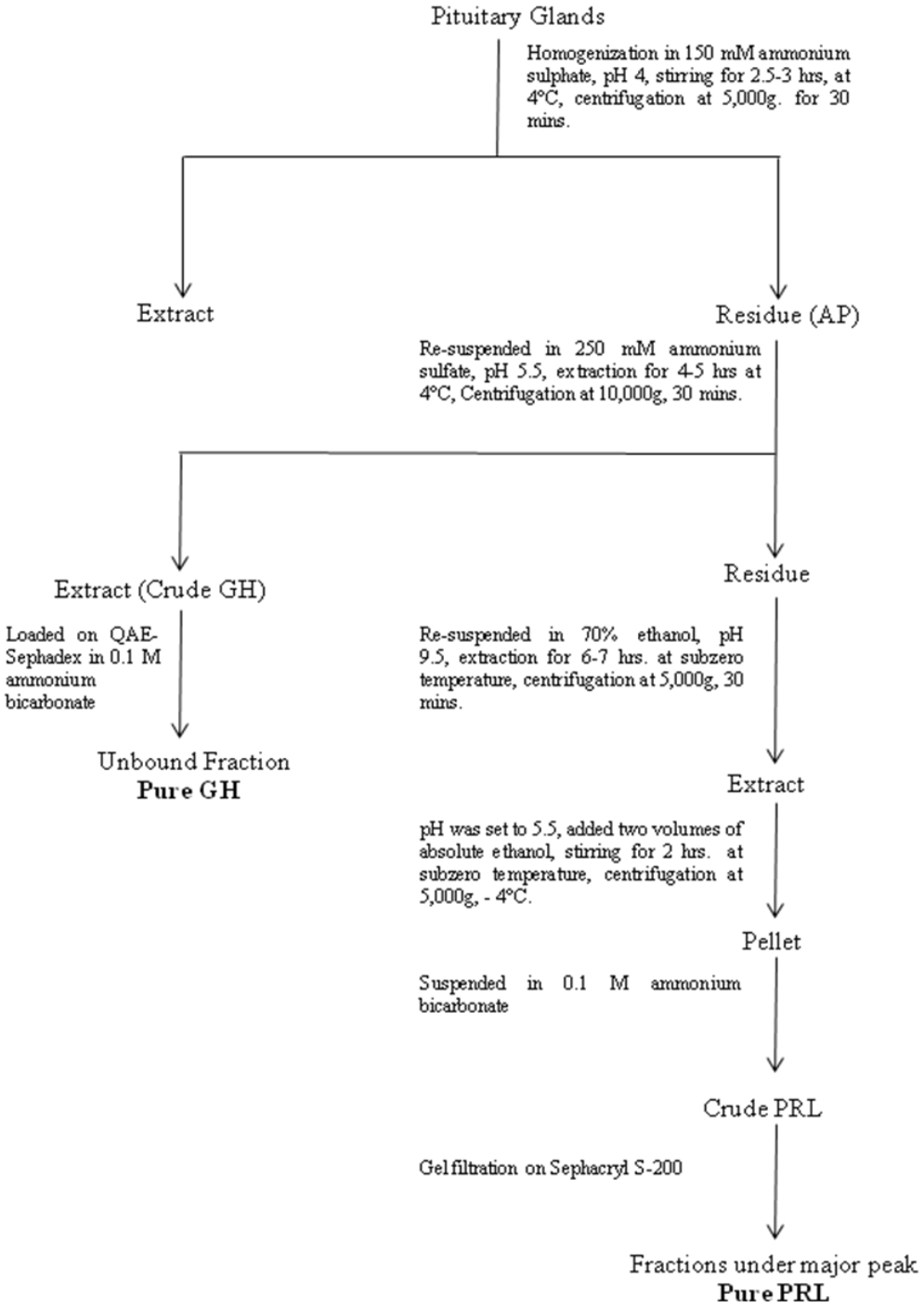

Figure 1. Flow diagram for obtaining pure goat GH and PRL

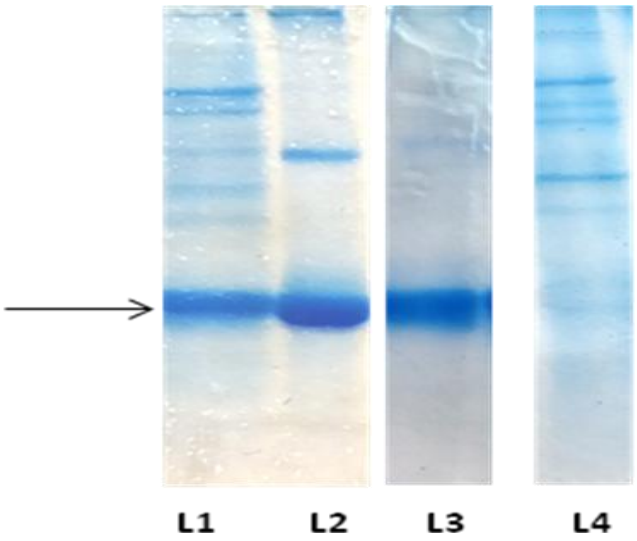

Figure 2: SDS-PAGE of different fractions obtained during purification of goat GH. Lane L1: 'crude goat GH', L2: pure buffalo GH taken as standard, L3: pure goat GH and L4: represents fractions bound to QAE-Sephadex which was eluted with 1M NaCl. Arrow points to the position of monomeric (major form) band corresponding to goat and buffalo Growth Hormones.

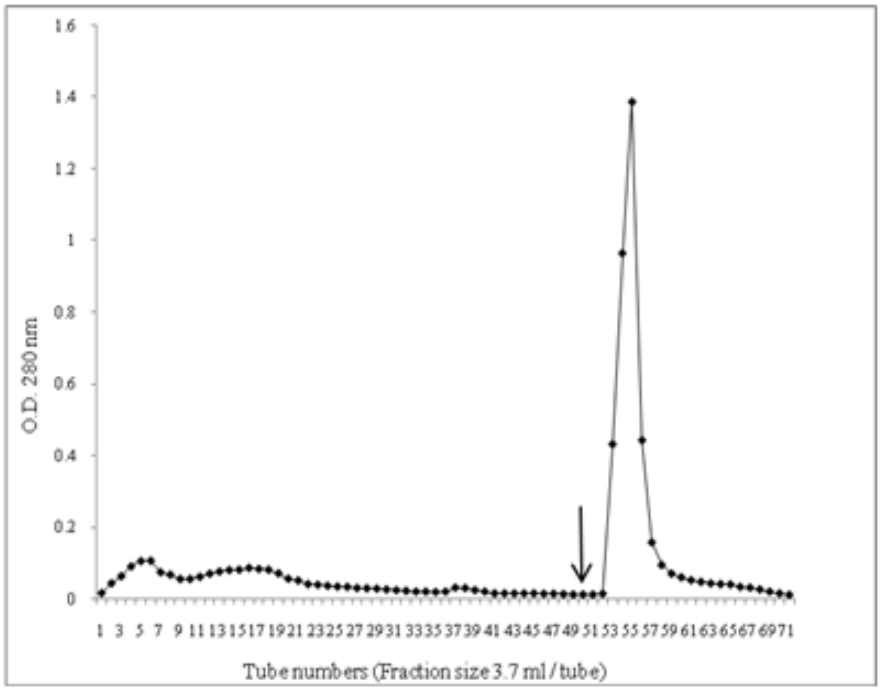

Figure 3: Chromatography of crude GH on QAE-Sephadex. Crude GH was loaded on the column in ammonium bicarbonate buffer and the elution of bound proteins was done with high concentration of NaCl (see text). Tubes with significant O.D and lying between tubes numbers 1 to 50 were pooled (pure GH). Arrow points to the tube where the buffer for the elution of bound proteins was applied.

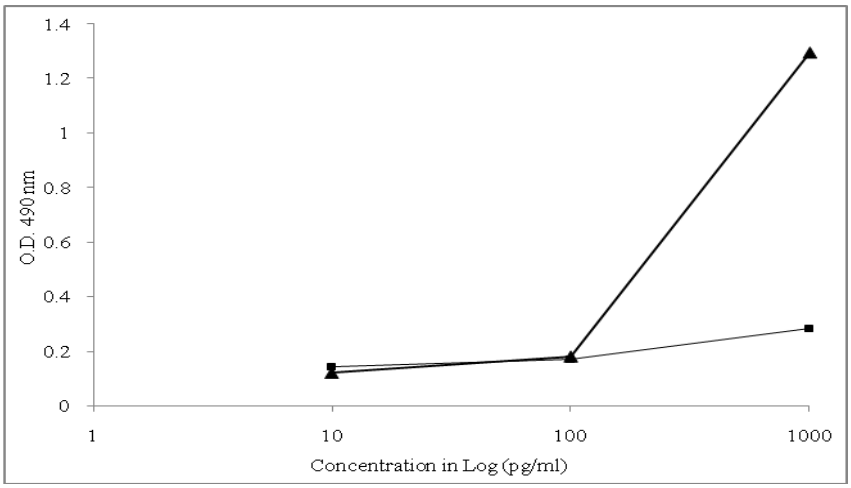

Figure 4: Immuno-reactivity of pooled QAE-unbound (pure GH) and QAE-bound fractions as measured by direct binding ELISA. The primary antibody used was raised against purified buffalo growth hormone. ▲ Denotes to immune-reactivity of QAE-unbound and ■ to immune-reactivity of QAE-bound pool.

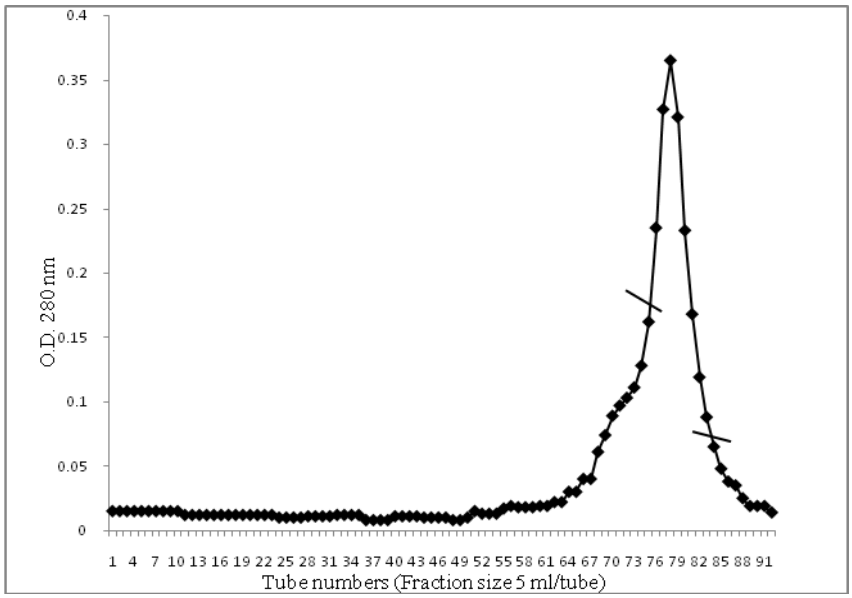

Figure 5: Sephacrly S-200 Chromatography of crude PRL. Fractions marked between oblique lines and falling under the main peak were pooled. The pooled fractions represent pure PRL.

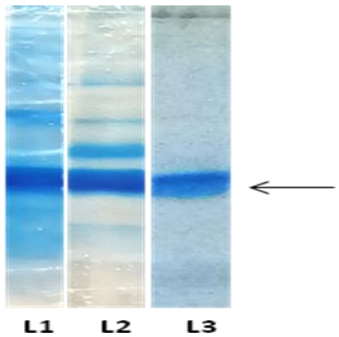

Figure 6: SDS-PAGE of different fractions obtained during purification of goat PRL. Lane L1: crude buffalo PRL taken as standard, L2: crude goat PRL and L3: represents pure goat PRL obtained after Sephacryl S-200 gel-filtration chromatography. Arrow points to the position of bands corresponding to monomeric (major form) of goat and buffalo PRL.

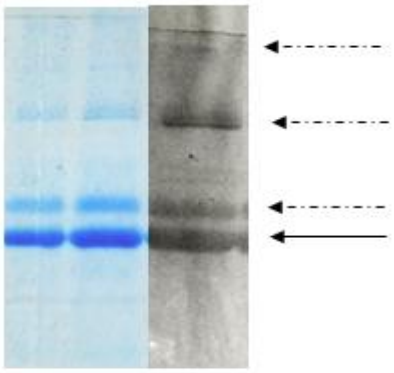

Figure 7: Western blot analysis of crude goat PRL, (A) SDS-PAGE done under non-reducing condition, (B) western blot of gel shown under 'A'. The blot was probed with antibody raised against highly purified buffalo PRL. Solid arrow pointing to the major (monomeric) form of PRL. Broken arrows pointing to the higher molecular size variant of goat PRL.

Amount of Protein in Crude GH (gms/Kg of glands)	Amount of Protein in Crude PRL (gms/Kg of glands)	Amount of pure GH (gms/Kg of glands)	Amount of pure PRL (gms/Kg of glands)
13.3 ± 1.26 (n=3)	3.41 ± 1.62 (n=3)	4.9	2.9

Table 1: Showing yields of GH and PRL.

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