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ASSESSMENT OF PURIFICATION PROTOCOLS FOR BUFFALO PITUITARY GROWTH HORMONE

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SUMMARY

Three different extraction protocols were applied to freshly frozen buffalo pituitaries collected from a local abattoir with a view to obtain a highly immunopotent preparation of buffalo GH. The 'enriched GH' obtained from each protocol was assessed on the basis of immunopotency and hormonal homogeneity. It was observed that, use of Ellis and Spitsberg protocols in tandem yielded the highest amount of immunoreactive prolactin free GH (ECS). Further, it was found to be relatively less contaminated with other protein impurities. Application of various chromatographic procedures on this enriched GH preparation (ECS) showed that Sephadex G-200 chromatography yielded most immunopotent GH preparation in high amounts. Further, the purification protocol was most convenient and reproducible procedure for obtaining bulk quantities of pure growth hormone from buffalo pituitaries.

Key words : buffalo, growth hormone, purification, immunopotency.

INTRODUCTION

Growth hormone (GH) is synthesized and secreted by the anterior pituitary lobe and is necessary for growth and development (1). In a classic experiment, it was shown that hypophysectomy stunted growth in animals and exogenous administration of pituitary extract or purified GH reversed this effect (2,3). GH has also been found to play an important role in the metabolism of proteins, carbohydrates and fats (4,5). Amino acid sequence of growth hormone from many species has been determined (6). It has been shown that GH isolated from pituitary glands is heterogeneous (7-9). A 20kD variant of human GH has been isolated, characterized and shown to have different biological, receptor binding and immuno activities from those of the 22 kD major form (10). Hart *et al.* (11) have shown that an isohormone of bovine GH possess more growth activity than immunoactivity although it has no diabetogenic

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activity. With recombinant DNA technology, homogeneous growth hormone of various species has been purified from both prokaryotic and eukaryotic expressing systems (12-21). Administration of bovine somatotrophin to buffaloes has been demonstrated to result in increase in the milk production (22,23). However, it would be advisable to use buffalo growth hormone for the purpose to avoid immune response to heterologous growth hormone. Hence, we undertook a study of different protocols for obtaining buffalo GH in high yield and homegeneity.

MATERIALS AND METHODS

The buffalo pituitaries were collected from a local abattoir. Aprotinin, phenyl methyl sulphonyl fluoride (PMSF), blue dextran, acrylamide, N, N- bisacrylamide, N, N, N', N'-tetramethylethylenediamine, SDS-PAGE protein markers (SDS-MW-70L kit), Coomassie brilliant blue R-250, Coomassie brilliant blue G-250, α-methyl-D-mannopyranoside, chloramine-T, Sephadex G-200, iminodiacetic acid (IDA) agarose, concanavalin A Sepharose, phenyl sepharose, nitrocellulose membrane, γ-Globulin were all obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Carrier free Na ^{125}I was obtained from Bhabha Atomic Research Center (BARC), India. Ovine growth hormone and anti-ovine growth hormone were gifts from Dr. S.M. Totey, National Institute of Immunology, India. Bovine growth hormone and anti-bovine growth hormone were obtained from National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, USA. All other chemicals were of reagents grade and were procured locally. Whole pituitary glands were excised from buffaloes of mixed sex and age within half an hour of their slaughter and were immediately frozen in liquid nitrogen for transportation to the laboratory where they were stored at -20°C till they were further processed.

Processing of buffalo pituitary glands

The entire extraction was carried out at 4°C till otherwise mentioned. Essentially, three extraction protocols were tried to obtain buffalo growth hormone. In one, the pituitary glands were subjected to the modified Ellis (24) and Spitsberg (25) protocols in tandem. Briefly, the freshly frozen buffalo pituitary glands were thawed in 1mM PMSF at pH 5.5, cleared of extraneous tissue, choped into small pieces and minced further using mortar and pestle. The minced tissue was weighed and homogenized in 1mM PMSF, pH 5.5 in a meat blender at maximum speed for total duration of 5 minutes taking care to avoid heating up of the jar. The pH of the homogenate was adjusted to 5.5 with 6N HCl. The homogenate was then stirred and centrifuged. The residual pellet was then subjected first to 100 mM ammonium sulfate extraction at pH 4.0 and then to 250mM ammonium sulfate at pH 5.5. The respective supernatants obtained were labeled as 'extract B' and 'extract C', respectively. This extract C was neutralized, dialyzed against distilled water and lyophilized. This was then subjected to modified Spitsberg protocol where it was suspended in 1mM ammonium bicarbonate buffer, pH 6.2-6.4 containing 250mM sucrose. It was stirred and then centrifuged (10000g, 15 minutes). The pellet was resuspended in 140mM ammonium bicarbonate buffer, pH 7.2-7.4 containing 1 mM EGTA. The extract was then centrifuged (1000 x g, 15 minutes) and the supernatant subjected to ammonium sulfate precipitation at 45% followed by at 40% saturation. The pellet was

resuspended in 100mM ammonium bicarbonate and dialyzed against the same. The dialysed solution was lyophilized and termed as 'ECS'.

In the second protocol, the glands were subjected directly to Spitsberg protocol (25). The glands were homogenized in 1 mM ammonium bicarbonate buffer, pH 6.2-6.4 containing 250mM sucrose and 1 mM PMSF and centrifuged (10000 x g, 15 minutes). The pellet was resuspended in the above buffer and again centrifuged (10000 x g, 15 minutes). The resulting pellet was suspended in 140mM ammonium bicarbonate buffer, pH 7.2-7.4 containing 1 mM EGTA; the remaining protocol is same as that described under protocol one. The material was labelled as 'GS'.

In the third protocol, Papkoff's protocol originally described for isolation of sheep LH and later used by Sharma *et al* for isolation of buffalo LH (26) was used along with Rand-Weaver's protocol (27) originally described for isolation of Atlantic Cod growth hormone. Briefly, the glands were homogenized in 150 mM ammonium sulfate, pH 4.0 containing 1 mM PMSF. The homogenate was stirred, proteins precipitated at pH 4.0 and pH 3.0, centrifuged each time at 10000 x g for 15 minutes to give a pellet called 'Acid Pellet'. This pellet was then suspended in ammonium acetate, pH 9.0 and stirred for 15 hours and then centrifuged. The resulting supernatant was then subjected to ammonium sulfate precipitation at 45% and 40% saturation to give a pellet which was taken in 100 mM ammonium bicarbonate to give us a material which was labelled as 'GAP'.

Ion Exchange Chromatography

Diethylaminoethyl (DEAE) –Sephadex Chromatography : Anion exchange chromatography was carried out on a 1.6 cm X 10 cm packed column of DEAE-Sephadex A-50 at 4°C. The resin was equilibrated with 100 mM ammonium bicarbonate buffer, pH 8.2. Sample was dissolved and dialyzed against the same buffer. The clear solution obtained after centrifugation was loaded on to the column. 2.0 ml fractions were collected at a flow rate of 10 ml/h using Atto Model Mini collector (Japan). Elution of unbound material was continued till the absorbance (A_{280}) of the effluent reached < 0.05. Different protein peak fractions were pooled separately, dialyzed and lyophilized.

Carboxymethyl (CM)-Sephadex Chromatography. Cation exchange chromatography was carried out on a 1.0 cm X 14 cm packed column of CM-Sephadex C-50 at 4°C. The resin was equilibrated with 5 mM-phosphate buffer, pH 6.0. Sample was dissolved and dialyzed against the same buffer. The clear solution obtained after centrifugation was loaded onto the column. 2.0 ml fractions were collected at a flow rate of 10 ml/h using Atto Model Mini collector (Japan). Elution of unbound material was carried out in the same buffer. The bound material was eluted stepwise using a buffer system of 10 mM phosphate buffer, pH 7.0; 25 mM borate buffer, pH 8.2 and 25 mM borate buffer, pH 8.2 with 200 mM NaCl, respectively. Elution of unbound material in each buffer was continued till the absorbance (A_{280}) of the effluent reached < 0.05. Different protein peak fractions were pooled separately, dialyzed and lyophilized.

Molecular Sieving Chromatography on Sephadex G-200 matrix: Sample was prepared, dialyzed against 100 mM ammonium bicarbonate buffer containing 1M urea and loaded on the column (1.88 cm X 37.5 cm) equilibrated in the same buffer. Elution was carried out in the same buffer. 3.0 ml fractions were collected at a flow rate of 12 ml/h and the absorbance was monitored at 280 nm. Different protein peak fractions were pooled separately, dialyzed and lyophilized. The void volume, Vo of the column was determined with 2% solution of Blue Dextran.

Immobilized Metal Affinity (IMA) Chromatography : Iminodiaceticacid Agarose matrix was suspended in 50mM copper sulfate solution for 3-4 h with 3-4 changes of the copper sulfate solution to chelate the copper ion (Cu^{2+}) to the matrix. A column of 1.2 cm X 3.0 cm was packed and free CuSO4 was washed away with distilled water. The column was equilibrated with 10 mM phosphate buffer, pH 7.8 containing 10 mM imidazole and later with 10 mM phosphate buffer, pH 7.8 containing 1 M NaCl and 1 mM imidazole. Protein sample was prepared in 10 mM phosphate buffer, pH 7.8 containing 1 mM NaCl, centrifuged and clear solution was loaded onto the column and eluted with a stepwise gradient of imidazole starting from 1 mM imidazole and then going to 5 mM, 10 mM, 50 mM, 250 mM and 500 mM, respectively. 1.5 ml fractions were collected at a flow rate of 15 ml/h. Elution of unbound material in each buffer was continued till the absorbance (A_{280}) of the effluent reached < 0.05. Different protein peak franctions were pooled separately, dialyzed and lyophilized.

Hydrophobic Interaction Chromatography : This was carried out according to the protocol of Hoefferer *et al.* (28). Phenyl sepharose resin column 1.2 cm X 3.0 cm was packed, washed and equilibrated in 50 mM ammonium bicarbonate containing 1 M ammonium sulfate. The protein sample was loaded in the same buffer. The unbound material was eluted out using the same buffer. The bound material was eluted out stepwise using 50 mM ammonium bicarbonate buffer and 50 mM ammonium bicarbonate buffer containing 50% ethylene glycol. 1.5 ml fractions were collected at a flow rate of 8.0 ml/h. Elution of unbound material in each buffer was continued till the absorbance (A_{280}) of the effluent reached < 0.05. Different protein peak fractions were pooled separately, dialyzed and lyophilized.

Protein Estimation : Estimation of protein was done using the method of Lowry *et al.* (29).

SDS-Polyacrylamide Gel Electrophoresis (PAGE) : Electrophoresis in polyacrylamide slab gels was performed using the discontinuous system of Laemmli (30).

Western Blotting and Immunoperoxidase Staining : The electrotransfer of proteins from polyacrylamide gels to nitrocellulose membranes was carried out using the method of Towbin *et al.* (31) and the NTC membrane was then taken for immunoperoxidase staining. The entire process of transblot was done at room temperature using the protocol of Khurana *et al.* (32).

Reverse Phase High Performance Liquid Chromatograpghy (RP-HPLC) : This analysis was carried out on a Shimadzu SPD 10A HPLC model using a Zorbax-OSD (0.46 cm X 15 cm)

C-18 column. 10 µg protein sample was prepared in 15 µl of distilled water and filtered through a Millipore 0.2 µ filter. The sample was eluted using a linear gradient of acetonitrile containing 1% TFA. The rate of the gradient was 4% acetonitrile increase per minute.

^{125} *I-Radioiodination* :The method of Greenwood *et al.* (33) was adopted.

Radioimmunoassay (RIA) : Basically, the protocol of Nanda *et al.* (34) was followed. One albino rabbit weighing around 2.5 Kg was immunized with buGH using method of Vaitukaitis *et al.* (35). The antiserum collected was checked for immunoreactivity by RIA. The immunopositive and characterized bleeds were stored, aliquotted with 0.01% azide at –20°C.

Purification of Antiserum : 100 µl of 1:10 normal buffalo serum (NbuS) was added to 1ml of the antiserum, mixed well and the tubes were incubated at 4°C for 16-18h. The precipitate formed was removed by centrifugation at 3000 rpm for 15 minutes at 4°C. The process of addition of 1:10 NbuS was repeated with the supernatant till no visible pellet was observed. The immunoadsorbed antiserum was pooled and ammonium sulfate precipitation of it was done at 50% saturation. The antiserum was aliquotted, lyophilized and stored at –20°C till further use. The antiserum was further purified on a Protein-A column. Briefly, Protein-A Agarose gel was equilibrated with 100 mM Tris buffer, pH 8.0 and packed into a column to give a bed volume of 1ml. The pH of the sample was adjusted by adding 1M Tris buffer, pH 8.0 to the antiserum at 1:10 dilution. The sample was loaded onto the column and the flow of the column stopped for 30-45 minutes so that the sample and gel matrix could interact properly. The column was then eluted with 10 bed volumes of 100 mM tris buffer, pH 8.0, 10 mM tris buffer, pH 8.0 and 100 mM glycine buffer, pH 3.2, respectively. Fraction size for buffers 1 & 2 was 1 ml while for buffer 3, it was 0.5ml and it also contained 50 µl of 1M tris buffer, pH 8.0 to bring the pH of the fractions back to normal. The absorbance of the effluent was monitored at 280 nm. The peak were pooled separately, dialyzed and lyophilized. Each peak was checked for immunopotency in a RIA.

RESULTS AND DISUCSSION

Buffalo pituitaries were subjected to three different protocols as mentioned in Materials and Methods section. Comparing the amount of protein obtained by each protocol per kg of pituitaries (Table-1), it could be seen that although yield of GS was the highest among the three, in SDS-PAGE it was found that ECS had the highest GH content (data not shown). Further, since prolactin, the most common GH contamination was absent in ECS (Fig – 1) it was taken as the reference material. This ECS was then subjected to various chromatographies.

When ECS was passed through DEAE-Sephadex column according to Spitsberg protocol (25), it was seen that GH starts to leach out after the initial 1/3rd bed volume and this continued till almost 5 bed volumes. The profile (not shown) was similar to that obtained by Spitsberg for bovine GH (25) and Wallis for ox growth hormone (36). The SDS-PAGE (Fig. – 2) shows that the leached material was a pure GH fraction.

On a cation exchange column the buffer system used was similar to the one used by Sharma *et al* for the purification of buffalo LH (26). The protein yield and immunopotency of each fraction is given in Table-2. It can be seen that the maximum protein and most immunopotent buGH fraction was found in buffer IV (ECSCM-4).

Since growth hormone, in general, is believed to be a hydrophobic protein, we tried to purify it using hydrophobic interaction chromatography on a phenyl sepharose matrix. The buffer system used had a stepwise gradient of increasing hydrophobicity. The elution profile (Fig.-3) and immunopotency (Table-2) show that GH is obtained in buffer III confirming the fact that GH is highly hydrophobic in nature. The immunopotency of buGH eluting in buffer III was found to be comparatively lower than those obtained by other techniques, probably because glycerol was present which we found was difficult to remove even by dialysis.

The technique involving the immobilization of a chelating metal ion, like Cu^{2+} was also employed to check its efficacy for purifying buffalo GH (37,38,39). The buffer system used had a stepwise gradient of increasing concentration of imidazole. It was seen from the elution profile (Fig.-4) and immunopotency (Table-2) that buGH was obtained in almost all fractions but with different immunopotencies. The most immunopotent GH rich fractions were found to be eluting out in buffer II (ECSIMAC-2) and IV (ECSIMAC-4). This indicates the presence of microheterogeneity in buffalo GH. The SDS-PAGE profile of different fractions obtained in this chromatography essentially confirmed this idea (data not shown).

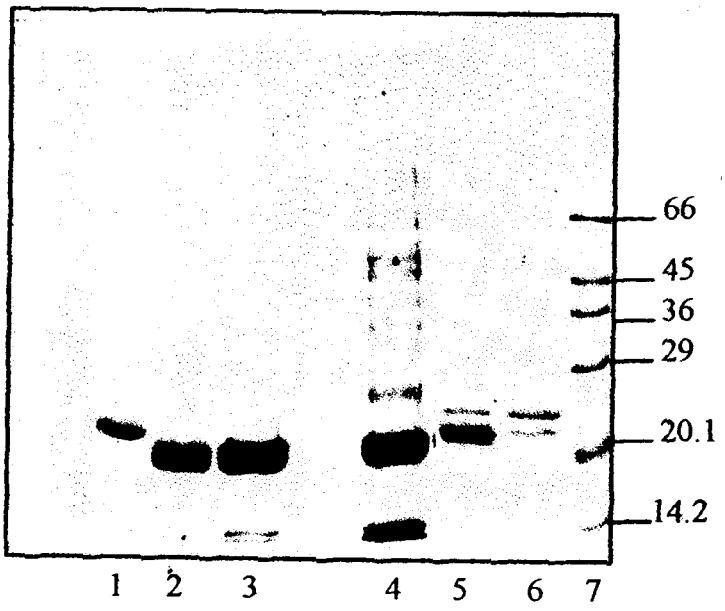


Figure 1 : A 12% SDS-PAGE showing that EC obtained in protocol I is free from prolactin contamination unlike GS and GAP obtained in protocols II and III, respectively. Lane 1 : Standard buffalo prolactin, Lane 2 : ECS (10 g), Lane 3 : ECS (20 g), Lane 4 : EC, Lane 5 : GS, Lane 6 : GAP, Lane 7 : Molecular size markers (Molecular sizes are indicated in KD).

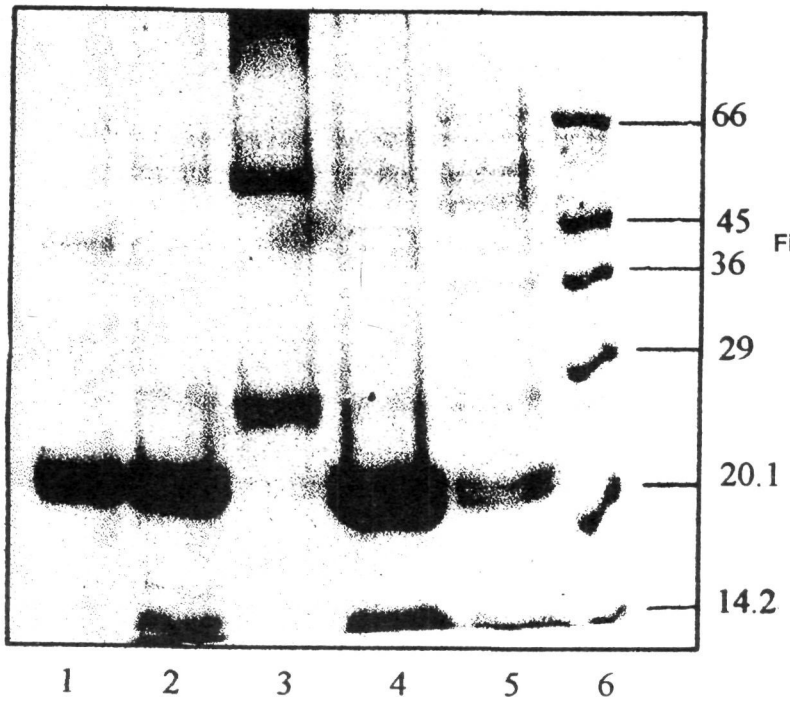


Figure 2 : A 15% SDS-Page of different protein peak fractions obtained in DEAE-Sephadex chromatography, Lane 1 : ECSD-2 (10 g), Lane 2 : ECSD-2 (20 g), Lane 3 : ECSD-1, Lane 4 : ECS, Lane 5 : EC, Lane 6 : Molecular size markers (Molecular sizes are indicated in KD).

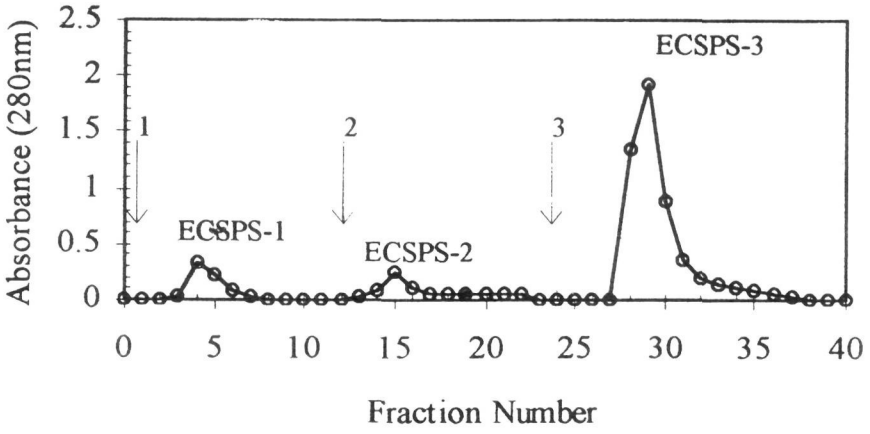


Figure 3 : Elution profile of ECS on Phenyl Sepharose column. The pooled protein fractions are labeled as ECSPS-1, ECSPS-2 and ECSPS-3. The buffers used are as, 1 : 50 mM ammonium bicarbonate buffer containing 1M ammonium sulfate, 2 : 50 mM ammonium bicarbonate buffer, 3 : 50 mM ammonium bicarbonate buffer containing 50% ethylene glycol.

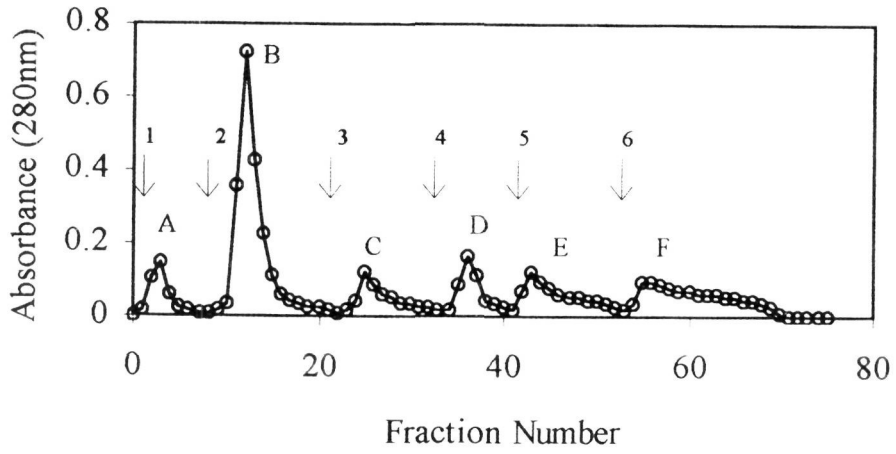


Figure 4 : Elution profile of ECS on IMAC column. The pooled protein fractions are labeled as A: ECSIMAC-1, B: ECSIMAC-2, C: ECSIMAC-3, D: ECSIMAC-4, E: ECSIMAC-5 and F: ECSIMAC-6. The buffers used are as; 1 : 10mM phosphate buffer, pH 7.8 containing 1 M NaCl and 1 mM imidazole, 2 : 10 mM phosphate buffer, pH 7.8 containing 1M NaCl and 5 mM imidazole, 3 : 10 mM phosphate buffer, pH 7.8 containing 1M NaCl and 10 mM imidazole, 4 : 10 mM phosphate buffer, pH 7.8 containing 1M NaCl and 50 mM imidazole, 5 : 10 mM phosphate buffer, pH 7.8 containing 1M NaCl and 250 mM imidazole, 6 : 10 mM phosphate buffer, pH 7.8 containing 1 M NaCl and 500 mM imidazole.

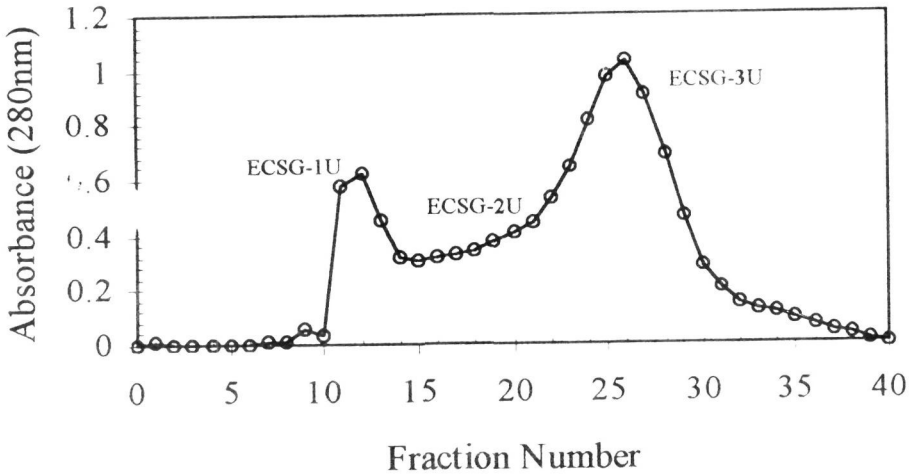


Figure 5 : Elution profile of ESC on Sephadex G-200 column. The pooled protein fractions are labeled as ECSG-IU, ECSG-2U and ECSG-3U. The buffer used was 100 mM ammonium bicarbonate buffer, pH 8.2 containing 1M urea.

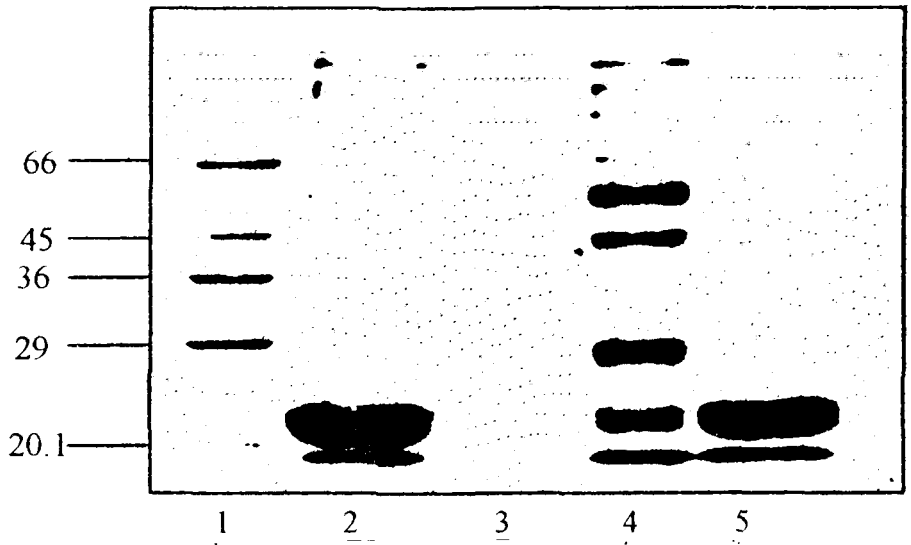
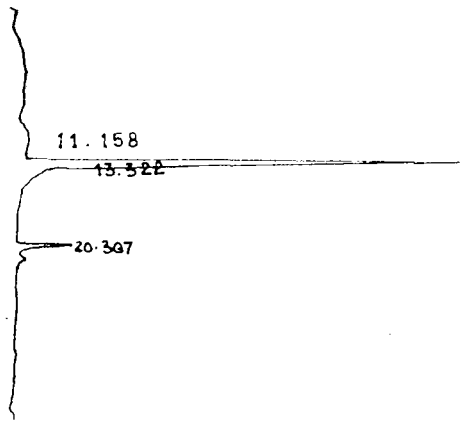


Figure 6 : A 15% SDS-PAGE of different protein peak fractions obtained in Sephadex G-200 chromatography. Lane 1 : Molecular size markers (Molecular sizes are indicated in kD), Lane 2 : ECS, Lane 3 : ECSG-IU, Lane 4 : ECSG-2U, Lane 5 : ECSG-3U.

START
00/00/00 00/00/00



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1.	11.158	5829			7.9233	
2.	13.322	61116	SV		83.0674	
3.	20.307	6628			9.0093	
	TOTAL	73574			100	

Figure 7 : Reversed phase HPLC profile of *buffalo growth* hormone (ECSG-3U).

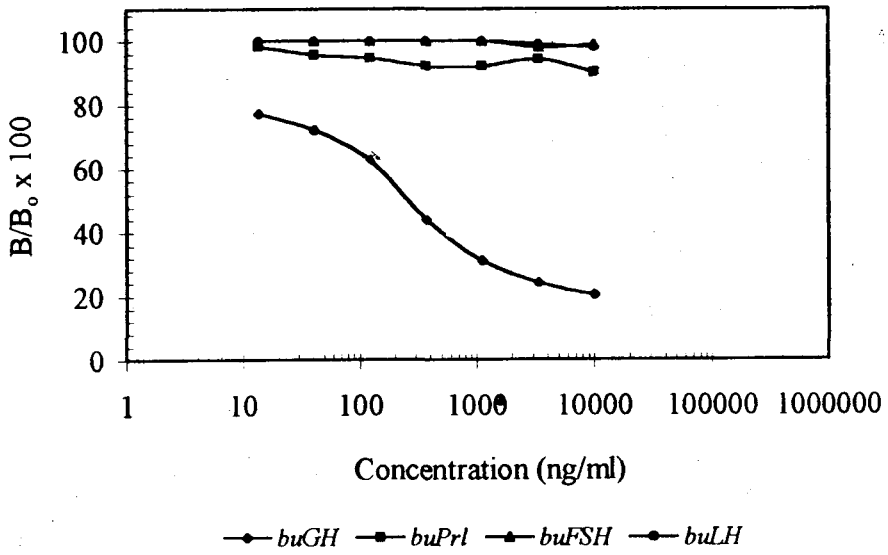


Figure 8 : A competitive RIA showing that the antibody to buffalo GH raised in rabbit is specific to buffalo GH and does not bind to buffalo prolactin, FSH and LH. ^{125}I-buGH was used as tracer. Anti-buGH was used at 1:1000 dilution.

On a Sephadex G-200 matrix, it was seen that two peaks were obtained (Fig.-5) having V_e/V_o values of 1.01 and 2.05. It was seen that the buGH rich fraction elutes after V_e/V_o value of 1.67 corresponding of the K_{av} value of 0.41. SDS-PAGE (Fig.-6) and Western blot (data not shown) of these three fractions obtained also confirmed the purity of ECSG-3U fraction. Immunopotency of these three fractions also showed the ECSG-3U was the most immunopotent fraction (Table-2). Table-2 shows that ECSG-3U is not only the most immunopotent fraction but is also obtained in a relatively high yield compared to other purification chromatographies. The homogeneity of our preparation was also checked on a reverse phase C-18 column on a HPLC. The HPLC profile (Fig.-7) shows that ECSG-3U was almost 83% monomeric preparation. Hence, this procedure is most appropriate to obtain high yields of pure GH from buffalo pituitaries.

Table 1 Comparison of yields of semi-crude GH obtained from the three protocols used.

S. No.	Protocol Used	Semi-Crude buGH Fraction	Yield / Kg. of Pituitary (mg)
1	I	EXTRACT - C ECS	3200 540
2	II	GS	2500
3	III	GAP	2800

Table-2 : Protein yield and immunopotency of various 'GH' fractions obtained from different chromatographies*.

S. No.	Fraction	Yield/Kg of Pituitary Immunopotency **	Relative
1	ECS	540	0.068
2	ECSCM-1	127	0.113
3	ECSCM-2	125	0.243
4	ECSCM-3	61	0.0085
5	ECSCM-4	227	0.358
6	ECSPS-1	75	0.002
7	ECSPS-2	60	0.0017
8	ECSPS-3	405	0.089
9	ECSIMAC-1	40	0.113
10	ECSIMAC-2	360	0.567
11	ECSIMAC-3	23	0.061
12	ECSIMAC-4	32	0.34
13	ECSIMAC-5	4	0.034
14	ECSIMAC-6	45	0.038
15	ECSG-IU	100	0.061
16	ECSG-2U	160	0.142
17	ECSG-3U	280	1

*For explanation of abbreviation see text/figure legends.
**The ED_{50} value of purest GH preparation has been assigned an arbitrary immunopotency unit of 1.

Table 3 : Table comparing the cross-reactivities of oGH, bGH and buGH antisera.

S. No.	Lable	Antiserum	Antigen	ED_{50} Value (ng/ml)
1	^{125}I-oGH	Anti-oGH	oGH buGH	10 40
2	^{125}I-bGH	Anti-bGH	bGH buGH	0.7 5
3	^{125}I-buGH	Anti-buGH	oGH buGH	650 170

The purification was monitored throughout by immunoassay. The antiserum used was raised against ECSG-3U. It was subsequently absorbed with normal buffalo serum to remove non-specific antibodies. As this antiserum cross-reacted with anti-ovine GH (Table-3) and also an anti-ovine GH serum cross-reacted with ECSG-3U (Table-3), it is believed that the preparation of buGH (ECSG-3U) is pure. It can also be seen that anti ECSG-3U (anti-buGH)

serum did not corss-react with buffalo prolactin, FSH or LH (Fig.-8). Subsequent detailed characterization of this preparation indicated that even the minor bands in SDS-PAGE below 20kD are growth hormone variants (Kapil Maithal *et al*-manuscript under preparation).

So, it can be concluded that a pure, immunopotent preparation of buffalo GH and its antibody have been obtained and the availability of sufficient quantities of these would permit undertaking of basic structural studies and also physiological studies in live animals.

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