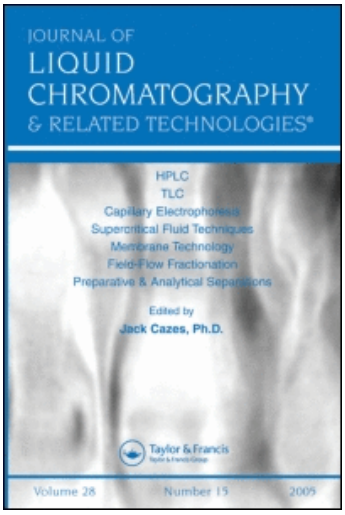

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CHROMATOGRAPHIC SEPARATION OF THYROID STIMULATING HORMONE FROM LUTEINIZING HORMONE

Taruna Arora[a]; Nidhi Vashistha[a]; K. Muralidhar[a]
[a] Hormone Research Laboratory, Department of Zoology, University of Delhi, Delhi, India

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CHROMATOGRAPHIC SEPARATION OF THYROID STIMULATING HORMONE FROM LUTEINIZING HORMONE

Taruna Arora, Nidhi Vashistha, and K. Muralidhar
Hormone Research Laboratory, Department of Zoology, University of Delhi, Delhi, India

□ *A new preparative scale procedure is described for the efficient purification of Thyroid stimulating hormone (TSH) from buffalo pituitary glands. The methodology involves the use of hydrophobic interaction chromatography for the separation of TSH from LH which is more abundant than TSH in the pituitary gland. The two hormones were isolated from the pituitary extract by ammonium sulphate precipitation and were then separated on a Phenyl-Sepharose column. The fractions obtained from Phenyl-Sepharose chromatography were assessed using a Uno-Q column on FPLC where enrichment of the TSH in the PS-2 fraction was evident. The final removal of the trace amounts of the LH was done by ion exchange chromatography in tandem on CM-Sephadex and DEAE-Sephacel columns. All the chromatography fractions were assessed in a heterologous direct binding ELISA using bTSH and buLH as the reference hormones and anti ovine TSH beta and anti bovine LH beta as the antisera. The purified fraction (DEAE-0.5 M peak) when checked exhibited no LH immuno reactivity in a western blot against anti bovine LH beta antiserum. Forty two milligrams of a 200 fold purified buTSH per kg wet glands was obtained.*

Keywords buLH, buTSH, hydrophobic interaction chromatography, purification

INTRODUCTION

Pituitary thyroid stimulating hormone (TSH), regulates synthesis and secretion of thyroid hormone.[1] Thyroid hormone, in turn, affects many diverse tissues and influences major processes such as metabolism, growth, differentiation, reproduction, and lactation.[2] There appears to be a disturbance in the pituitary-thyroid axis underlying summer related amenorrhea observed in many mammals. Thyrotropin has been purified and characterized from several species,[1,3–6] but there is no published work on the purification of buffalo TSH, except for the report on a partially purified preparation of TSH from our laboratory.[6] TSH has always been

Address correspondence to Taruna Arora, Hormone Research Laboratory, Department of Zoology, University of Delhi, Delhi, India. E-mail: tarunaarora27@rediffmail.com; kambadurmurali2001@rediffmail.com

a difficult hormone to purify from any species owing to a number of factors, which include low abundance and yields, interference of other glycoproteins with a very similar chemical nature in the pituitary extract, and the presence of a persistent contaminant hormone, Luteinizing hormone (LH) to different degrees in the thyrotropin preparations.[11]

The two anterior pituitary hormones i.e., TSH and LH are structurally and biochemically very similar to each other. Both are composed of two subunits the α and β, in which α subunit is identical in a given species and β subunit shows 60% sequence similarity.[1] Segregation of TSH from LH has been a difficult task. Lack of a strong anionic or cationic character, considerable charge heterogeneity leading to microheterogeniety within each has made this a daunting task.[7]

Buffalo hormones and their respective probes are not available at present from any commercial source. Thus, studies on hormonal profiles in animals have not been carried out with homologous immunoassay systems till now. The present study reports purification of buffalo thyroptropin but free of buffalo LH.

EXPERIMENTAL

Materials

Buffalo pituitary glands, freshly frozen in liquid nitrogen were obtained from the local abattoir. Bovine TSH and anti beta ovine TSH were obtained from the National Institute of Arthritis, Diabetes, Digestive, and Kidney diseases (NIADDK) Bethesda, USA. Phenyl Sepharose, CM-sephadex, and DEAE sephacel were obtained from Sigma-Aldrich Pvt. Ltd., USA. Anti beta bLH was a kind gift from Dr. J.G. Pierce, USA. Goat anti-rabbit IgG-HRP guinea pig IgG-HRP conjugate was purchased from Jackson Immuno Research laboratories, Inc. ELISA plates were purchased from Greiner, Germany. All other chemicals and reagents were of AnalaR grade.

Methods

Extraction of Semi Crude TSH from Whole Buffalo Pituitary Glands

All the steps were done at 0–4°C. Pituitary glands were processed as described by Chaudhary and Muralidhar.[8] Briefly, pituitary glands were first homogenized in 0.15 M $(NH_4)_2SO_4$, pH 4, and stirred for 3–4 hours. The solid phase was removed by centrifugation and the pH of the supernatant was set to 3 using 0.2 M meta phosphoric acid. After the pellet was removed by centrifugation, pH was adjusted to 6.0–7.0 using 1 M NaOH. Then, ammonium sulphate was added to the supernatant to achieve 50%

saturation. The 50% ammonium sulfate pellet was dialyzed extensively and lyophilized. This fraction (ASP) was used as the starting material for the purification of TSH.

Hydrophobic Interaction Chromatography

Pre swollen phenyl sepharose was packed in a 20 mL glass column and equilibrated with the loading buffer (50 mM Phosphate buffer containing 1 M $(NH_4)_2SO_4$) by passing ten bed volumes of the loading buffer at the rate of 25 mL/hour. Fifty percent $(NH_4)_2SO_4$ pellet fraction (25 mg) was dissolved in 1 mL of loading buffer and applied to the column. Unbound proteins were washed out using the same buffer. Subsequent elution of the bound proteins were done using 50 mM phosphate buffer and 80% ethylene glycol stepwise until the O.D. came to 0.01. Three milliliter fractions were collected using an automatic fraction collector. All the pooled protein peak fractions obtained were subsequently dialyzed against water and lyophilized.

CM-Sephadex Chromatography

CM-Sephadex was swollen in the loading buffer (5 mM phosphate buffer, pH 6.0) for 48 hrs at 4°C, with three changes of the buffer. The matrix was packed in a 25 mL glass column and equilibrated with the loading buffer by passing 4–5 bed volumes of the loading buffer at the rate of 25 mL/hour. Of the PS-2 fraction, 20 mg (phenyl sepharose peak eluted using 50 mM phoshate buffer pH 7.0) was dissolved in 2 mL of the loading buffer and applied to the column. Unbound proteins were washed out using the same buffer. Subsequently, the bound proteins were eluted using 10 mM phoshate buffer pH 7.0, 25 mM borate buffer, pH 8.2, and 25 mM borate buffer pH 8.2 containing 1 M NaCl stepwise until the O.D. came to 0.01. Fractions of 3.5 mL were collected using an automatic fraction collector. All the four eluted peaks were pooled, dialyzed, and lyophilized.

DEAE-Sephacel Chromatography

DEAE-sephacel was swollen in the loading buffer (0.1 M Na glycinate buffer pH 9.5) for 48 hrs at 4°C, with four changes of the buffer. The matrix was packed in a 15 mL glass column and equilibrated with the loading buffer by passing 4–5 bed volumes of the loading buffer at the rate of 25 mL/hour. Fifteen milligrams of CPS-2-4 fraction (CM-Sephadex peak eluted using 25 mM borate buffer pH 8.2 containing 1 M NaCl) was dissolved in 1.5 mL of loading buffer and applied to the column and unbound proteins were eluted out using the same buffer. Later the bound proteins were eluted using 0.2 M, 0.5 M Na glycinate buffer pH 9.5 and 0.5 M Na glycinate buffer containing 1 M NaCl stepwise until the O.D. came to 0.01. Two

milliliter fractions were collected using a fraction collector. All the four eluted peaks were pooled, dialyzed, and lyophilized.

FPLC

Biorad FPLC system was used to run a Uno Q1 column (1.3 mL), which was equilibrated with the loading buffer (20 mM Tris buffer, pH 8.0) (buffer-A). Two milligrams of PS-2 (phenyl sepharose peak eluted using 50 mM phoshate buffer pH 7.0) was dissolved in 1.2 mL of the loading buffer and filtered through a 0.2 μm syringe filter. The filtered sample was injected into the column using a 50 μL and a 120 μL loop. The unbound was eluted using the same buffer. Subsequently, a linear gradient was applied using buffer-B containing 300 mM NaCl in 20 mM Tris buffer, pH 8.0. One milliliter fractions were collected using an automatic fraction collector.

Immunoreactivity

ELISA

All the fractions obtained during various chromatographies were assessed for the presence of immunoreactive TSH and LH using anti beta ovine TSH (NIADDK, USA) and anti beta bovine LH (Kind gift from Dr. J.G. Pierce, USA); bovine TSH (NIADDK, USA) and buffalo LH (reference preparation purified in our own lab) were used as the standard proteins using direct binding ELISA. Antigen was serially diluted in triplicate using 0.1 M ammonium bicarbonate solution, pH 7.6–7.8, and incubated for 3 hrs. The antigen was coated within the concentration range of 1 μg to 100 pg. This was followed by the blocking of the nonspecific sites using 1% casein in 0.1 M phosphate buffer pH 7.0 for 30 minutes. Primary antibody was used at the dilution of 1:25,000 and incubated overnight at 4°C, while the secondary antibody (Goat anti-rabbit IgG-HRP for LH and Goat anti-Guinea pig IgG-HRP conjugate for TSH) was used at the dilution of 1:2,000. All the other incubations except the primary antibody step were performed at 37°C. Washing of the wells was done using phosphate buffer (10 mM, pH 7.0) and tween-20 (0.05%). O.P.D. (1 mg/mL) and H_2O_2 (0.02%) in 50 mM citrate buffer, pH 5.5 were used as the substrate. The plates were read at 490 nm in an ELISA reader. The O.D. of the triplicates were averaged.

Western Blot

This was essentially done according to the method described by Towbin et al.[9] After the electrotransfer, the blocking of the rest of the sites was done using 1% casein in 0.1 M phosphate buffer pH 7.0 for 30 minutes.

This was followed by the incubation of the blot in the primary antibody (anti beta bLH) at the dilution of 1:1000. Subsequently, the blot was incubated in the secondary antibody (Goat anti rabbit IgG HRP conjugate) at a dilution of 1:500. Washing of the wells was done using phosphate buffer (10 mM, pH 7.0) and all the incubations were performed at room temperature. Development of the blot was done using 4-chloro-α-naphthol and H_2O_2 as the substrate.

Protein Estimation

This was done according to the method of Lowry et al.[10] Bovine serum albumin was used as the standard protein in the assay. The reaction mixture (protein, both standard and unknown + alkaline copper solution + phenol calcateau reagent) was incubated for 30 min at room temperature.

RESULTS

Buffalo pituitary glands were processed. Analysis of the various side fractions obtained showed that both LH and TSH were found to get concentrated in the 50% ammonium sulfate pellet, which yields about 1 g of buLH and about 10–15 mg of buTSH per Kg wet pituitary glands (data not shown).

A heterologous direct binding ELISA system, which can clearly discriminate between LH and TSH was used. About 15% cross reactivity was observed in both the systems and the ratio of TSH/LH immuno reactivity was determined to assess the level of purity (Fig. 1).

The 50% ammonium sulfate pellet (ASP), taken as the starting material for the purification of buTSH was loaded on a phenyl sepharose column (Fig. 2). TSH and LH were found distributed in both PS-2 (50 mM PB, pH 7.0 peak) and PS-3 (80% ethylene glycol peak) fractions of the phenyl sepharose column; however, it was observed that PS-2 fraction with a 4 fold enrichment of TSH was found to have higher TSH content than LH (Fig. 9), unlike the PS-3 fraction where buLH was being enriched (Fig. 3). This was further confirmed when the PS-2 peak was loaded on a Uno Q column in FPLC chromatography and eluted using a gradient of NaCl (0–0.3 M) in Tris buffer (Fig. 4).The eluted fractions were assessed for TSH and LH immuno reactivity, wherein the fractions obtained using the NaCl gradient (Tube 12 onwards) exhibited higher TSH immunoreactivity than anti beta bLH (Fig. 5). During phenyl sepharose chromatography, the PS-3 fraction also had some amount of TSH besides LH in it; however, the LH content was 10 fold more than TSH, thus requiring sacrifice of that TSH content. The protocol at several stages requires a sacrifice of some amount of thyrotropin so as to yield us a LH free TSH preparation.

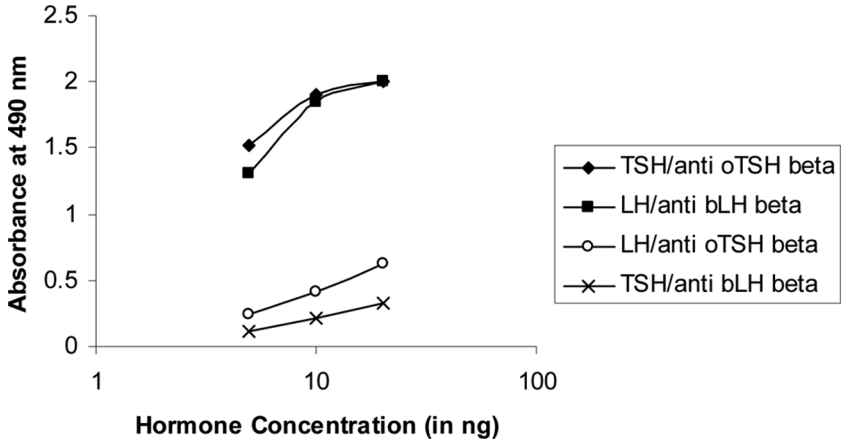

FIGURE 1 Analysis of the immuno reactivity and cross reactivity of bovine TSH (bTSH) and buffalo LH (buLH) with anti ovine TSH beta antiserum and anti bovine LH beta antiserum. The antigen was coated in triplicate on the ELISA plate and 5 ng, 10 ng and 20 ng of the antigen was coated per well. The antiserum was used at the dilution of 1:25,000.

FPLC chromatography on a Uno Q column reflected the possible pathway for the isolation, but it was not pursued and an alternative preparative pathway was searched so as to get good yields of the hormone, which an analytical FPLC column was unable to provide.

Therefore, phenyl sepharose chromatography was serving a dual purpose of enrichment as well as partially segregating TSH from LH. Further,

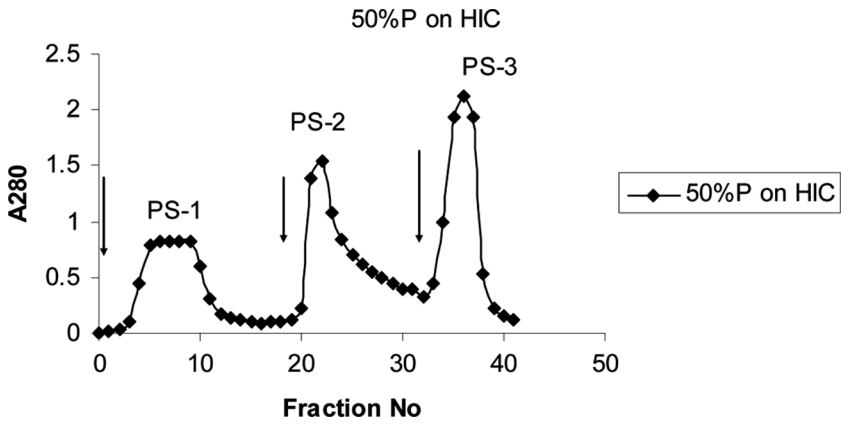

FIGURE 2 Chromatogram showing the elution profile of 50% ammonium sulphate pellet loaded on phenyl sepharose column in 50 mM Phosphate buffer, pH 7.0 containing 1 M $(NH_4)_2SO_4$ (Buffer-A). PS-1 is the unbound protein peak eluted using buffer-A.PS-2 is the bound protein peak eluted using 50 mM Phosphate buffer, pH 7.0 (Buffer-B) while PS-3 is the bound protein peak eluted using 80% Ethylene glycol (Buffer-C). Arrow heads point to the tube number where the buffer was changed.

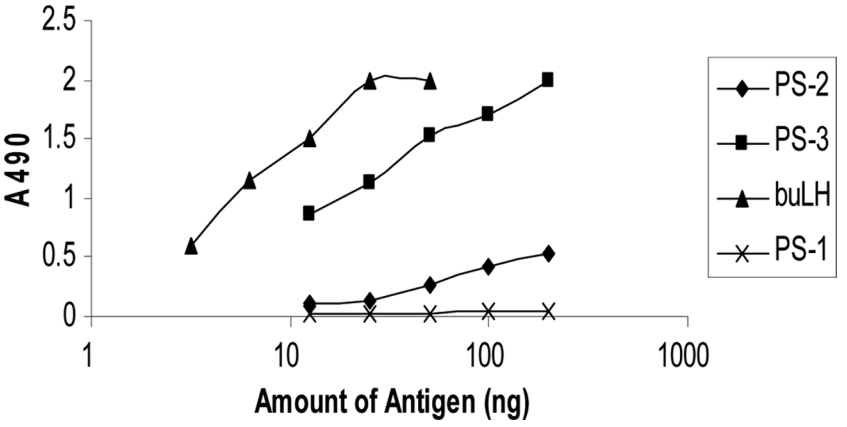

FIGURE 3 Comparative immuno reactivity of PS-1, PS-2 and PS-3 using anti bovine LH beta in a quantitative direct binding ELISA. Buffalo LH (Purified) was used as the standard antigen. The antigen was coated in triplicate on the ELISA plate in nanogram quantity per well. The antiserum was used at the dilution of 1:25,000.

as the loading buffer of the phenyl sepharose chromatography involves the use of 1 M $(NH_4)_2SO_4$, 50% ammonium sulphate fraction can be loaded as such without dialysis, therefore, the dialysis causing loss of TSH could be avoided.

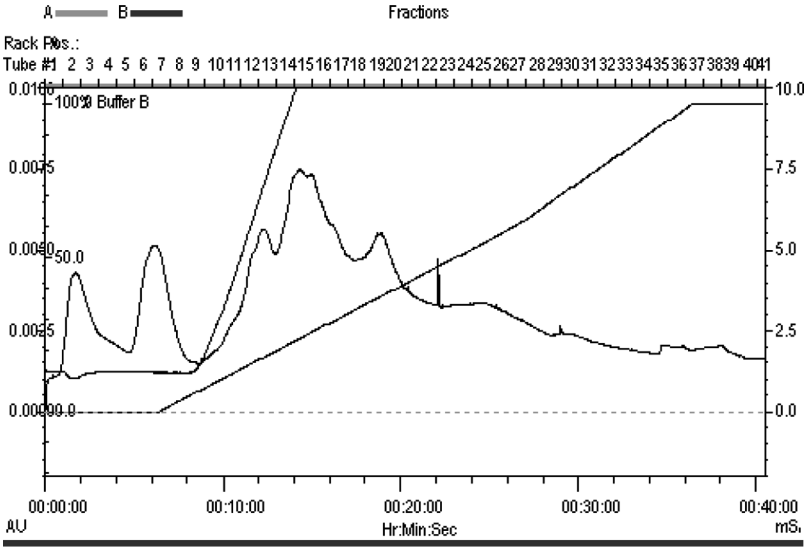

FIGURE 4 FPLC chromatographic profile showing the elution of PS-2 (50 mM PB, pH 7.0 peak of phenyl Sepharose column) on a Uno Q column. It was loaded in 20 mM Tris buffer, pH 8.0 (Buffer-A). The unbound protein was eluted using buffer-A. Subsequently the bound protein was eluted a linear gradient of 0–0.3 M with the help of buffer-B containing 300 mM NaCl in 20 mM Tris buffer, pH 8.0. 1 mL fractions were collected.

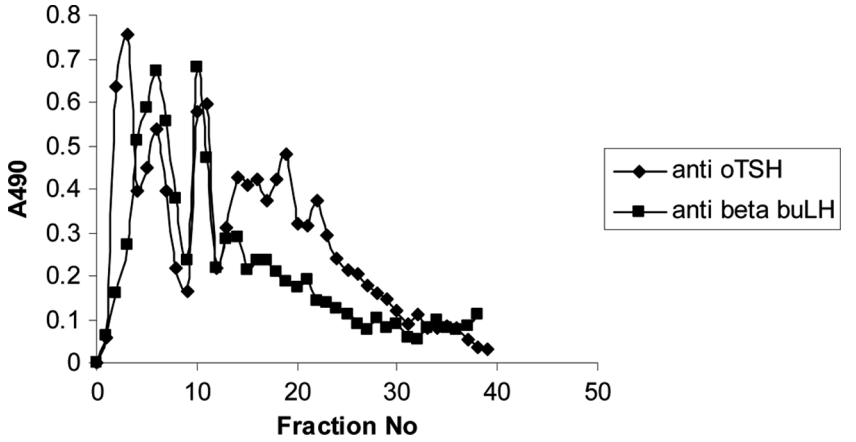

FIGURE 5 Comparative immuno reactive profile of the eluted FPLC fractions obtained by loading PS-2 on the Uno Q column. Equal amounts of each fraction was coated on the ELISA plate in triplicate and assessed for their TSH and LH immuno reactivity using anti ovine TSH beta antiserum and anti bovine LH beta antiserum. The antiserum was used at the dilution of 1:25,000.

The phenyl sepharose column was followed by a CM-Sephadex column chromatography (Fig. 6). The peak obtained using 25 mM borate buffer, pH 8.2 containing 1 M NaCl had maximum TSH immunoreactivity in it (Fig. 6). This step led to further enrichment of buTSH (four fold) in the

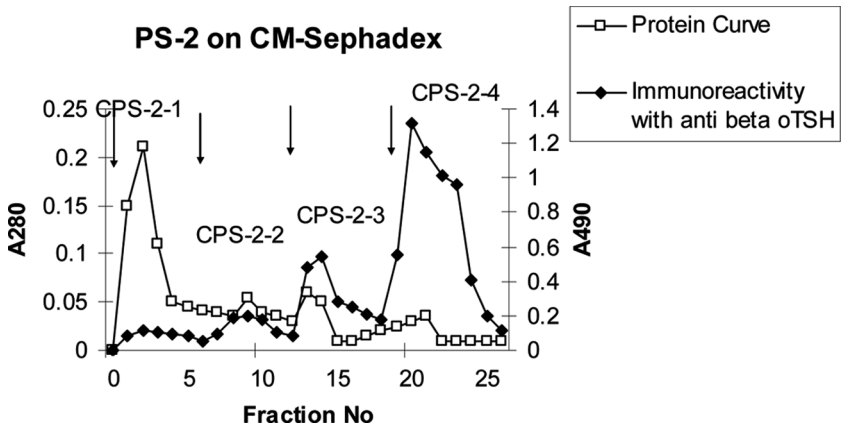

FIGURE 6 Chromatogram and the immuno reactive profile of PS-2 (50 mM PB, pH 7.0 peak of phenyl Sepharose column) on CM-Sephadex column PS-2 was loaded in the loading buffer (5 mM Phosphate buffer, pH 6.0) (Buffer-1). Unbound protein peak (CPS-2-1) was washed out using the same buffer. Subsequently the bound proteins were eluted using 10 mM Phosphate buffer pH 7.0 (Buffer-2) (CPS-2-2 peak), 25 mM Borate buffer, pH 8.2 (Buffer-3) (CPS-2-3 peak) and 25 mM Borate buffer pH 8.2 containing 1 M NaCl (Buffer-4) (CPS-2-4 peak). Arrow heads point to the tube number where the buffer was changed. All the four peak fractions were coated in equal amount (10 µl/well) and assessed for their immuno reactivity using anti beta ovine TSH antiserum at a dilution of 1:25,000.

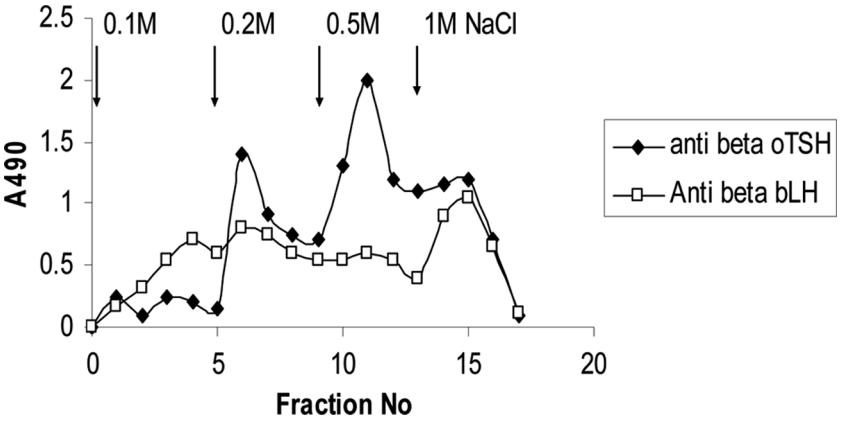

FIGURE 7 Comparative immuno reactive profile of the eluted fractions obtained by loading CPS-2-4 on the DEAE Sephacel column. Equal amounts of each fraction was coated on the ELISA plate in triplicate and assessed for their TSH and LH immuno reactivity using anti ovine TSH beta antiserum and anti bovine LH beta antiserum. The antiserum was used at the dilution of 1:25,000.

1 M NaCl eluate, i.e., CPS-2-4 ((Fig. 9). In terms of TSH/LH ratio, a factor of two was achieved till CPS-2-4, thus exhibiting more enrichment of buTSH than buLH.

This fraction (CPS-2-4) was bound to DEAE sephacel and could be eluted using varying concentrations of sodium glycinate buffer (Fig. 7). This yielded a buTSH preparation (0.5 M sodium glycinate, pH 9.5 peak), which was further enriched 2.5 fold (Figs. 8 and 9). This fraction

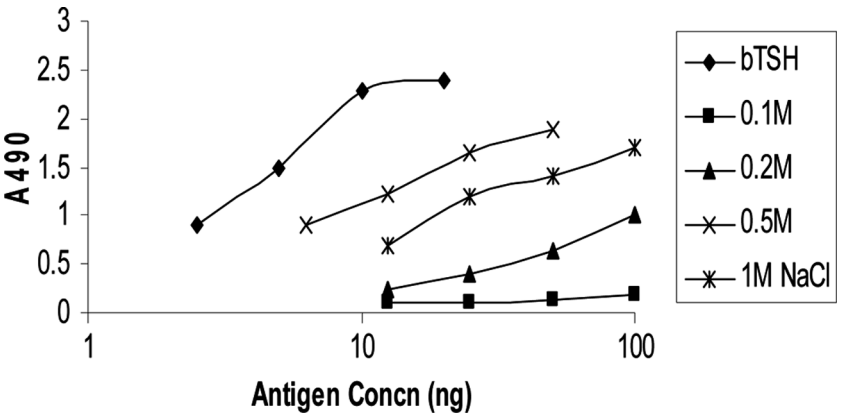

FIGURE 8 Comparative immuno reactivity of the peaks eluted using 0.1 M Na Glycinate buffer, pH 9.5, 0.2 M Na Glycinate buffer, pH 9.5, 0.5 M Na Glycinate buffer, pH 9.5 and 0.5 M Na Glycinate buffer, pH 9.5 containing 1 M NaCl using anti ovine TSH beta in a quantitative direct binding ELISA. Bovine TSH was used as the standard antigen. The antigen was coated in triplicate on the ELISA plate in nanogram quantity per well. The antiserum was used at the dilution of 1:25,000.

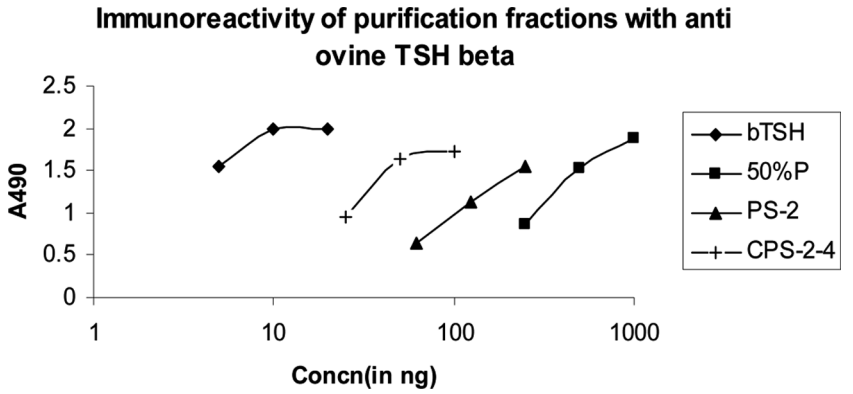

FIGURE 9 Quantitation of immuno reactive buTSH content in various fractions obtained during the purification protocol using anti ovine TSH beta in a direct binding ELISA. Bovine TSH was used as the standard antigen. The antigen was coated in triplicate on the ELISA plate in nanogram quantity per well. The antiserum was used at the dilution of 1:25,000.

(DEAE-0.5 M) was completely free of buLH (Fig. 10). The western blot of DEAE-0.5 M fraction with anti beta bLH didn't show any reactivity with the antiserum (Fig. 11). However, it can be seen that there are two non specific bands at higher molecular weight regions (as shown by SDS-PAGE), which will require a further additional step of gel filtration so as to get an absolutely pure buTSH preparation.

In terms of protein content as much as 42 mg of buTSH per kg wet glands could be obtained. This amount would further decrease when the DEAE fraction would be further sized so as to remove the non specific contaminants. In terms of fold purification, more than 200 fold purification has been achieved till now (Table 1).

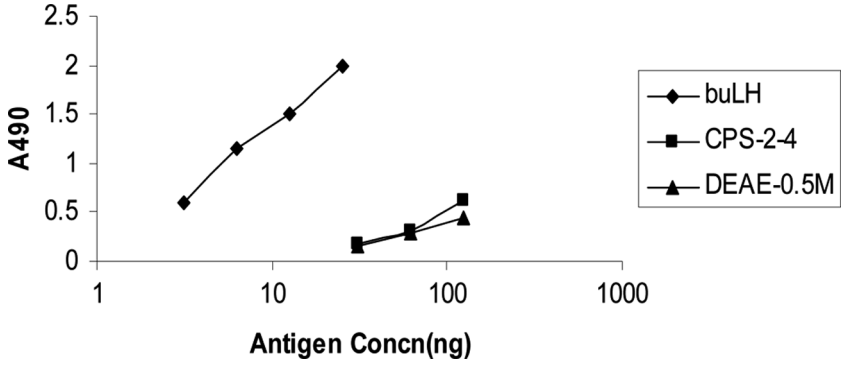

FIGURE 10 Quantitative analysis of immuno reactive buLH content in various fractions obtained during the purification protocol using anti bovine LH beta in a direct binding ELISA. Buffalo LH was used as the standard antigen. The antigen was coated in triplicate on the ELISA plate in nanogram quantity per well. The antiserum was used at the dilution of 1:25,000.

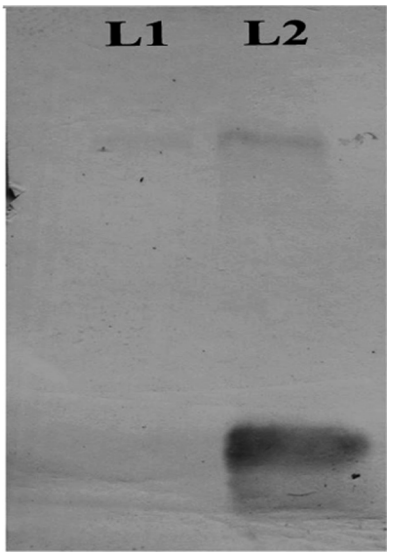

FIGURE 11 Western blot of DEAE-0.5 M (Lane-1) and PS-3. (Lane-2) The SDS-PAGE electro transferred was done in reducing condition, and at 13.5% concentration of the gel. The primary antibody used for probing the presence of buLH was against bovine LH beta subunit.

TABLE 1 Percentage Immuno Reactive TSH Content, Protein Content/kg of the Gland as Well as the Fold Purification at Various Stages of Purification of TSH from the Pituitary Glands of the Water Buffaloes. The Immuno Reactive Content was Determined Using a Quantitative Direct Binding ELISA Using Anti Ovine TSH Beta Antiserum While the Protein was Estimated According to the Lowry's Method

Purification Profile of TSH from buffalo pituitaries				
Protein Samples	Protein (mg)/Gland	Protein (gm)/Kg of Gland	Immuno Reactive TSH Content(%)	Fold Purification
Homogenate	199.31	139.7	0.14	1
50% Pellet	25.17	16.78	0.65	5
PS-2	5.43	3.69	2.5	20
CMS-2–4	0.62	0.44	10	80
DEAE-0.5 M	0.057	0.042	25	200

Hence, we report here the preparation of LH free TSH from buffalo pituitaries.

DISCUSSION AND CONCLUSION

Satisfactory purification of TSH has always been haunted by the inability to separate TSH and LH owing to the similar chromatographic properties of both LH and TSH.[11,16] Several techniques which have

commonly been used include gel filtration, ion exchange, immuno affinity chromatography, etc.[6,12,17,18] Heterogeneity due to various charge forms and overlapping charges makes their complete separation difficult and laborious and, thereby, not only making it difficult to purify these hormones as a single fraction but also loss of these hormones in various side fractions.[13,19] This problem is further accentuated by the fact that LH is present in large quantities in buffalo pituitaries compared to TSH.

Segregation of bovine TSH and bovine LH usually involves the use of DEAE sephacel, wherein LH was non absorbed at very low ionic strength and pH necessary for absorption of TSH. Sodium glycinate buffer at pH 9.5 and a very low ionic strength was used for this purpose.[14,20–23]

During our previous effort we had used CM-sephadex and DEAE sephacel as well as gel filtration for the segregation of the two hormones from the buffalo pituitaries. However, despite the use of three steps of column chromatography to purify the hormone, 10% LH content was still observed in the final fraction of buTSH.[6]

Hydrophobic interaction chromatography has been used previously for the purification of glycoprotein hormones.[16,18] The technique holds promise owing to the lesser effect of charge on the separation. The methodology is able to achieve significant separation in a single step, which is otherwise not easy to accomplish with anion exchange chromatography. Further removal of the trace levels of the LH was achieved using CM sephadex and DEAE sephacel.

The separation methodology also requires the use of a specific antibody, which can discriminate significantly between TSH and LH. The two antiserum (anti ovine TSH beta and anti bovine LH beta) used by us were not only highly specific but also reflected only 15% cross reactivity with the other reference hormones. This could be attributed to significant structural and sequence similarity between the two beta subunits of TSH and LH.

The TSH fraction thus obtained is not only free of LH but has also been found to show TSH bioactivity in a homologous in vitro bioassay in buffalo thyroid cells as well as in radio receptor assay performed in human TSH receptor cell line(data not shown).

REFERENCES

1. Pierce, J.G.; Parsons, T.F. Glycoprotein hormones: structure and function. Ann. Rev. Biochem. **1981**, *50*, 465–495.
2. Field, J.B. Mechanism of action of TSH. In *The Thyroid,* Werner, S.C., Ingbar, S.H., Eds.; 4th ed. Harper and Row: Hagerstown, MD, 1955, 185–253.
3. Pierce, J.G.; Wynston, L.K. On the composition of sheep thyrotropic hormone. Biochim. Biophys. Acta **1960**, *43*, 538–540.
4. Condliffe, P.G. Purification of human thyrotrophin. Endocrinology **1963**, *72*, 893–896.
5. Pierce, J.G.; Liao, T.H.; Howard, S.M.; Shome, B.; Cornell, J.S. Studies on the structure of thyrotropin: its relationship to luteinizing hormone. Rec. Prog. Horm. Res. **1971**, *27*, 165–212.

6. Taruna, K. Biochemical and immunological studies on buffalo pituitary thyroid stimulating hormone, PhD thesis submitted to Delhi University, 1997.
7. Yora, T.; Matsuzuki, S.; Kondo, Y.; Ui, N. Changes in the contents of multiple components of rat pituitary thyrotropin in altered thyroid states. Endocrinology **1979**, *104*, 1682–1685.
8. Chaudhary, R.; Muralidhar, K. Use of SP-sephadex to fractionate and obtain semi-pure preparation from whole pituitaries of indian water buffaloes (Bubalus bubalis). Prep. Biochem. Biotech. **2004**, *34*, 315–329.
9. Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. J. Proc. Natl. Acad. Sci. **1979**, *76*(9), 4350–4354.
10. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **1951**, *193*, 265–275.
11. Arora, T.; Muralidhar, K. Microheterogeneity in buffalo thyroid stimulating hormone. J. Endocrinol. Reprod. **1999**, *3*(2), 70–80.
12. Wynston, L.K.; Free, C.A.; Pierce, J.G. Further chromatographic studies on beef thyrotropin and a comparison of beef, sheep, and whale thyrotropins. J. Biol. Chem. **1960**, *235*(1), 85–90.
13. Fontaine, Y.A.; Condliffe, P.G. Purification de L'Hormone Thyr'eothrope d'un t'el'eoste'en (l'anguille). Parall'ele entre la sp'ecificit'e zoologique de cette hormone et quelques-uns de ses caract'eres physicochimiques. Bull. Soc. Chim. Biol. (Paris) **1963**, *45*, 681–693.
14. Fontaine, Y.A. Zoologic specificity of pituitary proteins capable of stimulating the thyroid gland. Acta Endocrinol. (Copenhagen) **1969**, *60*, 136–140.
15. Pierce, J.G.; Liao, T.H.; Carlson, R.B. *Hormonal Proteins Pept.* Ed. Li, C.H. Academic Press: New York, 1973, Vol. 1, pp. 17.
16. Hiyama, J.; Surus, A.; Renwick, A.G.C. Purification of human pituitary LH and thyrotrophin by hydrophobic chromatography. J. Endocrinol. **1990**, *125*, 493–500.
17. Condliffe, P.G.; Bates, R.W. Chromatography of thyrotrophin on diethylaminoethyl-cellulose. Arch. Biochem. Biophys. **1957**, *68*(1), 229–230.
18. Hoferer, S.; Lecompte, F.; Mgallon, T.; Palmer, E.; Combarnous, Y. J. Induction of ovulation and superovulation in mares using equine LH and FSH separated by hydrophobic interaction chromatography. J. Reprod. Fertility **1993**, *98*(2), 597–602.
19. Wide, L. Median charge and heterogeneity of human pituitary FSH, LH and TSH II relationship to sex and age. Acta Endocrinol. (Copenhagen) **1985**, *109*, 181–189.
20. Shome, B.; Parlow, A.F.; Ramirez, V.D.; Elrick, H.; Pierce, J.G. Human and porcine thyrotropins: a comparison of electrophoretic and immunological properties with the bovine hormone. Arch. Biochem. Biophys. **1968**, *126*(2), 444–55.
21. Shome, B.; Parlow, A.F.; Ramirez, V.D.; Elrick, H.; Pierce, J.G. Bovine, human and porcine thyrotropins: molecular weights, amino- and carboxyl-terminal studies. Arch. Biochem. Biophys. **1968**, *126*(2), 456–468.
22. Pierce, J.G. Chemistry of thyroid stimulating hormone, in *Handbook of Physiology and Endocrinology*; Amer. Physiol. Soc.: Washington, D.C., 1974, *4*, 79–101.
23. Condliffe, P.G.; Mochzuki, M.; Fontaine, Y.A.; Bates, R.W. Purification and properties of thyrotropin from functional pituitary tumors in mice. Endocrinology **1969**, *85*, 453–464.