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OCCURRENCE OF FREE ALPHA SUBUNIT OF THE THYROID STIMULATING HORMONE FAMILY IN PITUITARIES OF WATER BUFFALOES (*Bubalus bubalis*)

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SUMMARY

Presence of free alpha subunit has been noticed in a side fraction obtained during the processing of buffalo pituitary glands for thyrotropin and gonadotropins using standard isolation and fractionation procedures. In addition to the gel chromatographic data (Ve/Vo), when the material was analyzed with different antisera, it clearly reacted with alpha subunit directed antibodies. The NaCl precipitation method of Sairam for the separation of the free subunits from each other was applied to the material. The yield of the alpha subunit by the NaCl precipitation method with respect to the immunogenicity and the content was higher than what was obtained by the counter current distribution.

The sugar content of the free alpha subunit was less (70 µg/mg) compared to the sugar content of the alpha subunit isolated by the dissociation from the intact hormone (204 µg/mg). Most of the free alpha subunit eluted as the unbound fraction from a Concanavalin A sepharose column thus supporting the idea that there was an alteration in carbohydrate structure in these naturally occurring free subunits.

Key words : Alpha subunit; Free subunit; Glycoprotein hormones; Thyrotropin.

INTRODUCTION:

In addition to the alpha subunit present in the glycoprotein hormones, significant amount of the free subunits have been reported to be present in the bovine and mouse pituitary fractions as well as the human placental tissue and serum (1). The concentrations of the glycoprotein hormone free alpha and free beta subunits in the extra embryonic coelomic fluid (EECF) far exceeds than in the amniotic fluid or the meternal serum. It is twice that of the intact hCG (2). Serum concentrations of the free alpha subunits are also elevated in postmenopausal women and patients with primary hypothyroidism. Most markedly elevated and unbalanced concentrations

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of circulating alpha subunit have been described in patients with pituitary tumors and certain malignant tumors (3).

Many procedures have been used for the separation of the subunits from the intact whole hormone and these include procedures ranging from elaborate CCD (4). Ion exchange chromatography following dissociation (5, 6) to more simple gel filtration (7), salt precipitation (8) or RP-HPLC (9, 10, 11).

In our work, it was observed that gel chromatography of a side fraction obtained from the buffalo pituitary glands being extracted according to the classical Papkoff's protocol (12) pointed to the presence of considerable quantities of proteins in the region corresponding to 20 kDa molecular size (13). Curiosity prompted us to investigate the nature of this protein fraction (called hereafter fraction-4). Results presented below indicated that buffalo pituitary glands have free alpha subunit in them.

MATERIALS AND METHODS

Buffalo pituitary glands were procured from a local abbatoir in a freshly frozen state. Buffalo LH (buLH) and rabbit antiserum to buffalo LH and beta subunits of buffalo LH were produced in our laboratory only. Antiserum to bovine TSH and bovine LH alpha subunit were kind gifts from Dr. J.G. Pierce. All the other chemicals and reagents used were of GR or Excelar grade.

Processing of the buffalo glands was done using the modified Papkoff's protocol (12). Briefly, the glands were serially extracted using 0.15 M ammonium sulfate followed by the adjustment of the pH to 4.0 (for PRL precipitation), readjustment of pH to 3.0 (inert protein precipitation) and then ultimately to 6.7 followed by precipitation LH at 50% ammonium sulfate saturation (12). The 50% ammonium sulfate precipitant thus obtained was then further sized on a sephacryl S-300 column (120 cm x 0.8 cm) wherein the material eluted after the Ve/Vo value of 1.8 was then pooled and labeled as fraction-4 (13). This fraction-4 was investigated for the presence of free subunits.

Size fractionation of the sample was redone on a sephadex G-200 column. The gel. Swollen and equilibrated in 0.125M ammonium bicarbonate was packed in a 90 X 1.8 cm column. The column was calibrated using the standard gel filtration markers and their respective Ve/Vo values determined. 0.125 M Ammonium bicarbonate was used as the elution solvent wherein 3 ml fraction were collected at a flow rate of 15 ml / hour. The eluted fractions were monitored for UV-light absorption at 280 nm.

SDS-PAGE was done essentially according to the procedure of Laemmili (14). Separation of the subunits was done essentially according to the procedure of Sairam and Li (8). Briefly, the sample was dissolved in 5 ml. Of 0.1 M sodium acetate buffer. pH 3.0 and then incubated for 6 hrs at 23°C. This was followed by precipitation using 3 M NaCl for 2 hrs at 23°C. The

separated pellet was washed twice with 5 ml sodium acetate buffer (0.1M. pH 3.0) containing 3 M NaCl. The supernatants and the washings were then pooled together and designated as the alpha (NaCl) subunit while the pellet was desalted and designated as beta (NaCl) subunit.

For the counter current distribution (CCD), the two subunits in a sample of intact hormone were separated essentially according to the protocol of Papkoff and Samy (4), wherein the solvent system used to achieve both the dissociation of the hormone and its separation are: butanol: isobutyrate: triethylamine: water:: 1: 2: 0.1: 3.

Ten transfers were performed and the upper and the lower phases were separately pooled and lyophilized.

The total carbohydrate content of the sample was estimated, using phenol sulfuric acid method (15). Glucose was used as the standard. Concanavalin A Sepharose column was packed and equilibrated with buffer-Ia (0.01 M tris HCl. pH 7.5 containing 0.3 M NaCl, 1 mM calcium chloride, 1 mM magnesium chloride and 1 mM manganese chloride). Samples were applied at RT in buffer-1 (0.01 M tris HCl, pH 7.5 containing 0.3 M NaCl), incubated for 1 hour and the unbound fraction eluted. The bound fraction was eluted using buffer-2 (0.01 M tris HCl buffer. pH 7.5 containing 0.3 M NaCl and 0.2 M methyl mannoside and buffer-3 (0.05 M acetate buffer. pH 5.0 containing 0.3 M NaCl, 0.3 M ammonium sulfate and 0.3M methyl mannoside).

RESULTS:

Buffalo pituitary glands were processed essentially according to the classical protocol of Papkoff *et al.* (12) and the 50% ammonium sulfate precipitation obtained was size fractionated on a Sephacryl S-300 column. Fractions which eluted after the 30 kDa material hereafter designated as fraction-4 i.e., at a Ve/Vo > 1.8 were pooled, dialyzed and lyophilized. The material was rechromatographed on a Sephadex G-200 column (90 x 1.8 cm) using 0.125 M ammonium bicarbonate as the solvent. As indicated in Fig. 1, there was no protein eluting before the Ve/Vo of 2.1. This is far beyond the Ve/Vo value of intact TSH or LH from different species (1.57-1.66).

The material was analyzed for respective immunoreactivity using the four antisera i.e., anti bTSH, Anti buLH, Anti alpha buLH and anti beta buLH wherein the fractions showed positive dose dependent reactivity at the given three doses of 100. 50 and 25 ng while the primary antibody was used at a single dilution of 1:5000 (Fig. 2), when analyzed on a SDS-PAGE. The protein bands corresponded to a molecular size of 18.7 kDa and 16.3 kDa (Fig. 3). These values were less than the molecular mass of the two dissociated subunits of either ovine LH (19.3 and 18.1 kDa) or buLH (19.3 and 17.6 kDa) (13). When calculated, each gram of the frozen wet gland yielded about 4 mg of the 50% ammonium sulfate pellet and 15% of the 50% ammonium sulfate pellet i.e., 0.6 mg represented the material with a Ve/Vo > 1.8. This was three times more compared to the yields of buLH which is 0.2 mg/g of the wet weight of the gland.

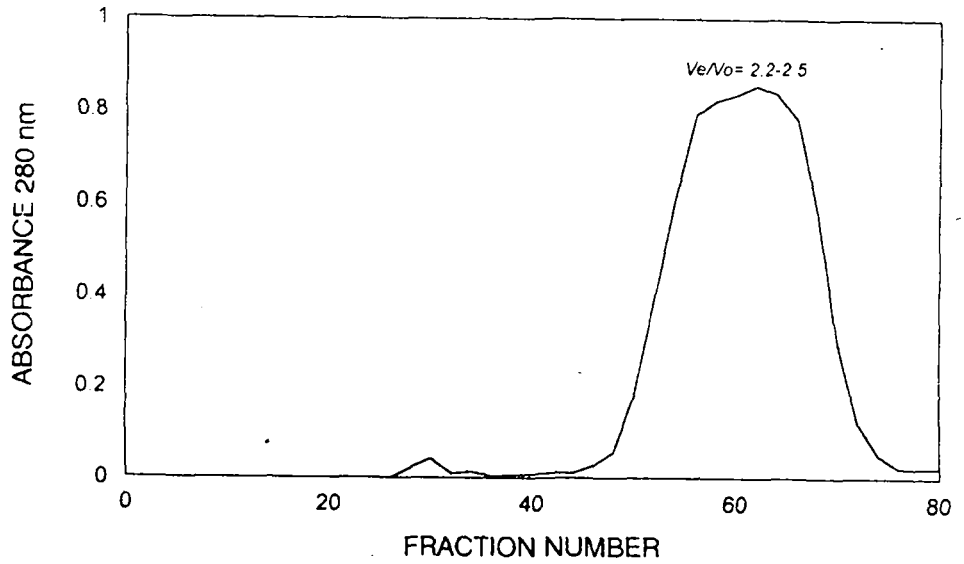

Figure 1 : Elution profile of fraction – 4 (material with a Ve/Vo >1.8) on a Sephadex G-200 column.

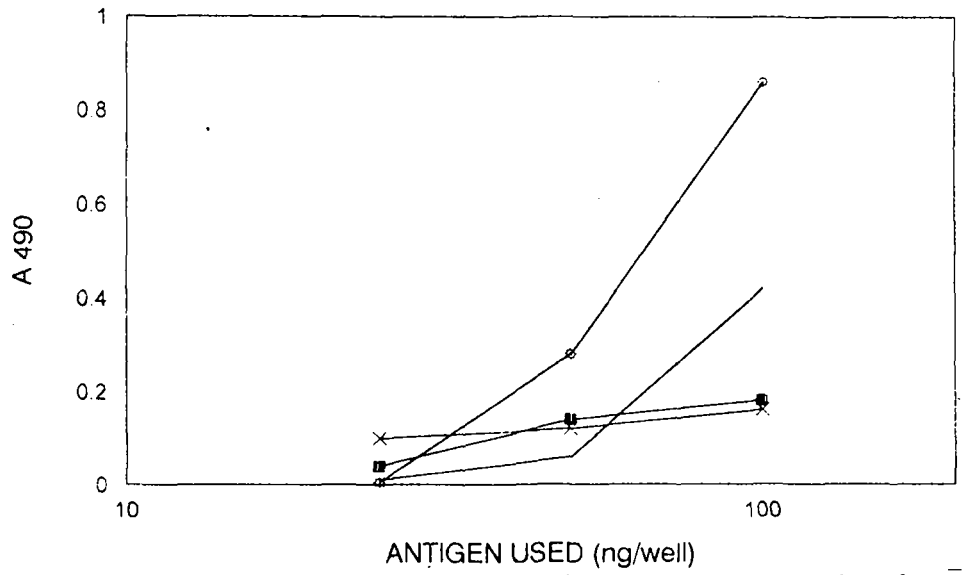

Figure 2 : Immunoreactivity of fraction-4 as determined using peroxidase based direct binding ELISA. The antisera used were anti bTSH (—), anti buLH (⋆), anti alpha bLH (⋆) and anti beta buLH (⋆) at a dilution of 1:5000.

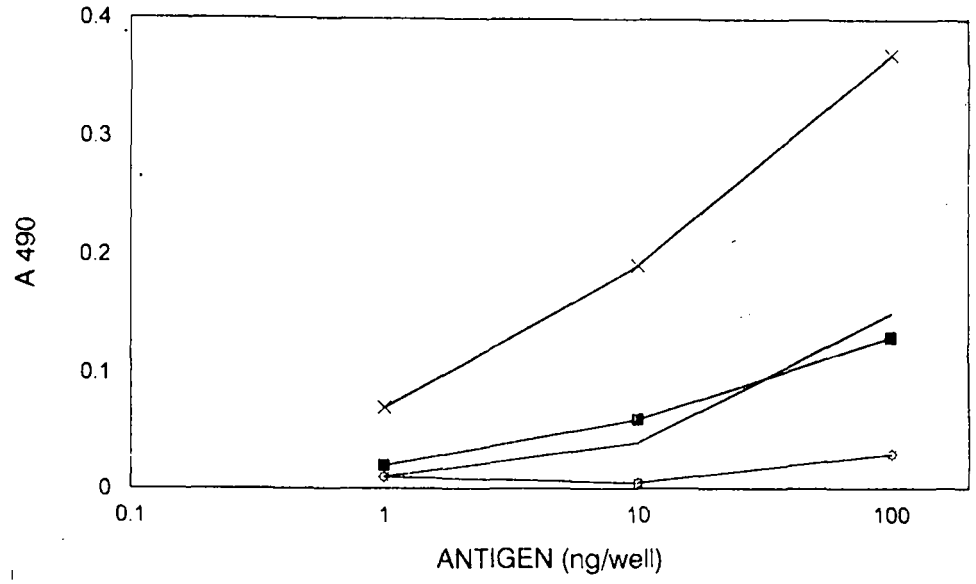

Figure 4 : Peroxidase based direct binding ELISA using anti alpha bLH at a dilution of 1:5000, to determine the presence of alpha subunit in various fractions as alpha CCD (—); alpha (NaCl) (*); beta CCD (•) and beta (NaCl) (■).

Free Subunits

The alpha (NaCl) and the beta (NaCl) subunits obtained from the material with Ve/Vo > 1.8 was compared in a direct binding peroxidase based ELISA with the alpha and beta subunits of the intact buLH separated using the counter current distribution method. These subunits have been designated as the alpha (NaCl), beta (NaCl), alpha (CCD) and beta (CCD). The different antisera used were anti alpha bLH serum and anti beta buLH serum. With anti alpha bLH serum, alpha (NaCl) showed very high reactivity compared to alpha (CCD), while the immunoreactivity of beta (CCD) was negligible compared to the beta (NaCl) which showed some reactivity (Fig.4). Similarly, when anti beta buLH serum was used, the immunoreactivity of beta (CCD) and beta (NaCl) at a single dose was almost equal while the immunoreactivity of alpha (NaCl) was higher compared to the immunoreactivity of alpha (CCD), thus suggesting a higher level of contamination in alpha (NaCl). The analysis was done at the doses of 100. 10 and 1 ng (Fig. 5). When alpha (NaCl) was analyzed for its total sugar content using the phenol-sulfuric acid method (15), the carbohydrate content was found to be comparatively less than the values mentioned in the literature for dissociated subunits (16). Thus alpha (NaCl) had 70 µg/mg protein as the carbohydrate content (Table-1).

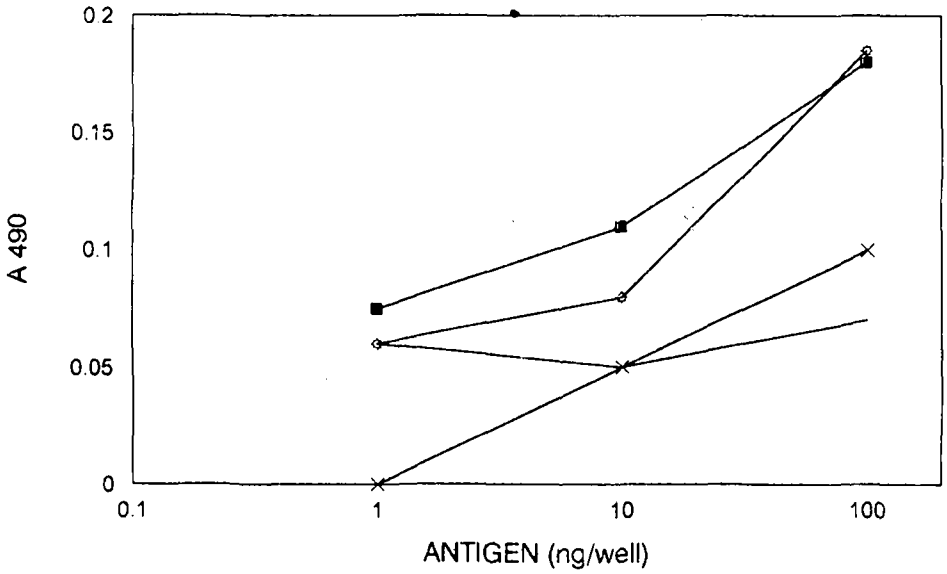

Figure 5 : Peroxidase based direct binding ELISA using anti beta buLH at a dilution of 1:5000, to determine the presence of alpha subunit in various fractions as alpha CCD (—); alpha (NaCl) (☆); beta CCD (✪) and beta (NaCl) (■)

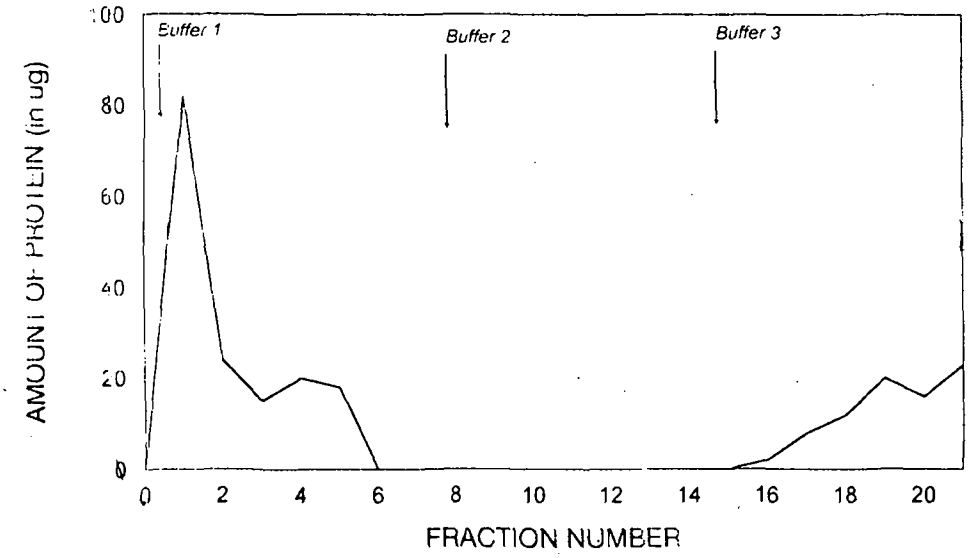

Figure 6 : Elution profile of alpha (NaCl) when passed through a Concanavlin A Sepharose column.

Table 1 : Total sugar content (µg/mg) in various fractions using phenol sulfuric acid method.

Fraction	Total Sugar Content (µg/mg)
Bovine LH alpha*	214
Ovine LH alpha*	204
Bovine LH beta*	92
Ovine LH beta*	97
Alpha (NaCl)	70
Beta (NaCl)	40

* From Literature (16)

When alpha (NaCl) was passed through a Con A column, most of the material was eluted as the unbound fraction thus confirming that the carbohydrate content of the free subunits is less and/or altered (Fig. 6).

DISCUSSION

Presence of free subunits has been reported earlier in the case of bovine species and in humans (17, 18, 1). An attempt was made towards assessing buffalo pituitary fractions for the plausible presence of free subunits. Sairam (19) reported the Ve/Vo values of native oLH, bLH and the alpha subunit are 1.55, 1.62 and 2.24, respectively. Thus, the Ve/Vo value of the fraction analyzed in this study was well within the subunit range which was reaffirmed when the same fraction was resized on a Sephadex G-200 column. The immunoreactivity of different fractions analyzed using anti alpha bLH, anti beta buLH, anti bTSH and anti buLH qualitatively showed the presence of subunits in the fractions. The general problem in quantitating the free alpha and free beta subunits by immunoassays is that serum developed to one subunit recognizes both the combined and the free subunit, thus preventing the independent quantitation in the presence of the dimer. Hence, it was difficult to determine the yields of the free alpha and beta subunit separately. All the above mentioned results indicate that the immunoreactive material are most likely the free subunits which are produced and secreted from the pituitary. The molecular size of the free subunits was in general found to be less than the subunits of the intact hormone respectively.

Immunoreactive LH material of size smaller than the subunits has also been found in the pituitary preparation (20) which further adds support to the theory that the circulating subunits were not derived from a peripheral degradation of the intact hormone (21). These free subunits could be pituitary secretions, metabolic degradation products or dissociation of the native molecule resulting from the

technical manipulations. The free subunit content far exceeded that of the native hormone In the buffalo pituitary. A large amount of the free alpha subunit has been observed when compared to the hTSH and hLH content (17, 20). This excess of alpha subunit is ascribed to a greater synthesis of this subunit than what is required for the assembly of the whole hormone. The sizing of the free subunit after going through separation suggests either aggregation or heterogeneity in the preparation. The starting material analyzed in this study, on the other hand could also be a smaller LH like the one reported by Ryan (20) in the case of human LH and it could also be a species without a full complement of sugar. The latter suggestion has been encountered during the isolation of bLH by Pierce (22). In addition, evidence for the presence of multiple forms of hLH in pituitary glands showing difference in the carbohydrate composition has also been reported (23).

While the alpha subunit from native LH has a carbohydrate content of 214 µg/mg protein (in the case of oLH) and 204 µg/mg protein (in the case of bLH), the free alpha subunit obtained in this study had the carbohydrate content of only about 70 µg/mg protein. Similarly, when the free subunit was passed through a Con A Sepharose column, most of it eluted as the unbound fraction, confirming the carbohydrate content in the free form of the subunit present in buffalo pituitaries was highly reduced.

In conclusion, buffalo pituitary glands appear to synthesize considerable amounts of free alpha and beta subunits which do not appear to form the heterodimeric intact hormone at least in vivo. Whether they circulate as such in blood and whether they have any biological role is being investigated.

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