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MICROHETEROGENEITY IN BUFFALO THYROID STIMULATING HORMONE

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SUMMARY

Microheterogeneity has been observed in the thyrotropin isolated from buffalo pituitary glands using the standard isolation and fractionation procedures. Crude thyrotropin was passed through CM-Sephadex column for enrichment, followed by sizing through Sephadex G-200 and ultimately DEAE-Sephacel was used for the segregation of TSH from LH. The buTSH obtained had 72% TSH immunoreactivity and a specific bioactivity of 71U/mg. It had only about 10% LH content in it. N-terminal analysis of buTSH revealed heterogeneity where the two N-termini were determined as Phe and Gly. The carbohydrate analysis of the buTSH fraction revealed the presence of three microheterogenous forms, out of which the form containing the biantennary carbohydrate structure had the highest immunoreactivity. However, buffalo TSH was found to be different from sheep TSH. The latter appears to have a high mannose/hybrid carbohydrate structure in the most immunoreactive fraction.

Key words : Buffalo TSH, glycoprotein hormones, heterogeneity, pituitary, sheep TSH, thyrotropin.

INTRODUCTION

Structural microheterogeneity in pituitary glycoprotein hormones has been described earlier (1). Both charge and size based heterogeneity has been described for these hormones (2, 3). The presence of heterogeneity also affects the isolation of biological macromolecules and hence, the homogeneity of the purified hormone varies based on its structural, immunological and biological characterization. The physiological significance of such heterogeneity is not yet clear.

Thyroid stimulating hormone (TSH, thyrotropin), the main regulatory hormone affecting the thyroid hormone synthesis, is a glycosylated heterodimeric protein consisting of a species specific alpha subunit and a hormone specific beta subunit noncovalently linked to each other. Thyrotropin from a number of animal groups like sheep, pig, cattle, horse, fish and human has

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been purified and characterized (4). Buffalo is not only an important Indian dairy animal but is also the preferred one, owing to its hardiness, resistance to tropical diseases and adaptability to a large number of environmental conditions. Buffalo hormones and their respective probes (i.e. antibodies, cDNAs) are at present not available from any source. This affects the hormonal profile studies of these animals as well as the MOET studies on these animals. Altered thyroid status is known to affect the rest of the body like the action of the other trophic hormones like LH and FSH (5). To understand this, the purification of buffalo TSH as well as the heterogeneity studies were undertaken.

MATERIALS AND METHODS

Buffalo pituitary glands were procured from a local abbatoir in a freshly frozen state. Bovine TSH, anti-ovine TSH and rat LH were obtained from National Institute of Arthritis, Diabetes, Digestive and Kidney diseases (NIADDK), Bethesda, USA. Buffalo LH (buLH) and rabbit antiserum to buffalo LH were produced in our laboratory only. Antiserum to bovine TSH, bovine LH alpha subunit and bovine LH beta subunit were kind gifts from Dr. J.G. Pierce, carrier free $Na^{125}I$ was obtained from Bhabha Atomic Research Center, India. All the other chemicals and reagents, used were of GR or Excelar grade.

Processing of Buffalo Glands

Freshly frozen (Liquid nitrogen) buffalo pituitary glands were processed and differentially extracted following the classical protocol of Ellis (6) with a few modifications. Briefly, the glands were homogenized in distilled water at pH 5.5 and centrifuged, to obtain extract-A, while the residual pellet was extracted with 0.1M ammonium sulfate, pH 4.0 and centifuged to yield extract-B. The residual pellet from this was further used for extracting growth hormone and prolactin differentially. The extract-B was used as the starting material for the purification of TSH (11).

Assays

Heterologous immunoassays such as ELISA and RIA, where antisera to bovine TSH, ovine TSH as well as buffalo LH beta subunit and bovine LH were used as the primary antibodies, were standardized and used to detect the presence of TSH and LH in various side fractions. SDS-PAGE was performed according to the Laemmili's procedure (7).

The modified method of McKenzie (9) using radioiodinated Iodine was followed to determine the thyrotropic activity of different samples. The Dansyl chloride protocol followed for the N-terminus analysis was essentially that of Gray (10).

The more sensitive bicinchoninic was performed (10) for protein estimation.

Concanavlin A Sepharose column was packed and equilibrated with buffer –1a (0.01M

tris-HCl, pH-7.5 containing 0.3M NaCl, ImM calcium chloride, ImM magnesium chloride and ImM manganese chloride). Samples were applied at RT in buffer-1 (0.01M 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 (0.01M Tris-HCl buffer, pH-7.5 containing 0.3M NaCl and 0.2 M methyl mannoside) and buffer-3 (0.05 M acetate buffer, pH-5.0 containing 0.3M NaCl, 0.3M ammonium sulfate and 0.3M methyl mannoside).

RESULTS

Freshly frozen buffalo pituitary glands were processed and differentially extracted following the classical protocol of Ellis with a few modifications (11). All the side fractions obtained were analyzed for the presence of TSH so that it could serve as a starting material for the purification of buffalo thyrotropin. Extract-B obtained using 0.1M ammonium sulfate, pH 4.0 solution was found to be reactive (data not shown). Also, heterologous RIA confirmed this wherein the TSH content in the extract-B was estimated to be around 30 microgram/mg protein. Hence, extract-B was considered as a good source for TSH purification (Table.1). This starting material was subjected to series of chromatographic procedures. The final step was a gradient elution from DEAE Sephacel. The 0.5M fraction was considered as the purified buTSH fraction (data not shown). N-terminal analysis of the fraction using the dansyl chloride and the DABITC method revealed the two N-termini of the two subunits of TSH to be phenylalanine and glycine (data not shown). This suggests that either the N-terminus of one of the two subunits is different or there is a nick or a cut at the third position in the alpha subunit leading to the exposure of glycine. This is based on bTSH primary structure.

Table-1 : TSH content in the various side fractions obtained during the processing of the buffalo pituitary gland using the Ellis protocol.

Fractions	TSH content (ug/mg protein)
Extract-A	2.8 ug
Extract-B	30 ug
Extract-C	3.2 ug

Purified 0.5M fraction (buTSH) from DEAE Sephacel was also analyzed on concanavalin - A Sepharose chromatography. Owing to the small fraction size, equal aliquots were assessed for TSH immunoreactivity as well as protein estimation. Three isoforms of buTSH fraction with almost equal TSH immunoreactivity were observed (Unbound (UB) = 32%, Weakly bound (B-1)=31% and Tightly bound (B-II)=37% (Fig.1). However, when the specific immunoactivity was checked, B-I was seen as the form of TSH which had got maximum specific immunoactivity for bTSH (70%) followed by B-II (20%) and then UB (10%). The B-II form is predominantly composed of biantennary carbohydrate structures (12-14).

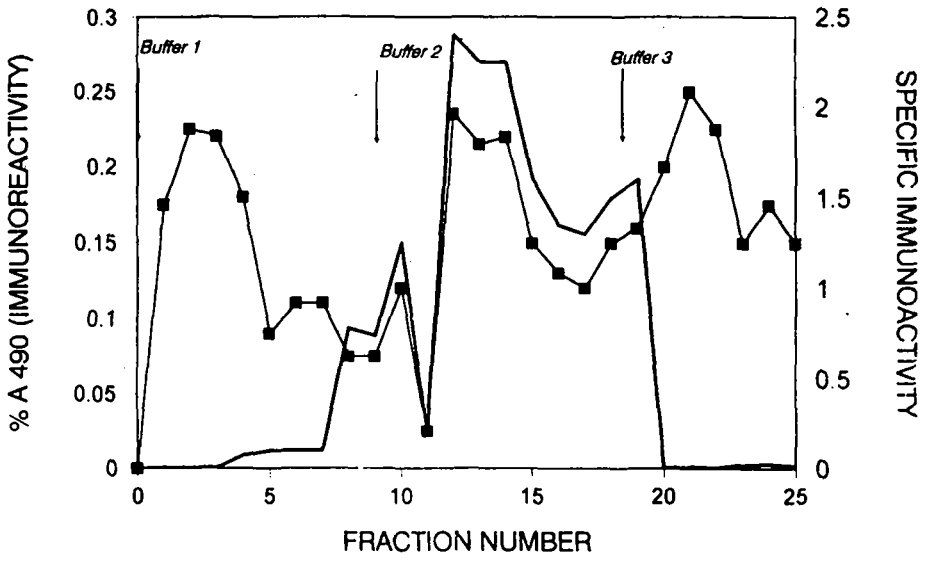

Figure 1 : Comparison of immunoreactivity and specific immunoreactivity profiles of different fractions obtained when buTSH was passed through a Con-A Sepharose column. The antiserum used for analyzing the fractions was anti bTSH at a dilution of 1:5000. Immunoreactivity; (■)Specific immunoactivity.

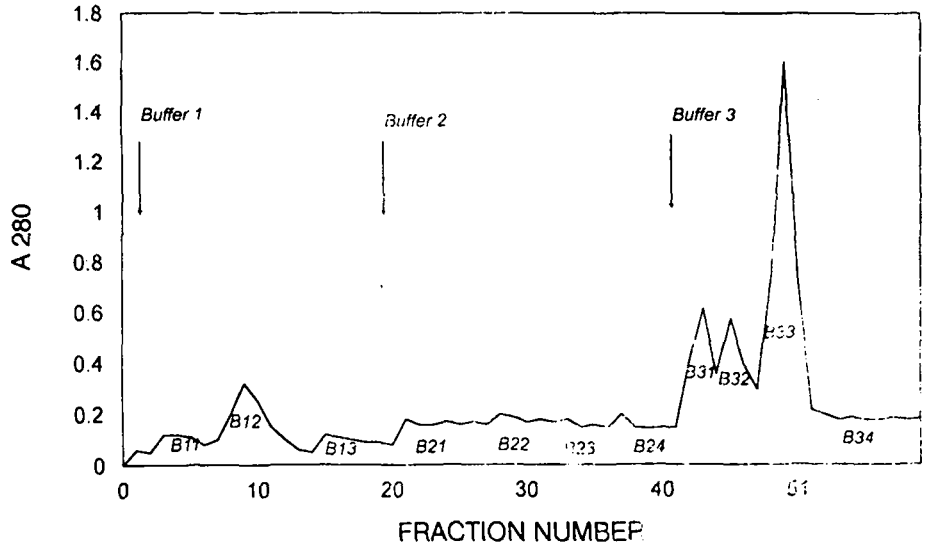

Figure 2a : Protein elution profile of extract-B (Buffalo) on a Con-A Sepharose column.

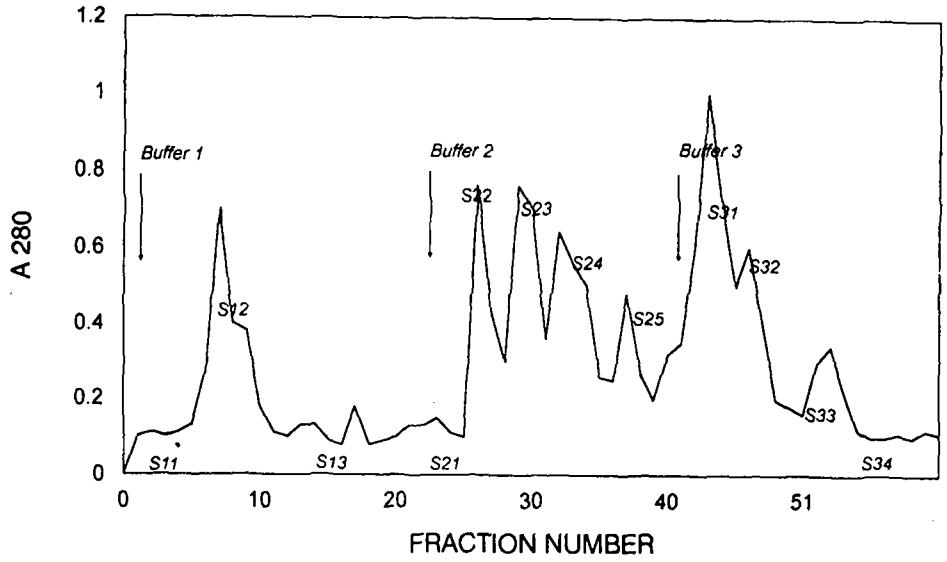

Figure 2b : Protein elution profile of extract-B (Sheep) on a Con-A Sepharose column.

Comparative studies of sheep and buffalo TSH were also performed where both the sheep and the buffalo glands were processed essentially according to the modified protocol of Ellis and one of the extracts i.e. extract-B (representing crude TSH) of both the species were run on a Concanavalin-A Sepharose column and their specific immunoreactivity compared. In the case of buffalo, the total protein, bound to the Con-A column was about 76% out of which most of it (55%) was very tightly bound to the column and was eluted as the B-II fraction. The remaining material was eluted almost equally as the unbound and the weakly bound fraction (23% each). In the case of sheep, most of the protein (80%) was bound to the Con-A column and eluted almost equally as the WB (B-I) and the B-II fraction (43% and 37%, respectively) (Fig.2 a and b). About 20% of the protein eluted as the UB fraction.

When the specific immunoreactivity of anti-bTSH and anti-oTSH with the eluted Con-A fractions of buffalo and sheep were compared, a distinct difference was observed. With antibovine TSH, the specific immunoreactivity of the UB, B-I and B-II fractions of buffalo were 4%, 54% and 42% and for the sheep were 23%, 17% and 59%, respectively (Fig.3 a and b). For antiovine TSH, the specific immunoreactivity were 18%, 55% and 29% and 18%, 33% and 49% for buffalo and sheep, respectively (Fig.4 a and b).

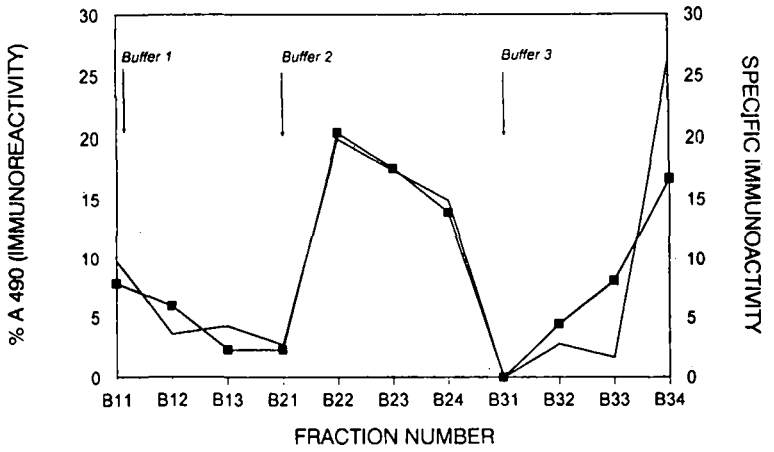

Figure 3a : Comparison of immunoreactivity (% A490) and specific immunoactivity profiles of different side fractions obtained when extract-B (buffalo) was passed through a Con-A Sepharose column. The antiserum used for analysing the fractions was anti bTSH at a dilution of 1:5000, B11, B12, and B13 represent the unbound fractions; B21, B22, B23 and B24 represent the weakly bound fractions and B31, B32, B33 and B34 represent the tighly bound fractions. ■ Immunoreactivity; — Specific immunoactivity.

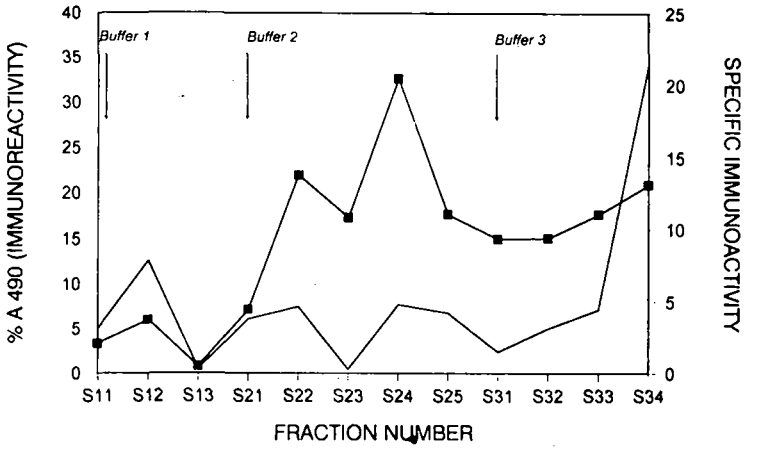

Figure 3b : Comparison of immunoreactivity (% A490) and specific immunoactivity profiles of different side fractions obtained when extract-B (sheep) was passed through a Con-A Sepharose column. The antiserum used for analysing the fractions was anti bTSH at a dilution of 1:5000. S11, S12 and S13 represent the unbound fractions; S21, S22, S23, S24 and S25 represent the weakly bound fractins and S31, S32, S33 and S34 represent the tightly bound fractions. ■ Immunoreactivity; — Specific immunoactivity.

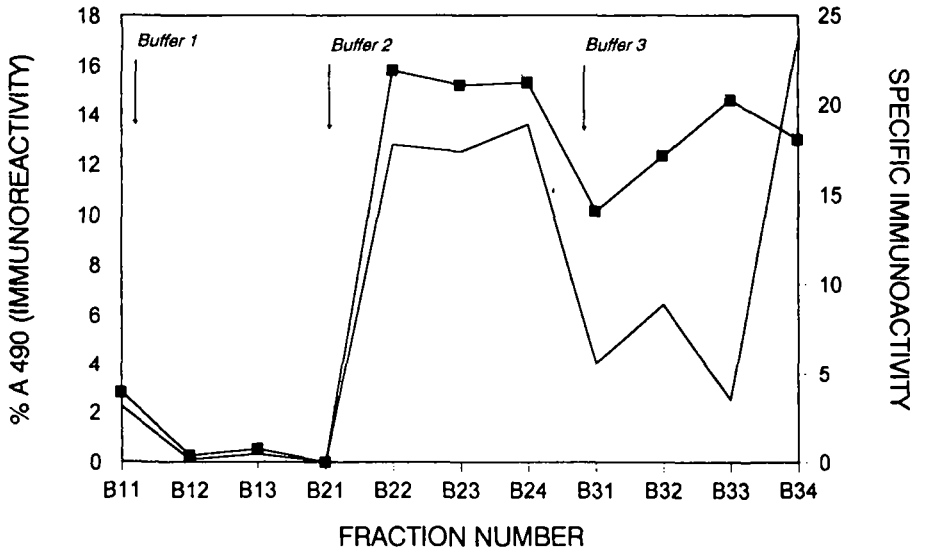

Figure 4a : Comparison of immunoreactivity (% A490) and specific immunoactivity profiles of different side fractions obtained when extract-B (buffalo) was passed through a Con-A Sepharose column. The antiserum used for analysing the fractions was anti oTSH at a dilution of 1:5000. ■ Immunoreactivity; — Specific immunoactivity.

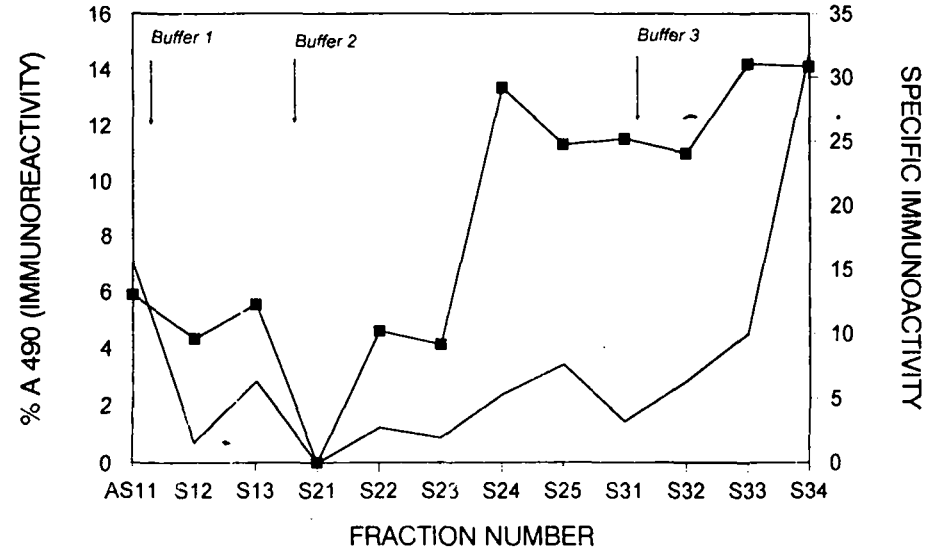

Figure 4b : Comparison of immunoreactivity and specific immunoactivity profiles of different side fractions obtained when extract-B (sheep) was passed through a Con-A Sepharose column. The antiserum used for analysing the fractions was anti oTSH at a dilution of 1:5000. ■ Immunoreactivity; — Specific immunoactivity.

DISCUSSION

Molecular heterogeneity of TSH has been observed in studies using physicochemical techniques such as ion exchange chromatography, starch gel electrophoresis and isoelectric focussing (2). This is essentially due to post-translational modifications such as deamidation, sulfation, acetylation and phosphorylation and even limited proteolysis. N-terminus of buTSH also showed heterogeneity. According to the literature, differences in the amide and aminoacid composition could be the cause of heterogeneity in TSH (15). Also while N-terminus of TSH-beta is highly conserved, some levels of heterogeneity have been observed in the case of alpha subunit (16). While bovine and the ovine species have got Phe as the N-terminus of both the two subunits, some level of heterogeneity in N-terminus till the sixth residue in the alpha subunit has been observed. Earlier too, heterogeneity in few aminoterminal residues has been observed in subunits owing to probable proteolysis during purification (17).

It has been observed that most of the isoforms of the glycoprotein hormones represent differences in the oligosaccharide structure and are referred to as the glycoforms. Affinity chromatography of glycopeptides on immobilized lectins is a powerful method for oligosaccharide fractionation (18). The oligosaccharides that interact with Con-A Sepharose have the following characteristics : Triantennary, tetraantennary and bisecting

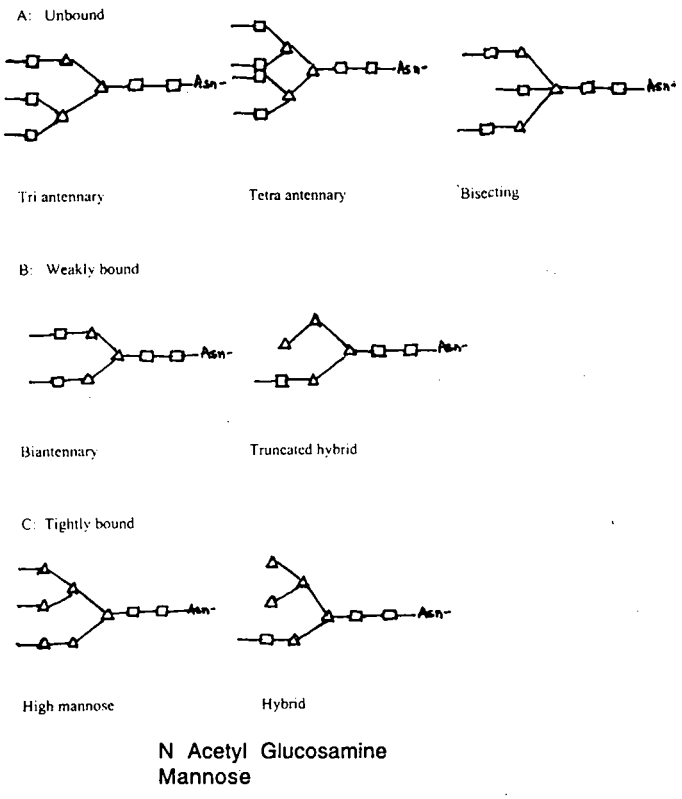

Figure 5 : Representative structures of oligosaccharides based on Concanavlin-A affinity chromatography; a: Tris buffered saline elutes the unbound trisaccharide, tetra saccharide and bisecting oligosaccharide structures; b: 200 mM alpha methyl mannopyranoside elutes the weakly bound biantennary and truncated hybrid oligosaccharide structures; c: 300 mM alpha methyl mannopyranoside elutes the firmly bound high mannose and hybrid structures.

oligosaccharide structures do not bind; biantennary and truncated hybrid oligosaccharide structures bind weakly while high mannose and hybrid structure bind firmly to Concanavalin-A Sepharose (12, 13, 14) (Fig. 5). Most of the available evidence indicates that while the epitopes of the glycoprotein hormones consist solely of peptide elements, the carbohydrate moiety may influence the conformation and reactivity of these elements by enhancing and/or inhibiting the binding of these epitopes and their antibodies by influencing the overall physicochemical properties of the hormone, effecting hydrophilicity thus serving to inhibit intermolecular aggregation (19).

The lectin binding studies suggest that the difference in the carbohydrate content may lead to the low B/I and high B/I (Biological activity/Immunological activity) forms of TSH. Multiple immunoreactive forms have been recognized in the crude preparation of mouse tumor (20), serum, medium and bovine pituitary TSH preparation (Sigma) (21, 22) as well as in the most highly purified bTSH (1). Whether some of the multiple forms observed in the bovine TSH were produced as a result of chemical changes during the hormone purification is not known but those present in the crude mouse TSH preparation probably represent various forms of TSH in different stages of carbohydrate processing (22, 23).

The effect on the carbohydrate moiety on the fractions obtained from buTSH elution through Con-A column was quiet apparent. Where eventhough the immunoreactivity was the same, their specific immunoreactivity was quite different. It was observed that normally the presence of a biantennary structure leads to high specific immunoreactivity. Also the heterogenous forms of TSH and LH which were differentiated based on their elution from DEAE Sephacel, can be further subdivided into microheterogenous based on their variation in carbohydrate composition. Selective purification thus further complicates the characterization of these heterogeneous forms including the structural analysis of their carbohydrate component (24). As these hormones exist in different isoforms, the majority of which appear to represent differences in their oligosaccharide structure that may appear to constitute 20-40% of the molecular mass of these hormones. This heterogeneity creates potential differences for the standardization of clinical assays for these hormones, because the specimens and the standards with which they are compared are likely to differ in their isoform composition (22).

Thus, it has been observed that most of the TSH like material with higher immunoreactivity elute as the weakly bound material in buffaloes thus pointing to the fact that most of the TSH has biantennary and truncated hybrid structure. However, in the case of sheep, more immunoreactive TSH was eluted as the tightly bound material, thus suggesting that most of the TSH is composed of high mannose residues or has a hybrid carbohydrate structure. Thus, buffalo and sheep thyrotropins show difference in their carbohydrate structure from each other.

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