



# Pseudo-affinity chromatographic approach to probe heterogeneity in buffalo pituitary luteinizing hormone: probable pseudolectin-like behavior of immobilized Cibacron Blue 3GA

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## ABSTRACT

The alpha ( $\alpha$ ) and beta ( $\beta$ ) subunits of buffalo pituitary luteinizing hormone (LH) were chromatographed on Cibacron Blue 3GA agarose and their immunoreactivity was quantitated using anti- $\alpha$  and anti- $\beta$  anti sera. Subsequent analyses showed  $\alpha$  subunits were relatively more hydrophilic than  $\beta$  subunits. Further, the naturally occurring free  $\alpha$  and  $\beta$  subunits were more hydrophobic than their native counterparts which were dissociated and isolated from heterodimeric LH. The lesser sugar content in freely occurring  $\alpha$  and beta subunits may be attributed for increased hydrophobicity and consequent upon the existence of their uncombined free forms. In order to ascertain putative sugar-dye interaction, crude LH carrying free subunits, pure LH, and non-glycosylated recombinant  $\beta$  subunit of LH were loaded separately on Cibacron Blue. Methyl mannoside was able to elute 33% of the bound protein in case of crude and pure LH, whereas there was little (3%) elution in case of recombinant LH  $\beta$  subunit. This study suggests a compositional heterogeneity in free and native subunits of LH from the buffalo pituitary. In addition, our findings reveal the pseudolectin-like behavior of Cibacron Blue.

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## 1. Introduction

Gonadotropins from pituitary gland of all vertebrates are required for growth and maintenance of differentiated functions of gonads in males and females [1]. Biochemically gonadotropins are glycoproteins and are heterodimers consisting of noncovalently associated  $\alpha$  and  $\beta$  subunits. The  $\alpha$  subunit of the three glycoprotein hormones are identical, species-specific and in most mammals consists of 92–96 amino acids. However, their  $\beta$  chains are unique, confer biological and hormonal specificity. Beta subunit of luteinizing hormone (LH) has 117–121 amino acids in different species while the molecular mass ranges between 27 and 40 kDa of which 16% is contributed by the carbohydrate [2]. The two subunits exhibit 10–40% sequence similarity with each other across various species [3].

The two subunits can be separated from intact hormone (native  $\alpha$  and  $\beta$  subunits) following dissociation using a variety of methods which include ion exchange chromatography [4,5], gel filtration

[6], salt precipitation [7], RP-HPLC [8–10] and counter current distribution (CCD) [11,12]. Significant amounts of  $\alpha$  and  $\beta$  subunits occurring in the free state without combining into the heterodimeric hormones have been reported in the pituitary of adult buffalo [13,14] and other species [15–19]. They are also reported to be present in human placenta [20] and in extra embryonic coelomic fluid [21].

Triazine dyes, a class of synthetic textile dyes, have been successfully used as ligand for pseudo-affinity chromatography. Gonadotropins especially buffalo LH (buLH) has been shown to interact with Cibacron Blue [22,23]. In the present study we have used Cibacron Blue in immobilized form to understand microheterogeneity in the freely occurring subunits of LH and in subunits dissociated from the native heterodimeric LH.

Furthermore it also evaluates the specificity of the dye-protein binding in terms of glycosylation as 0.3 M methyl mannoside was able to elute the bound protein from the Cibacron Blue dye [17].

## 2. Experimental

### 2.1. Materials

Buffalo pituitary glands from animals of mixed age and sex, were obtained from a local abattoir within half an hour of the slaughter and were transported frozen in liquid nitrogen to the

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laboratory. Anti- $\alpha$  bovine LH (bLH) and anti- $\beta$  bLH antisera were a kind gift from Dr. J.G. Pierce, USA. Cibacron Blue 3GA agarose, SP-Sephadex, Sepharose S-200 were obtained from Sigma–Aldrich, USA. Goat anti-rabbit IgG–HRP conjugate was purchased from Bangalore Genei Ltd., India. ELISA plates were purchased from Greiner, Germany. All other chemicals and reagents were of AnalR grade.

## 2.2. Methods

### 2.2.1. Purification of buffalo luteinizing hormone

The luteinizing hormone from buffalo pituitaries was essentially purified according to the purification protocol described by Chaudhary and Muralidhar [24]. The freshly frozen buffalo pituitary glands were homogenized in 0.15 M ammonium sulfate solution containing 0.001 M PMSF. The pH of the homogenate was set to 4.0 by using 1 M hydrochloric acid, centrifuged (10,000 rpm/30 min) and the supernatant was further processed. The pH of the supernatant was adjusted to 3.0 using meta phosphoric acid and centrifuged (10,000 rpm/30 min). The pH 3.0 supernatant was saturated to 50% with solid ammonium sulfate, stirred for 3 h and centrifuged (10,000 rpm/30 min). The pellet obtained was dissolved in and dialyzed against water and finally lyophilized. The crude LH (50% ammonium sulfate pellet) was then loaded onto a Sepharose S-200 column (2.42 cm  $\times$  145 cm) and 5.5 mL fractions were collected. The eluate fractions with a  $V_e/V_0$  value of 1.5–1.6 (30 kDa Fraction, F III) were pooled, dialyzed and lyophilized. This 30 kDa fraction was taken in 0.01 M disodium phosphate and loaded on the SP-Sephadex column (1.2 cm  $\times$  15 cm). The 0.1 M disodium phosphate eluate represented purified buLH. This purified buLH was used as the starting material for obtaining native subunits. Protein fractions having  $V_e/V_0$  of >1.8 (F IV), from the same S-200 column chromatography were separately pooled, dialyzed and lyophilized. This material was used as the starting material for isolation of free subunits which exist physically separate in this fraction [9,10]. The separation procedure is highly reproducible and the results have been further reaffirmed using SDS-PAGE [24]. Counter current distribution

The  $\alpha$  and  $\beta$  subunits of buLH were separated essentially according to the protocol of Liu et al. [12] wherein the solvent system used to achieve both the dissociation of hormone and its separation were butanol:isobutyrate:triethylamine:water in the ratios of 1:2:0.1:3. The solvents were mixed vigorously in a separating funnel and the mixture was allowed to stand for overnight at RT. After 16 h, the two separate phases (organic and aqueous) were observed and collected separately into two different conical flasks. 10 mg of buLH was dissolved in 2 mL of the lower phase in a tube (tube 1), to the same tube 2 mL of upper phase was added, vortexed and let stand at 37 °C for 1 h followed by centrifugation (5000 rpm/10 min/25 °C). Thereafter, the upper organic phase was carefully pipetted out and added to another tube (tube 2) containing 2 mL of fresh lower aqueous phase. 2 mL of fresh upper phase was further added to the tube 1. Eight more such serial transfers of the organic phase were performed. The two phases from all the tubes were separately pooled and lyophilized. Absorbance at 230 nm of both the phases was measured in a UV spectrophotometer. Immunoreactivity of the protein in each of the separated phases was assessed against anti- $\alpha$  and anti- $\beta$  antibodies.

### 2.2.3. Cibacron Blue Sepharose

A 3 mL column of Cibacron Blue 3GA agarose gel was prepared, equilibrated with 8–10 bed volumes of 0.05 M phosphate buffer, pH 7.0. The column was maintained at a constant flow rate of 20–25 mL/h. The sample dissolved in the equilibration buffer was loaded onto the gel. After the sample entered the gel the flow was stopped. Thirty minutes later the gel was washed with the same

buffer at a constant flow rate to wash all the unbound proteins. Subsequently the bound proteins with ionic interactions were eluted with 0.05 M PB pH 7.0 containing 1 M NaCl. Finally the column was washed with 80% ethylene glycol, to elute out the hydrophobically bound protein to the column. Fractions (2 mL) of unbound and bound protein were collected and their absorbance was measured at 280 nm.

In a separate experiment to elute bound proteins, 0.3 M methylmannoside in 0.05 M PB pH 7.0 was initially used. Following this, two other buffers were used, the order of elution being 0.05 M PB pH 7.0 containing 1 M NaCl followed by 80% ethylene glycol in 0.05 M PB pH 7.0. Experiments were repeated at least two more times. Protein was estimated by the procedure of Lowry et al. [25] using BSA as calibration standard. ELISA

Immunoreactivity of all protein fractions from CCD and pseudo-affinity column chromatography was measured in triplicates using direct binding ELISA [26]. Highly purified homologous buLH was used to obtain standard immunoreactivity curve. Primary antibody developed against bLH  $\alpha$  and  $\beta$  subunits was used at the dilution of 1:15,000 (standardisation data not shown). Goat anti-rabbit IgG–HRP conjugate was diluted to 1:1500. A solution of ortho phenylene diamine (1 mg/mL) and 0.06%  $H_2O_2$  in 0.05 M citrate buffer pH 5.5 was used as substrate. Plates were read at 490 nm in an ELISA reader (ECIL, India). Percentage immunoreactivity was calculated using the following formula:

$$\text{percentage immunoreactivity (\%)} = \frac{\text{pooled A 490 of a fraction}}{\text{additive A 490 of all the bound and eluted fractions}} \times 100$$

## 3. Results

In order to understand the compositional differences between the native and the freely occurring subunits of buLH, Cibacron Blue dye based pseudo-affinity chromatography was used. The  $\alpha$  and  $\beta$  subunits, both native (dissociated from intact LH) and freely occurring forms were separated by CCD. The native subunits were dissociated from purified buLH while the free subunits were isolated from size fractionated protein peak ( $V_e/V_0 > 1.8$ ) from S-200 chromatography of crude buLH (Flowchart 1 and Fig. 1). The upper and lower phases from CCD analysis of both were pooled, desalted and lyophilized. The immunological activity of the two types of subunits (both native as well as free) was determined using anti- $\alpha$  bLH and anti- $\beta$  bLH antisera. ELISA results depicted that the upper

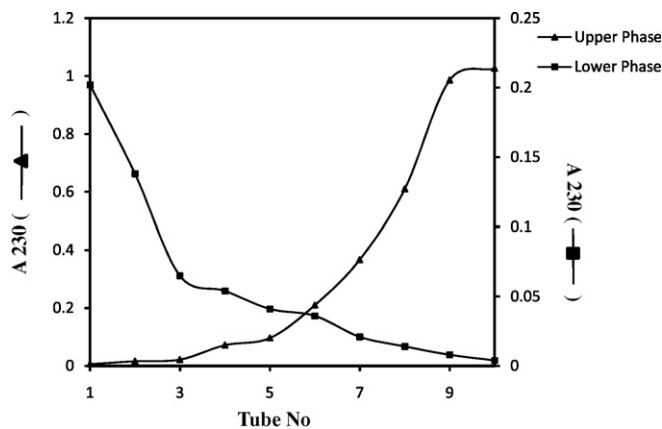
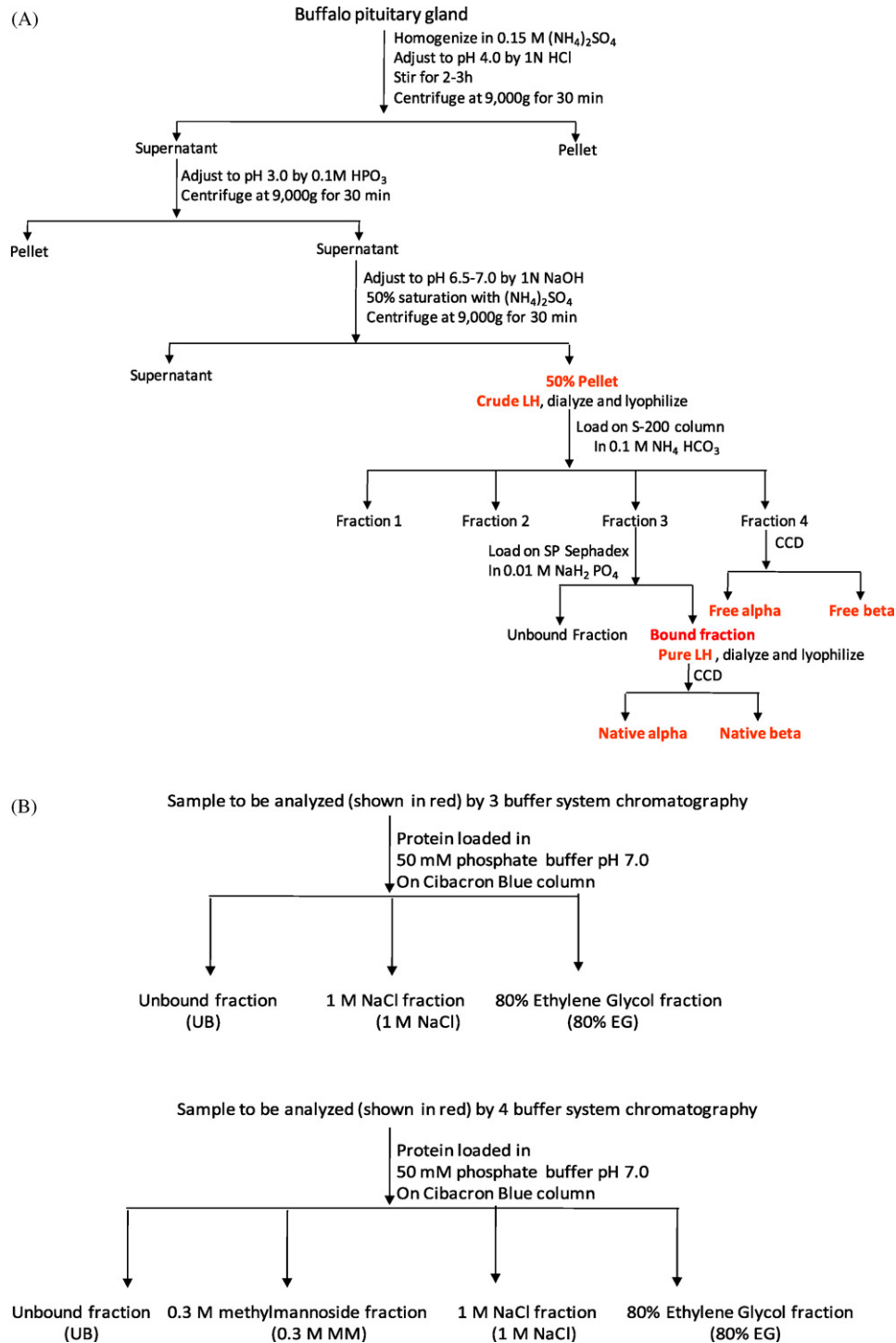


Fig. 1. Counter current distribution of F IV (the size fractionated peak of 50% ammonium sulfate pellet of buffalo pituitary glands with a  $V_e/V_0$  of >1.8) for the separation of free alpha and beta subunits from each other. Similar protocol was followed to segregate native alpha and beta subunits of LH.



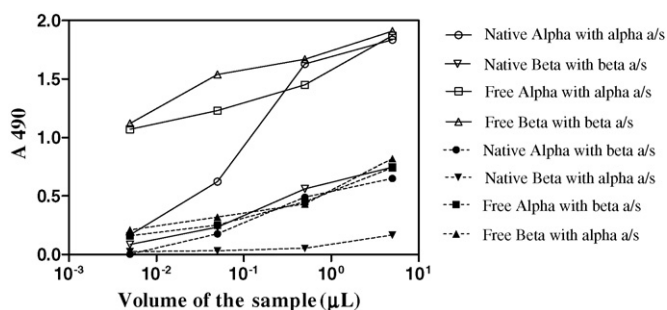
**Flowchart 1.** Indicating the samples analyzed (A) and the strategy of experimental design (B).

phase fraction had more reactivity against anti- $\beta$  while the lower phase fraction exhibited more reactivity against anti- $\alpha$  bLH antisera. Therefore,  $\beta$  subunit of LH is concentrated in upper phase and similarly  $\alpha$  subunit in the lower phase. In general the  $\alpha$  and  $\beta$  subunits of pituitary glycoprotein hormones share 20–40% sequence similarity which could be reflected in immunological cross reaction between the two subunits. Hence 25% cross reactivity was observed in case of both the antisera (Fig. 2).

Subsequently the separated subunits were loaded individually on Cibacron Blue column and eluted in tandem with elution buffer (0.05 M phosphate buffer pH 7.0, 1 M NaCl and 80% ethylene glycol).

All the eluted fractions were analyzed for their respective immuno reactivity. Based on the nature of the eluant it can be said that the unbound fraction is an extremely hydrophilic and nonionic fraction, while the fraction eluted using 1 M NaCl is an ionic fraction. Finally the fraction eluted using ethylene glycol is an extremely hydrophobic fraction.

The native LH  $\alpha$  subunit appeared in all three fractions, i.e. 48.5% as unbound, 39.1% as ionic and 12.35% as hydrophobic fraction. In the case of freely occurring  $\alpha$  subunit, the unbound fraction represented only 31% while there was an increase in the hydrophobic fraction from 12.35% to 29.7% (Fig. 3). Hence the native  $\alpha$  subunits



**Fig. 2.** Immunoreactive profiles of free and native subunits (alpha and beta) using anti- $\alpha$  bovine LH (bLH) and anti- $\beta$  bLH antisera to assess the cross reactivity of the individual preparations.

are predominantly hydrophilic molecules while the freely occurring  $\alpha$  subunit exhibits a little more hydrophobicity.

In the case of  $\beta$  subunit, the native  $\beta$  subunit had 13.49% unbound, 37.5% ionic and 49% hydrophobic fractions. In the freely occurring  $\beta$  subunits also, there was an increase in the hydrophobic molecules as the percentage hydrophobic fraction of the  $\beta$  subunit increased from 49% to 59% (Fig. 3) thereby further asserting the above mentioned hypothesis.

Hypoglycosylation of the free forms of the subunits could be responsible for this observation. In an additional serendipitous experiment an attempt was made to elute bound LH with sugar. To our surprise almost 25% of the total protein was eluted with methyl mannoside (Fig. 4B–D). The same was observed when pure intact LH (28%) was applied and eluted using methyl mannoside. In both the cases there was almost no change in the percentage of hydrophobic fraction. To ascertain whether it was actually sugar recognition and not any other interaction, recombinant LH  $\beta$  subunit expressed in *Escherichia coli*, a non-glycosylated protein, was loaded on Cibacron Blue and was eluted using methyl mannoside. Very little (3%) of the bound protein was observed in the methylmannoside eluted fraction (Fig. 5). Hence the binding of the Cibacron Blue to buLH was possibly through sugar residues.

#### 4. Discussion

It has been observed that  $\alpha$  and  $\beta$  subunits of glycoprotein hormones are physico-chemically distinct from each other [1]. While the  $\alpha$  subunit is known to be hydrophilic in nature,  $\beta$  subunit is a predominantly hydrophobic molecule [27]. Alterations in the structure in terms of carbohydrate residues have been observed [27–33]. The presence of both a large and a small form of subunits and a changing large/small free  $\alpha$  subunit ratio has been shown in the var-

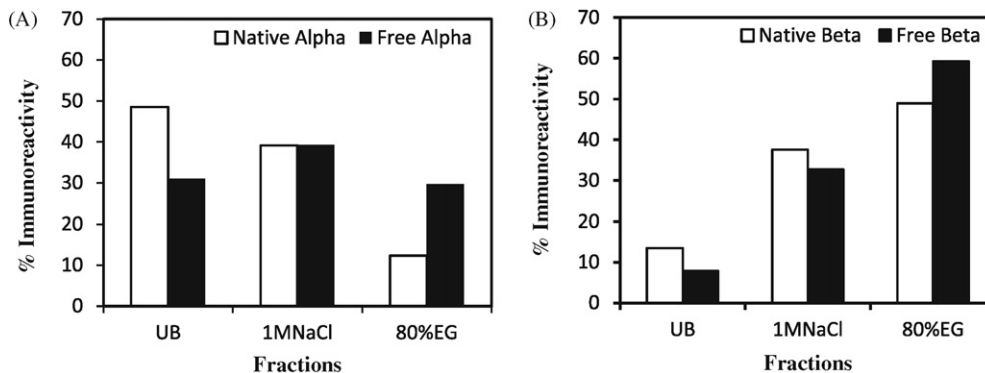
ious extracts [9,34]. Further, heterogeneity in terms of the amount of glycosylation has been observed in free and heterodimeric pituitary common  $\alpha$  and TSH  $\beta$  subunits [35].

The increase in the 'hydrophobic fraction' (i.e. eluted with 80% ethylene glycol) in the freely occurring subunit as observed in the present study enables us to attribute this to decreased glycosylation [13]. Cibacron blue obviously could distinguish these two grossly different forms.

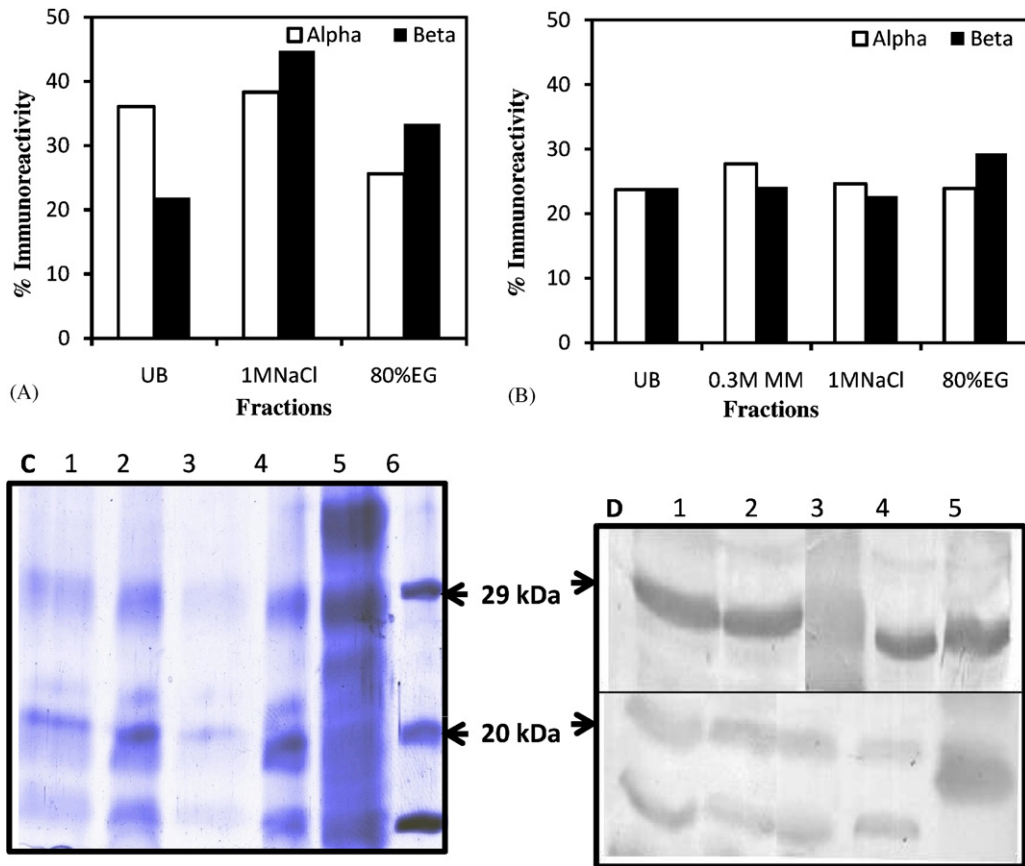
Cibacron Blue, a sulphonated triazine dye is known to interact with proteins via ionic interaction and hydrophobic interaction. So it has been shown that ionic interaction of Cibacron Blue as a ligand is due to the sulphonic acid groups in its structure while the hydrophobic interaction owes it to the presence of polycyclic aromatic rings in the dye structure (data not shown). Since sugar residues are known to increase the hydrophilic index of a protein molecule [27], hypoglycosylation of the free subunits is possibly leading to a relative lowering of the polar species on these molecules and therefore a concomitant increase in the hydrophobic character of these free subunits is observed.

To further understand whether Cibacron Blue was actually able to recognize the structural changes occurring on the protein tertiary structure due to the sugar residues and can segregate them on this basis, crude as well as pure LH was loaded on the immobilized dye and the bound hormone was eluted with a sugar. We could elute a significant amount (almost 1/3) of the bound protein using methyl mannoside. To abolish the changes in ionic behavior of Cibacron Blue, a constant pH and ionic concentration in the eluants (0.05 M PB, pH 7.0) was maintained though out the elution of bound protein. The specificity of the interaction was further confirmed when almost no elution was observed in the case of recombinant LH  $\beta$  molecule which was a bacterial expression product and thus non-glycosylated in nature.

Microheterogeneity in pituitary glycoprotein hormones has been described well in literature. The present study shows that even when pure LH was loaded on Cibacron Blue dye column and eluted differentially, it redistributed itself into four subpopulations of LH. These represent the unbound, the sugar elutable, the salt elutable and the ethylene glycol elutable subpopulations. As the percentage of ethylene glycol fraction, involving hydrophobic interaction, of the bound LH increased when subunits from intact LH, freely occurring subunits and bacterially expressed recombinant subunits were loaded in the same order. This same order was therefore ascribed to decreased content of sugar in LH subunits. Even though there are several reports in the literature involving the use of Cibacron Blue in the purification of various glycoproteins [36–39], none of them provide evidence for the binding of Cibacron Blue dye to proteins through their sugar residues. For example Miribel and Arnaud [36] suggested the presence of a het-



**Fig. 3.** Percentage immunoreactivity profiles of the eluted peak fractions of alpha (native and free) and beta (native and free) subunits loaded on Cibacron Blue 3GA agarose. Three peak fractions obtained using 0.05 M phosphate buffer pH 7.0 (unbound (UB)), 1 M sodium chloride (NaCl) and 80% ethylene glycol (EG) were assessed with anti- $\alpha$  and anti- $\beta$  bLH antisera. The percentage immunoreactivity of fractions was calculated as given under methods section.



**Fig. 4.** Percentage immunoreactivity profiles of fractions from the 50% ammonium sulfate pellet (Crude LH) loaded on Cibacron Blue 3GA agarose. Unbound protein was washed off using 0.05 M phosphate buffer pH 7.0 (UB). (A) bound proteins were subsequently eluted with 1 M sodium chloride (NaCl) and 80% ethylene glycol (EG) while in the other experiment (B) bound proteins were eluted sequentially with 0.3 M methyl mannoside (0.3 M MM), 1 M sodium chloride (NaCl) and 80% ethylene glycol (EG). All the obtained fractions were assessed with anti- $\alpha$  and anti- $\beta$  bLH antisera. The percentage immunoreactivity of fractions was calculated as given under methods section. (C) SDS-PAGE of the above fractions stained with coomassie blue R-250 where lane 1 is UB, 2 is 0.3 M MM, 3 is 1 M NaCl, 4 is 80% EG and 5 is crude LH. Lane 6 is molecular weight marker. (D) Western blot of (C).

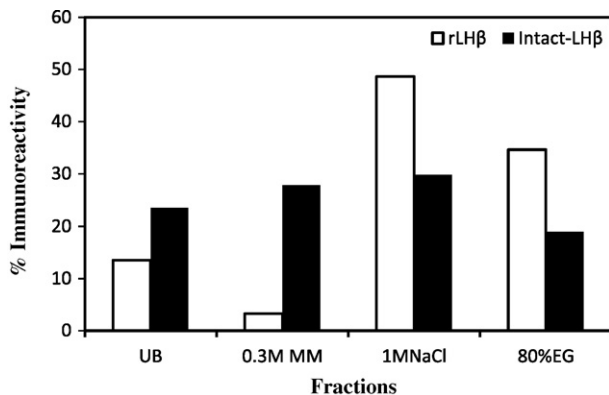
erogeneous population of human  $\alpha_1$ beta glycoprotein by virtue of their ability to bind to Cibacron Blue and differential elution from the column. However no sugar was used as an eluant. Similarly, Gianazza et al. [37] reported preferential binding of one allele of group specific component (Vitamin-D binding protein) over the other alleles. Both of them hypothesized the role of ter-

tiary structure and sugar content in Cibacron-protein binding but did not provide direct evidence for sugar-dependent bindings. Further Patterson et al. [38] did comparative analysis of donkey and horse  $\alpha_1$ B-glycoprotein on Cibacron Blue and observed the presence of new alleles and further echoed on the same hypothesis. However no proof to substantiate their statement was presented. Hattori et al. [39] tried to purify the envelope (Env) glycoproteins of human immunodeficiency virus (HIV) from the culture supernatants of CHO-Sec cells by removal of unwanted albumin through the latter. Unexpectedly, Env proteins as well as albumin were bound to the column. They indicated that triazine dyes bind to the functional regions of Env proteins of HIV-1 that play important role(s) for HIV infection though the reason of this binding was not investigated.

The present study however clearly demonstrates that sugar residues of LH indeed participate in interaction with Cibacron Blue dye. Further it accentuates the fact that microheterogeneity exists in a pure and homogeneous preparation of bLH.

## 5. Concluding remarks

The specific binding of Cibacron with sugar residues has not been explored, though a few papers have hypothesized the role of tertiary structure or sugar content being responsible for the heterogeneity or differential binding of various protein molecules with this triazine dye. The present study reports for the first time evidence for the pseudolectin-like behavior of Cibacron Blue 3GA agarose.



**Fig. 5.** Percentage immunoreactivity profiles of pure intact LH and recombinant LH  $\beta$  loaded on Cibacron Blue 3GA agarose. Unbound protein was washed off using 0.05 M phosphate buffer pH 7.0 (UB), bound proteins were eluted with 0.3 M methyl mannoside (0.3 M MM), 1 M sodium chloride (NaCl) and 80% ethylene glycol (EG). All the obtained fractions were assessed with anti- $\beta$  bLH antisera. The percentage immunoreactivity of fractions was calculated as given under methods section.

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