Journal of Chromatography B, 878 (2010) 2679–2684

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

Taruna Arora[a], Nidhi Vashistha[a], Rajesh Chaudhary[b], Kambadur Muralidhar[a,*]

[a] Department of Zoology, University of Delhi, Delhi, India
[b] Department of Biomedical Science, AND College, Delhi University, Delhi, India

ARTICLE INFO

Article history:
Received 24 April 2010
Accepted 29 July 2010
Available online 5 August 2010

Keywords:
Buffalo
Cibacron Blue
Free subunits
Glycoprotein hormones
Pseudolectin

ABSTRACT

The alpha (α) and beta (β) subunits of buffalo pituitary luteinizing hormone (LH) were chromatographed on Cibacron Blue 3GA agarose and their immunoreactivity was quantitated using anti-α and anti-β anti sera. Subsequent analyses showed α subunits were relatively more hydrophilic than β subunits. Further, the naturally occurring free α and β subunits were more hydrophobic than their native counterparts which were dissociated and isolated from heterodimeric LH. The lesser sugar content in freely occurring α 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 β 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 β 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.

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 α and β subunits. The α subunit of the three glycoprotein hormones are identical, species-specific and in most mammals consists of 92–96 amino acids. However, their β 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 α and β 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 α and β 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

* Corresponding author at: Hormone Research Laboratory, Department of Zoology, University of Delhi, Delhi 110007, India. Tel.: +91 011 27667443x105; fax: +91 011 27667985.
E-mail addresses: kambadur@zoology.du.ac.in, kambadur@hotmail.com (K. Muralidhar).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.07.025

laboratory. Anti-α bovine LH (bLH) and anti-β 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 × 145 cm) and 5.5 mL fractions were collected. The eluate fractions with a V_e/V_o 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 × 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_o 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 α and β 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-α and anti-β 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 methyl mannoside 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 α and β 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 α and β 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_o > 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-α bLH and anti-β bLH antisera. ELISA results depicted that the upper

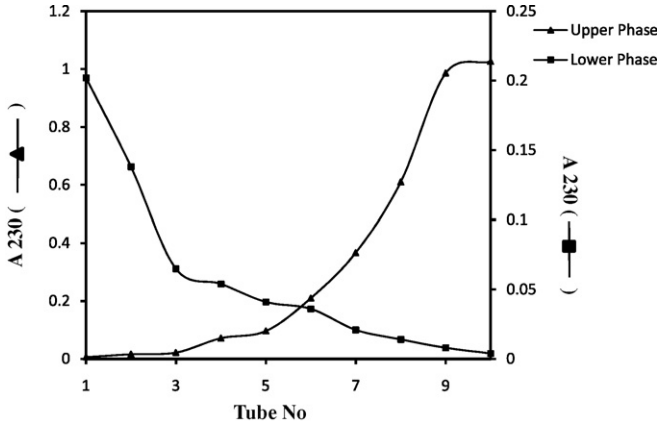

Fig. 1. Counter current distribution of F IV (the size fractionated peak of 50% ammonium sulfate pellet of buffalo pituitary glands with a V_e/V_o of >1.8) for the separation of free alpha and beta subunits from each other. Similar protocol was followed to segregate native alpha and beta subunits of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