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Short communication

Purification of bubaline luteinizing hormone by gel filtration chromatography in the presence of blue dextran

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Abstract

A luteinizing hormone (LH) enriched fraction from the whole pituitary glands of buffaloes has been obtained by a simple, economical and scalable protocol using two gel filtration steps. An extract of pituitary glands was mixed with blue dextran and applied to the first Sephacryl S-200 gel filtration column. Blue dextran fractions eluting in the void volume of the column together with bound LH were pooled, reduced in volume and re-loaded on to the second gel filtration column, pre-equilibrated with 50 mM phosphate buffer pH 7.3 containing 1 M KCl. In the presence of 1 M KCl, LH dissociated from the blue dextran and eluted in the same elution volume as in the case of dimeric native form of buffalo LH, while blue dextran eluted in the void volume of the column. The protein obtained from the peak in the second gel filtration was found to be highly immunorective against bovine LH β subunit specific antiserum and it was 46-folds purer over the starting material as indicated by the direct binding ELISA. SDS-PAGE of the purified LH showed two major bands of LH, which was confirmed by western blot analysis. The yield of LH was found to be 262 mg LH/kg of whole pituitary glands.

Keywords: Blue dextran; Buffalo luteinizing hormone; Dye affinity chromatography; Gel filtration; Immunoreactivity; Protein purification

1. Introduction

Luteinizing hormone (LH) is a pituitary hormone which is classified as a gonadotropin. The biological roles of LH are: growth of the ovarian follicle including ovulation, differentiation of granulosa to luteal cells, regulation of corpus luteum formation in ovaries and stimulation of interstitial cells and function in testis [1–3]. Due to its direct effect on gonads, LH like hormone (e.g. human chorionic gonadotropin, hCG) is used as a thereupatic agent to cure disorders related to infertility caused by the insufficient amount of endogenous gonadotropins and to cause superovulation in the farm animals. Hence, enriched preparations of gonadotropins, including LH from farm animals and humans are in demand.

Conventionally, pituitary gonadotropins were isolated from the urine, a readily available and cheap source. In later years, pituitaries collected from abattoirs have been used more frequently [4]. The purification procedures for LH have relied upon multistep protocols involving precipitation, ion exchange chromatography and gel filtration [1,4–8]. For example, a purified LH preparation from bovine pituitaries was obtained by salt precipitation, pH precipitations, gel filtration, ion exchange chromatography and a lectin column [5]. The classical protocol for isolation of buffalo LH from pituitaries consists of seven steps which utilize pH precipitation, salt fractionation, ion exchange chromatography and gel filtration [8]. Lately, it has been realized that one obvious strategy to improve the yield in a protein purification protocol is to reduce the number of unit processes. This minimizes the collective loss of biological activity inherent in various steps [9–10]. The outcome of this realization has been the increasing use of affinity-based separations for purification of biologically important proteins.

In the present study, a short and simple protocol to purify LH from the whole pituitaries of Indian water buffaloes has been described. The protocol exploits the earlier observation that many gonadotropins show affinity towards the textile dye cibacron blue F3GA [7,11–13].

Textile dyes constitute robust pseudoaffinity ligands for a variety of proteins [14–16]. For example, cibacron blue F3GA has been widely used for purification of nucleotide dependent enzymes. Here, its binding to such enzymes originates in the structural homology with NAD^+ coenzyme. The dye also has

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been used as an affinity ligand in several cases where no such clear-cut biological relationship with the target proteins can be established.

In such cases, it is believed that the structure of the dye allows multiple interactions (which include electrostatic bonds, H-bonds and hydrophobic interactions) with the target protein [14]. In several cases, binding constants and kinetics have been extensively studied. In fact, dye based affinity chromatography is now considered a valuable tool in large-scale bioseparations [15].

The present protocol does not use dye based affinity chromatography in the traditional sense. Unlike earlier workers who have used dye-linked matrices for purification of glycoprotein hormones [7,12–13], it exploits cibacron blue–LH interaction in another manner. It has been reported earlier that prothrombin and factor IX when gel filtered in the presence of blue dextran, co-eluted with the latter in the void volume [17]. Similar behavior has been shown by human FSH [11]. The explanation of these observations is rather simple. Cibacron-blue present in the blue dextran binds to these proteins and the elution of these proteins in void volume separates these from others on the basis of twin mechanisms of affinity and gel filtration. The present protocol utilizes this less exploited strategy for purification of LH.

2. Materials and methods

2.1. Chemicals and biological materials

Buffalo pituitary glands were procured from local abattoir and the bovine LH (bLH) β antiserum was a kind gift from Dr. J.G. Pierce (Department of Biological Chemistry, UCLA, USA). Sephacryl S-300, S-200, blue dextran-2000 were purchased from Pharmacia Bio-Tec. Ltd., Sweden and was fractionated on Sephacryl S-300 (blue dextran 2000 fraction eluting in the void volume only was lyophilized and used). Goat anti-rabbit IgG-HRP conjugate was purchased from Banglore-Genei Ltd., India. Plastic wares including ELISA plates were purchased from Tarson Ltd., India. SDS-PAGE was performed in an Atto make mini gel system obtained from Atto Japan. Ortho phenylene diamine (OPD), 4-chloro-α-napthol and human chorionic gonadotropin (hCG) were purchased from Sigma Co., USA. Human menopausal gonadotropin (hMG) was purchased from Serono Laboratories, USA. Immature rats (24-days old) of Holtzman strain were obtained from departmental animal facility. All other chemicals used were of analytical grade.

2.2. Extraction of acid supernatant from the whole pituitary glands of buffaloes

Frozen (stored at −20 °C) whole buffalo pituitary glands (10 gm) were homogenized in 0.15 M ammonium sulfate containing 1mM Phenyl methyl sulfonyl fluoride, using a kitchen blender at 4 °C. Tissue mass to salt solution ratio was kept 1:4 (w/v). The pH of the homogenate was adjusted to 4.0 using 1N HCI and it was stirred for 2 h at 4 °C. Separation of insoluble material was done by centrifugation of the homogenate at 3000 × *g* for 30 min at 4 °C. The pellet so obtained was labelled as ''acid pellet'' and a slight turbid supernatant was called as acid supernatant (AS) containing bulk of pituitary LH and FSH. This was dialysed and lyophilized and called dialysed acid supernatant (ASD).

2.3. Gel filtration chromatography of ASD mixed with fractionated blue dextran

All the steps were performed at 4 °C. Lyophilized ASD (77 mg) was dissolved in 5 ml of 10 mM phosphate buffer pH 7.3 containing 0.15 M NaCl (PBS) and 30 mg of the fractionated blue dextran was added. This solution was loaded on to the S-200 column (150 cm × 2.4 cm) pre-equilibrated with the PBS. Elution of the column was done with the same buffer at the flow rate of the 25–30 ml h^{-1}. Five milliliter fractions were collected and their absorbance was measured at 612 nm (Fig. 1).

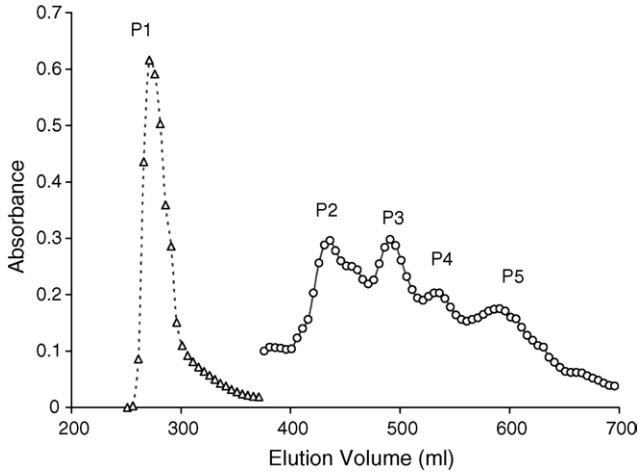

Fig. 1. Chromatogram of dialyzed acid supernatant (ASD) on gel filtration column (S-200) in presence of fractionated blue dextran. Peak P1 is the peak of blue dextran containing proteins bound to it together with bulk of pituitary LH, also represents the void volume of the column. Dotted line shows absorbance at 612 nm (Δ) and solid line shows absorbance at 280 nm (○).

2.4. Gel filtration chromatography of the proteins bound to the blue dextran under high salt conditions

Lyophilized blue dextran protein complex (20–25 mg) obtained from peak P1 of the gel filtration chromatography (Fig. 1) was dissolved in 4 ml of 50 mM Phosphate buffer pH 7.3 containing 1 M KCl and set aside for 30 min at 4 °C. This solution was loaded on to the second gel filtration column S-200 (150 cm × 2.4 cm), pre-equilibrated with the same buffer. Elution of the column was also done with the same buffer at the flow rate of 25–30 ml h^{-1} and 3.1 ml fractions were collected. Absorbance of the fractions was measured at 612 and 220 nm. The fractions under P2 (Fig. 2) were pooled dialyzed against distilled water and lyophilized. This material represented the enriched buLH preparation (LHBD).

2.5. Enzyme linked immunosorbent assay (ELISA)

This was carried out by standard procedure as reported elsewhere [18].

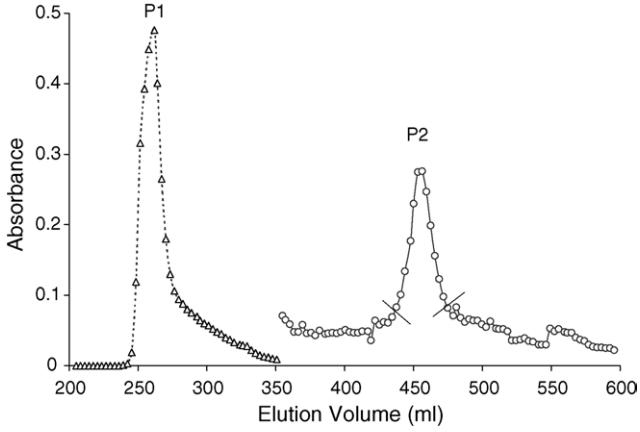

Fig. 2. Chromatogram of material obtained from peak P1 (Fig. 1) on gel filtration column (S-200) in the presence of 1 M KCl in PBS. Dotted line shows absorbance at 612 nm (Δ) and solid line shows absorbance at 220 nm (○).