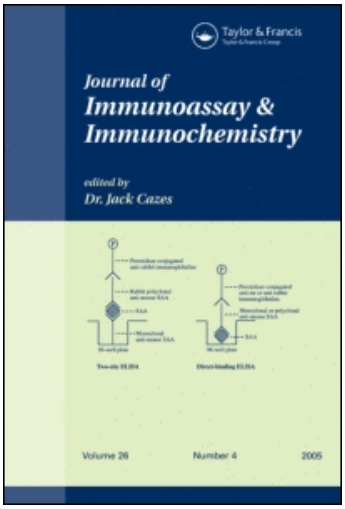

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Rathnam, P. and Saxena, E. B.(1990) 'Removal of Residual Luteinizinc Hormone by the Use of a Specific Receptor and Antibody Coupled to Glass Beads', Journal of Immunoassay and Immunochemistry, 11: 2, 147 — 158
To link to this Article: DOI: 10.1080/01971529008053266
URL: http://dx.doi.org/10.1080/01971529008053266

JOURNAL OF IMMUNOASSAY, 11(2), 147-158 (1990)

REMOVAL OF RESIDUAL LUTEINIZING HORMONE BY THE USE OF A SPECIFIC RECEPTOR AND ANTIBODY COUPLED TO GLASS BEADS

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ABSTRACT

We describe here, a novel and effective use of a specific receptor for Luteinizing Hormone (LH) coupled to controlled pore-glass beads (CPG) and LH antibodies to glass beads (GB), in selective removal of residual LH from purified Follicle Stimulating Hormone (FSH) and Thyroid Stimulating Hormone (TSH). The LH receptor was prepared from bovine corpora lutea. LH antisera were raised in rabbits and purified. FSH and TSH preparations were purified by treatment with the LH receptor coupled to CPG-beads; and/or LH-antibody coupled to glass beads. This procedure avoided dilution of the FSH or TSH during purification.

KEY WORDS: Receptor, Antibody, LH, FSH, TSH, Glass beads, Purification.

INTRODUCTION

Hormones of the highest purity are required for biological and clinical studies. In the treatment of infertility due to a specific hormonal deficiency, there is a need for pure and biologically active hormones. The use of pure hormones is also vital to the

development of specific polyclonal and monoclonal antibodies against the hormones, for immunoassays.

Human FSH, LH, TSH and hCG have nearly identical α-subunits and share homologous regions in the amino acid sequences of their β-subunits (1). Overlapping hormonal activities among highly purified human FSH, LH, and TSH are either due to structural similarities or actual contamination with each other. Conventional methods of purification of hormones do not remove the last traces of the contaminating hormones. We describe here the use of LH-hCG receptor coupled to controlled pore glass beads and LH-hCG antibody coupled to glass beads to remove residual LH and its subunits from hFSH and hTSH. This procedure also avoids dilution of the hormones.

MATERIALS AND METHODS

Hormones:

FSH, LH, and TSH were purified from acetone preserved human pituitary glands, provided by the National Pituitary Agency, MD by procedures already published (2-4). Human FSH preparations used in this study contained 5,000-9,000 2nd-IRP-HMG units/mg. Human LH contained 5233 2nd-IRP-HMG units/mg. Human TSH preparations contained 3.3 - 3.8 International Units/mg. The hCG preparations, containing 10,000-12,000 Units/mg, were gifts of Dr. O.P. Bahl (State University of New York, Buffalo, N.Y.) and of Dr. R.E. Canfield (Columbia University, New York, N.Y.). Bioassays of FSH and LH were performed by an ovarian augmentation method (5), and by the ovarian ascorbic acid depletion method (6), respectively.

Radioreceptorassay (RRA) of LH was performed as described earlier (7), and radioimmunoassay (RIA) was performed by the use of kits obtained from Cambridge Medical Corp., Boston,MA.

Antibodies against LH :

Antisera against hLH were raised in rabbits by multiple site intradermal injections of human LH. Antisera with high titer and affinity were pooled and used for the isolation of γ-globulin by the method of Horejsi and Smetana (8). Protein content was determined by the method of Lowry et al. (9).

LH Receptor :

Plasma membranes from bovine corpora lutea, containing the LH-specific receptor, were prepared as follows. Fresh ovaries from pregnant cows were obtained in dry ice from Roth Products (Lansdale, PA). Corpora lutea were immediately excised, weighed, ground in a meat grinder and homogenized twice with 60 sec bursts at low and high speeds in a Waring blender (Model CB-4), in 10 mM Tris-HCl buffer of pH 7.2 containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% sodium azide (NaN_3), 0.5 mM phenylmethylsulfonylfluoride (PMSF), 900 KIU aprotinin per liter, and 25% glycerol. The tissue to buffer ratio was 1:7.5 (w/v). The homogenate was filtered through two layers of cheese cloth and the filtrate was centrifuged at 1000 x g in a GS-#3 rotor using a Sorvall RC2-B centrifuge (Dupont Co., New Town, CT). The supernatant was recentrifuged at 7000 x g

for 1 hour. The 7000 x g supernatant was diluted with an equal volume of Tris-HCl buffer, and concentrated in an Amicon DC-10 ultrafiltration unit using a hollow fiber filter HI-50 with an exclusion limit of Mr 50,000. The concentrate was adjusted to a volume yielding a protein concentration of 10 mg per ml. The concentrate was made 0.5% in Triton X-100 and solubilized overnight by stirring. All procedures were performed at 1°C. The solubilized receptor was extracted with an equal volume of chilled petroleum ether by constant shaking for 1 hour to remove neutral lipids.

The mixture was centrifuged at 10,000 x g for 1 hour. Five hundred ml of the aqueous phase, containing 5 gms protein of the solubilized receptor, was fractionated on a 10 x 100 cm column of Sepharose-6B in the Tris-HCl buffer, containing 0.5% Triton X-100 and 10% glycerol. The column was eluted with the same buffer with a flow-rate of 1 ml per min. through a Uvicord Recorder at 280 nM. The partially purified receptor, eluted in the unretarded fraction due to aggregation, contained a specific binding activity of 288 pM of $_{125}$I-hCG/mg protein.

Preparation of LH receptor-coupled CPG beads :

An aliquot of 5 ml of a 5% aqueous solution of gamma-aminopropyltriethoxysilane was reacted with 1 g of CPG beads (Electronucleonics, Inc.) for one hour by shaking at 80°C. The supernatant was decanted, the CPG beads were washed three times with water and

dried overnight in vacuo. The amino groups on CPC beads were activated by treatment with 20 ml of a 2.5% aqueous solution of glutaraldehyde. CPG beads were stirred for 2 hrs on a magnetic stirrer until an off-white to light pink color was visible on CPG beads. The suspension was centrifuged at 3500 rpm for 3 minutes. The supernatant was decanted. The CPG beads were washed three times with distilled water on a Buchner funnel and dried overnight.

Activated CPG beads were then coupled with bovine LH-hCG receptor as follows: An aliquot of 10-15 mg of purified receptor was diluted with 6 ml of 10 mM Tris-HCl buffer of pH 7.2, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% BSA (RRA buffer). 1.5 g of CPG beads were added and the coupling reaction was allowed to occur overnight at 4°C. The CPG beads coupled to the receptor were centrifuged, and the supernatants were discarded. The LH-hCG receptor-coupled CPG beads were washed three times with chilled distilled water. After vacuum drying, the CPG beads were incubated with 0.5M glycine for 2 hrs at 4°C to block uncoupled reactive sites. The CPG beads were washed, vacuum dried, and stored in the RRA buffer.

Antibody-Coupled Glass Beads :

The anti-LH immunoglobulin was coupled to CPG as described by Post et al.(10). Glass beads of 3 mm radius were sandblasted,(60-grit particle pressure, 75 lb/in^2) for 10 min to increase the surface area. Reactive groups were generated by the treatment of

beads with 10% gamma-aminopropyltriethoxysilane in toluene for 7 hrs at 3°C. The liquid was decanted off and the beads were washed with distilled water. The beads were air dried completely and treated with 2.5% glutaraldehyde in 0.01M phosphate buffer of pH 7.0, for 2 hrs at room temperature, to activate the amino groups. The solution was decanted, and the beads were washed with distilled water. The activated beads were suspended in 0.005% anti-LH γ-globulin, diluted in 0.01M phosphate buffer, and incubated overnight at 4°C with gentle shaking. The antibody solution was decanted, and the beads were washed three times with chilled phosphate buffer. The antibody coupled beads were treated with 1M glycine for 2 hrs at 4°C to block uncoupled alkylamino groups. The beads were finally washed with chilled phosphate buffer and stored in small test tubes in the freezer.

Binding Characteristics of the receptor coupled CPG beads and antibody coupled glass beads:

Aliquots of 50,000 cpm of ^{125}I-hCG were incubated with different quantities of receptor-coupled CPG beads and antibody-coupled glass beads, in 400 ml of RRA buffer, for 1 hour, at 37°C in a total incubation volume of 500 µl, in the absence and in the presence of 100 ng of unlabelled hCG in 100 µl of RRA buffer. The tubes were centrifuged for 30 minutes at 3500 rpm. The supernatants were aspirated. The beads were suspended in 1 ml of RRA buffer, and counted in an auto gamma counter (Micromedic System 4/600) with an

efficiency of 61% for ^{125}I. Specific binding was calculated as percent of ^{125}I-hCG displaced in the presence of excess unlabeled hCG.

Purification of hormones:

Separate aliquots of 10 mg FSH or TSH in one ml of Tris-HCl buffer (10 mM Tris-HCl buffer of pH 7.2, containing 1 mM each of $CaCl_2$ and $MgCl_2$), were added to 200 mg of receptor-coupled CPG beads or to antibody-coupled glass beads or to a mixture of the same quantities of CPG beads and glass beads together. The suspensions were incubated at 37°C for 1 hr. in a Dubanoff shaker. At the end of the incubation, the beads were allowed to settle. The supernatants were gel filtered through a 1 x 100 cm column of Ultrogel AcA-44, equilibrated with 0.1 M ammonium bicarbonate to remove buffer salts. The fractions eluted from the columns were collected and assayed for the respective hormone and for LH by RRA and RIA.

RESULTS AND DISCUSSION

Receptor-coupled CPG beads and antibody-coupled glass beads specifically bound upto 52.6% and 64% of ^{125}I-hCG.

The results of assays of FSH and TSH preparations before and after purification are presented in Tables I and II. After purification, almost 80% of the starting materials were recovered. There was no dilution of the hormones after purification.

Purification of FSH preparations (Table I):

Two batches of FSH preparations were purified. Unretarded fractions from Ultrogel AcA-44 columns contained FSH or TSH activity. After purification, FSH preparations showed a 1.1 fold increase in the activity with concommitant removal of most of the LH activity. Receptor-coupled CPG beads removed 67% of the LH contamination as determined by RRA and almost all the LH as determined by RIA from Batch #1. From Batch #2, the receptor-coupled beads removed 20% of LH by RRA and 63% as determined by RIA. When Batch #2 FSH was purified with antibody-coupled glass beads, a similar result of 50-75% removal of LH was obtained. However, when Batch #2 was purified by the combination of receptor-coupled CPG beads and antibody-coupled glass beads, as much as 70-100% of LH was removed. Overnight incubation did not significantly increase the removal of LH. Trace amounts of LH activity in the purifed FSH may be due to structural similarities between LH and FSH or trace amounts of free subunits, which may crossreact in RIA using polyclonal antibodies.

Purification of TSH preparations:

Treatment of TSH preparation, Batch #1 with receptor-coupled CPC beads removed 36% of LH as determined by RRA and 67% as determined by RIA, (Table II). From Batch #2 of TSH, the antibody-coupled glass beads removed 56% (RRA) to 29% (RIA) of LH. The variation in the estimation of LH by RIA and RRA may be due to the

TABLE I

Removal of LH From FSH

FSH	FSH ACTIVITY IU/mg	LH-ACTIVITY RRA IU/mg	RIA
Batch # 1 (Untreated)	5,677±600(3)[a]	60±10 (3)	15±6 (3)
Receptor-coupled CPG beads (Treated)	6,438±738(3)	20±4 (3)	Trace (3)
Batch # 2 (Untreated)	8,850±865(3)	10±3 (3)	8±2 (3)
Receptor-coupled CPG beads (Treated)	10,000±568(3)	8±2 (3)	3±2 (3)
Antibody-coupled Glass beads (Treated)	" "	5±3 (3)	< 2 (3)
Receptor-coupled CPG beads + Antibody-coupled Class beads (Treated)	" "	3±1 (3)	<0.005(3)

[a]: Mean ± S.E (number of determinations)

TABLE II

Removal of LH From TSH

TSH	TSH ACTIVITY IU/mg	LH ACTIVITY RRA IU/mg	LH ACTIVITY RIA IU/mg
Batch # 1 (Untreated)	5.7±0.9 (3)[a]	11±4 (3)	6±2 (3)
Receptor-coupled CPG beads (Treated)	6.1± 1 (3)	7±4 (3)	2±1 (3)
Batch # 2 (Untreated)	3.3±0.4 (3)	185±11(3)	24±2 (3)
Antibody-coupled Glass-beads (Treated)	3.8±0.7 (3)	82±15(3)	17±1 (3)
Receptor-coupled CPG beads + Antibody-coupled Glass beads	4.1±0.5 (3)	6±1.5(3)	<0.005(3)

[a]: Mean ± S.E (number of determinations)

measurement of immunological versus biological activity. Once again, the combination of the receptor-coupled CPG beads and the antibody-coupled glass beads was most optimal by removing 97% or more of the contaminating LH activity. The TSH activity of the purified material also showed a slight increase. After receptor purification approximately, 80% of the starting protein was

recovered, indicating little loss of material during the procedure. A little LH activity of the receptor purified TSH is probably due to a comparatively greater structural similarity between LH and TSH than between LH and FSH.

These findings demonstrate the use of LH-specific receptors as an effective and novel method of selective removal of contaminating activities from FSH and TSH, which due to the presence of microheterogeneity or isohormones, are difficult to achieve by most of the sophisticated methods of protein separation based on differences in physicochemical properties of biologically active proteins. The method has potential use in removing native hormones from the subunits of LH and hCG, and is of interest in raising hormone-specific antisera, especially for use in current approaches to contraception by active immunization of women with hCG-β.

ACKNOWLEDGEMENTS

This work was supported by Grant #HD06543 - 05, from the National Institute of Health, Bethesda, MD. Reprint requests should be made to Dr.P.Rathnam, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

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