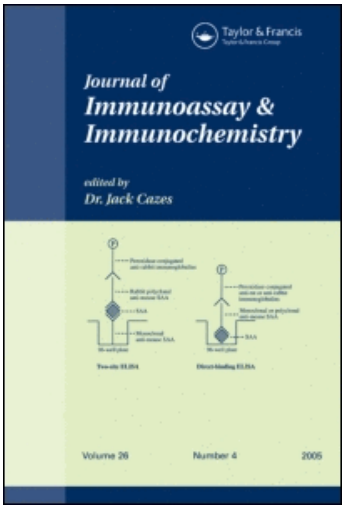

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Isolation of α- and β-Subunits of Peak-I hCG and Generation of Highly Specific Polyclonal Antisera

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Abstract: Development of polyclonal antisera is still a choice in some hard-pressed budget laboratories. In the present study, an attempt was made to isolate α- and β-subunits from peak-I hCG, generation of polyclonal antisera and their characterization. The anti-hCG-α antisera showed titres of 1 : 8000 and anti-hCG-β antisera 1 : 16,000 at 50% binding to radiolabelled hCG in RIA. Studies on specificity using anti hCG-β antiserum demonstrated no cross-reaction with several hormones tested in the present study, except for hCG-β and hCG, thus eliciting a highly specific hCG-β antiserum.

Keywords: Peak-I hCG, Anti hCG-α, Anti hCG-β

INTRODUCTION

Several investigators have developed polyclonal antisera against hCG subunits.[1–4] However, all of them exhibited varying cross-reactions with other glycoprotein hormones. The polyclonal antisera are sometimes

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non-specific against given antigen; therefore, they find limited applications in the development of immunoassays. A problem of non-specificity have been resolved with the advent of hybridoma technology.[5] But, this technique is highly expensive, time consuming, and requires sophisticated infrastructure. Therefore, development of polyclonal antisera is still a choice of some of the hard-pressed budget laboratories.

In order to develop immunodiagnostic assays for measuring hCG or β-subunit of hCG and characterization of the hCG molecule, both polyclonal and monoclonal antibodies are frequently used. The α- and β-subunits of hCG were isolated from peak-I[6] with a view to develop highly specific polyclonal antisera against each subunit.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA), blue dextran, trizma base, urea, glycine, sodium dodecyl sulphate (SDS), N,N,N′,N′-tetramethyl ethylenediamine (TEMED), coomassie blue G-250, 4-chloro-1-napthol, diethylaminoethyl (DEAE)-Sephacel, Protein-A-Sepharose, thimerosal (mercury-[(O-carboxyphenyl)thio] ethyl sodium salt), polyethylene glycol (PEG) and visking tubing (No. 3) were all purchased from Sigma Chemical Company, St. Louis, USA. Sephadex G-100 and DEAE-Sephadex A-25, were procured from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade and were purchased locally in India.

Preparation and Purification of hCG Subunits

Peak-I of hCG was isolated from pregnancy urine (45–75 days post LMP)[6] and was used for the preparation of α- and β-subunits following a published protocol.[7]

Peaks-I(A) and I(B) were pooled and 300 mg of hCG was taken in 10 M freshly recrystallized urea solution (40 mg/ml). Normality (pH) was adjusted to 4.5 using HCl followed by incubation at 40°C for an hour. It was then diluted to a urea concentration of 6 M with 0.3 M glycine and the pH was adjusted to 7.5 with 0.1 N NaOH. The chromatography was performed on a DEAE-Sephadex A-25 column. The α-subunit was eluted with equilibration buffer, 6 M urea-0.3 M glycine, pH 7.5. After passing 200 mL of effluent, the buffer was changed. The hCG-β subunit was eluted using 0.5 M tris-HCl buffer, pH 7.5. Fractions of 10 mL were collected. Protein-containing peak fractions were pooled separately and acidified with aldehyde-free acetic acid to pH 5.0 in order to inhibit cyanate formation. Dialysis was performed for

24 hours at 4°C against 4 liters of 1% acetic acid, followed by two changes of distilled water. The glycoprotein solution was lyophilized and stored at −20°C.

The purification of each subunit on the Sephadex G-100 column resulted in fractions (pooled) comprising a single peak. The α- and β-subunits of hCG (200 μg each) were dissolved separately in 20 mL of 1% ammonium bicarbonate, and was then chromatographed on a column of Sephadex G-100 using the same buffer at 4°C. Protein containing fractions were pooled, lyophilized, and stored at −20°C. These fractions were characterized by dot-blot analysis.

Characterization of hCG Subunits: Dot-Blot Analysis of α- and β-Subunits of hCG

At three different points on a nitrocellulose strip 20 μL each of hCG peak-IA, hCG-β (pool), and negative control (growth hormone) were loaded and allowed to dry at room temperature (RT). For non-specific blocking of additional protein binding sites, the strip was dipped into 50 mM PBS containing 1% BSA, pH 7.4 for 2 hrs. The strip was then washed five times with 50 mM PBS and then dipped in primary antibody solution, i.e., anti-hCG-β (1 : 1000) raised in rabbit and shaken for 5 hrs at RT. After washing 5 times in PBS, the strip was dipped in a second antibody-enzyme conjugate solution (i.e., ARGG-HRP conjugate, 1 : 200) and shaken for 2 hrs at RT, followed by 5 times washing with PBS. Finally, a color reaction was performed using 4-chloro-1-napthol. Similarly, another set of dot blot analysis was performed for hCG-α subunits. In this case, the primary antibody used was anti hCG-α (1 : 50) raised in rabbit. The remaining procedure was same as described for hCG-β subunit. Specificity for binding antibody was also tested using β-subunit antibody for α-subunit and vice-versa.

SDS-PAGE Electrophoresis

Fractions containing α- and β-subunits were tested for their molecular size using 10% polyacrylamide gel by following the procedure of Laemmli.[8]

Enzyme Immunoassay

Enzyme immunoassay of α- and β-subunits of hCG was performed as described earlier.[6]

Generation and Purification of Polyclonal Antisera

Four New Zealand white male rabbits of three months age were divided into two groups of two each. Both groups were separately immunized with

α- and β-subunits of Peak-I hCG. In brief, the subunits of hCG (200 μg each) were emulsified with 2.0 mL of Freund's complete adjuvant and normal saline mixed together in a 1 : 1 proportion. This suspension was injected at 50–80 sites, intradermally, on the back of each animal. The first booster of 100 μg was given after one week of immunization at 20 different sites. Thereafter, an intramuscular booster injection was administered after one month and the animal was bled between the 10th to 14th days after the injection and, subsequently, every week. The serum was collected and characterized for antibody titre and specificity by RIA. The booster injections were given whenever the titre was found to be low. The anti hCG-α and anti hCG-β antiserum were purified following the procedure described elsewhere.[9]

Determination of Titre of Polyclonal Anti-hCG-α and Anti-hCG-β Antisera

The titre was checked by RIA using a double antibody precipitation method.[9]

Determination of Specificity of Polyclonal Antisera

The specificities of hCG-α and hCG-β antisera were determined by performing cross-reaction studies by setting up standards of hCG and of other hormones (i.e., hCG-α, hCG-β, hLH, hFSH, hTSH, hPRL and human growth hormone) likely to cross-react over a range of 0.5 ng to 100 ng. The percent cross-reaction of hormone was then calculated as follows:

$$\% \text{ Cross-reaction} = \frac{X}{Y} \times 100$$

where X = Dose of hCG required to displace 50% of ^{125}IhCG bound to the antibody; Y = Dose of other related hormone required to displace 50% of ^{125}IhCG bound to the antibody.

RESULTS

Preparation of Subunits of hCG and their Characterization

As is evident from Fig. 1, peak fractions from 8 to 15 which comprised hCG-α and hCG-β subunits (from 47 to 54) were pooled separately and analysed by ELISA. Subsequently, the subunits of hCG were purified on separate columns of Sephadex G-100. Figure 2 clearly indicates that the pooled fractions from 12–37 constituted hCG-α subunit, while pooled fractions of 10–37 had hCG-β (Fig. 3). Dialysis, followed by lyophilization, yielded 20 mg of hCG-α and 1.76 mg of hCG-β. Both α- and β-subunits of hCG were found to be free of each other by SDS-PAGE (Fig. 4).

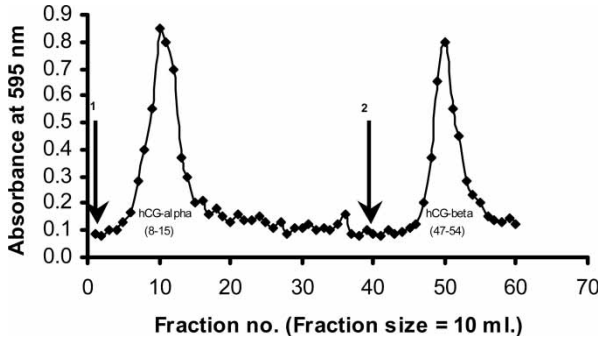

Figure 1. Elution profile of hCG subunits from DEAE-Sephadex A-25 column. Arrow No. 1 indicates fraction No. from which elution with 6M urea-0.3 M glycine, pH 7.5, was initiated, while arrow No. 2 indicates starting point of elution with 0.5 M Tris-HCl buffer, pH 7.5. Fractions showing activities of hCG-α and hCG-β subunits were pooled separately. Values in the parentheses indicates fraction numbers pooled for each subunit.

As is shown in Fig. 5(a), the hCG-β pool showed the presence of immunoreactive hCG-β subunit when subjected to dot blot analysis. Similarly, Fig. 5(b) revealed the presence of immunoreactive hCG-α subunit in the hCG-α pool. Negative results were obtained when the α-subunit was tested in hCG-β antibody coated dots and β-subunit tested with hCG-α antibody coated dots.

Characterization of Polyclonal Antisera Raised Against α- and β-Subunits of hCG

Titre of Polyclonal Antisera

The titres of hCG-α and hCG-β antisera developed in rabbits are shown in Fig. 6. The anti-hCG-α antisera showed titres of 1 : 8,000 and anti-hCG-β antisera 1 : 16,000 at 50% binding to the radioactive hCG in the RIA system.

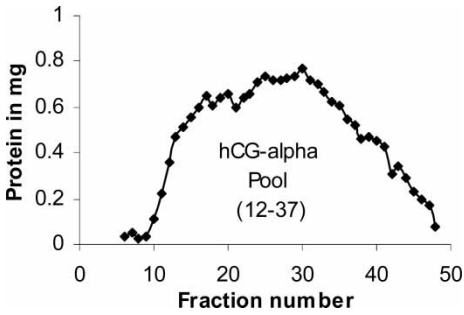

Figure 2. Size exclusion chromatography of hCG-α subunit preparation on Sephadex G-100 column. Values in the parentheses indicates fractions pooled for hCG-α subunit.

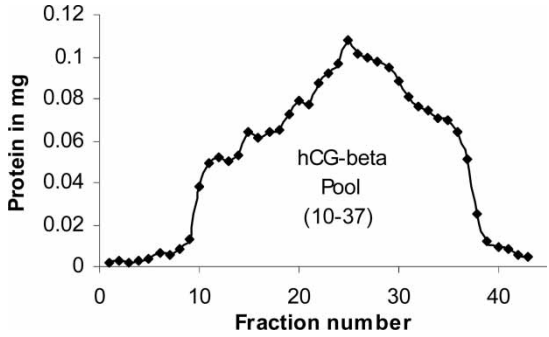

Figure 3. Size exclusion chromatography of hCG-β subunit preparation on Sephadex G-100 column. Values in the parentheses indicates fractions pooled for hCG-β subunit.

Specificity of Polyclonal Antisera

Anti-hCG-α antiserum and anti-hCG-β antiserum were checked for their specificity in RIA using 0.5 to 100 ng of hCG-α, hCG-β, hCG, hTSH, hFSH, hLH, hPRL, LER 907, and growth hormone. As shown in Fig. 7, both hCG-β and growth hormone showed no cross-reaction with anti-hCG-α. Similar studies on specificity using anti-hCG-β antibodies demonstrated no cross-reaction with any of the hormones except for hCG-β and hCG, thus eliciting a highly specific hCG-β antiserum (Fig. 8). The cross-reactivities of anti-hCG-α and anti-hCG-β are shown in Table 1.

DISCUSSION

The method adopted for preparation of α- and β-subunits of hCG was similar to that reported by other investigators.[1,7,10,11] The molecular weights of α- and β-subunits of hCG were reported to be approximately 15–20 and

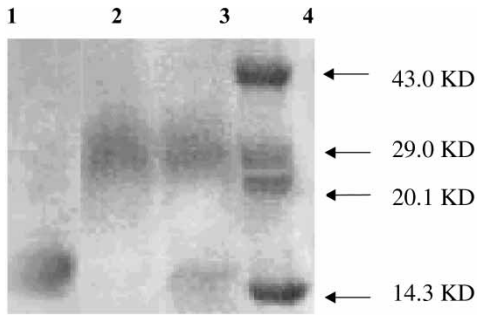

Figure 4. SDS-PAGE of subunits of hCG. Lanes: 1. hCG-α; 2. hCG-β; 3. Sigma hCG; 4. Molecular weight markers.

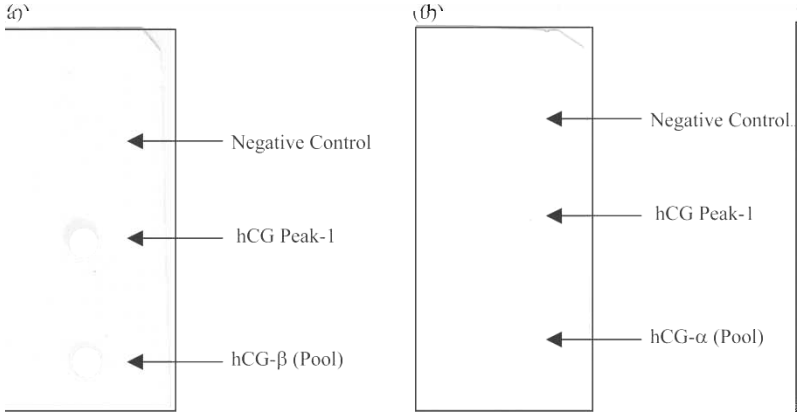

Figure 5. a) Dot blot analysis of hCG-β subunits; b) dot blot analysis of hCG-α subunits.

25–30 kilo Daltons, respectively.[12–17] The molecular weights of subunits reported in the present study, i.e., 19,539 Daltons for hCG-α and 28,870 Daltons for hCG-β, are in congruence with earlier reports.[12–17]

The results of this study suggest that high titer polyclonal antisera against α- and β-subunits of hCG could be produced without any conjugation. Other investigators have also shown that high titer antisera could be produced against intact hCG without any conjugation, but such antisera could not discriminate between hLH and hCG.[18] Vaitukaitis et al.[19] found that hCG-specific antisera can be generated when the native β-subunit of hCG is used as an immunogen. It is well known that covalent coupling to protein carriers enhances antigenicity.[20] Therefore, Swaminathan et al.[1] utilized

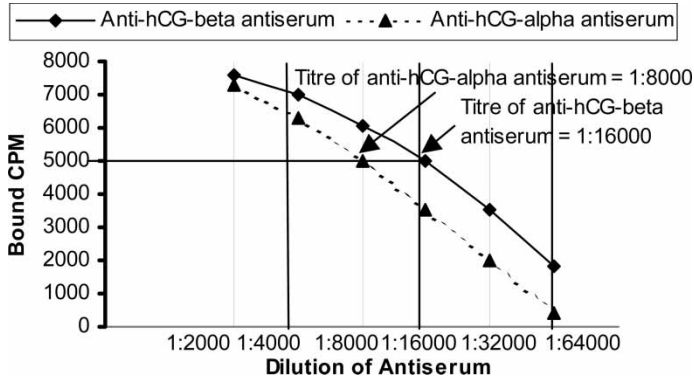

Figure 6. Determination of titer of anti-hCG-α and anti-hCG-β antisera by RIA. Initial dilution of 1 : 8,000 and 1 : 16,000 were found to be the titer for anti-hCG-α and anti-hCG-β, at which 50% of ^{125}IhCG was bound to the anti-hCG-α and anti-hCG-β antisera respectively.

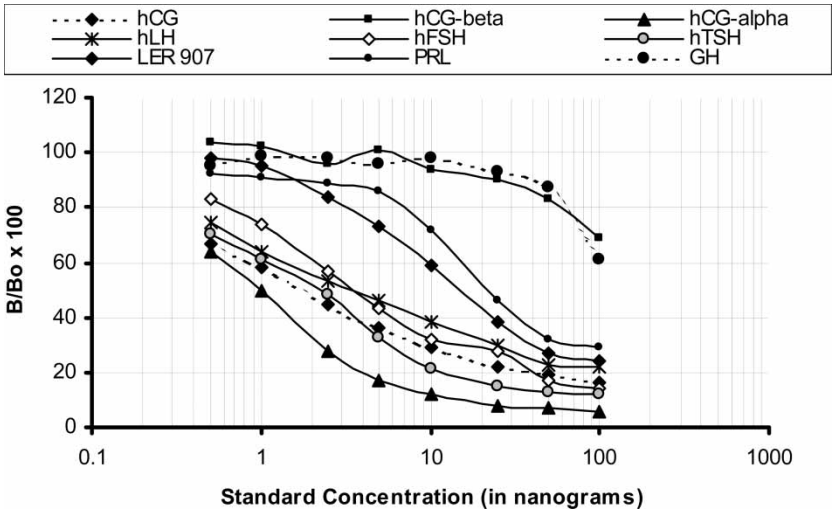

Figure 7. Cross-reactivity studies of hCG-α antisera with some of the protein hormones by RIA (displacement analysis).

an advantage of conjugating the antigen hCG to the carrier protein hemocyanin, which elicited a better antibody response. It was shown that half life of native sub-units of hCG is substantially shorter compared to intact hCG.[21] Linking to a carrier protein may increase the half-life of a β-subunit, or may delay its adsorption from the immunization site, thus increasing the exposure of immunogen.

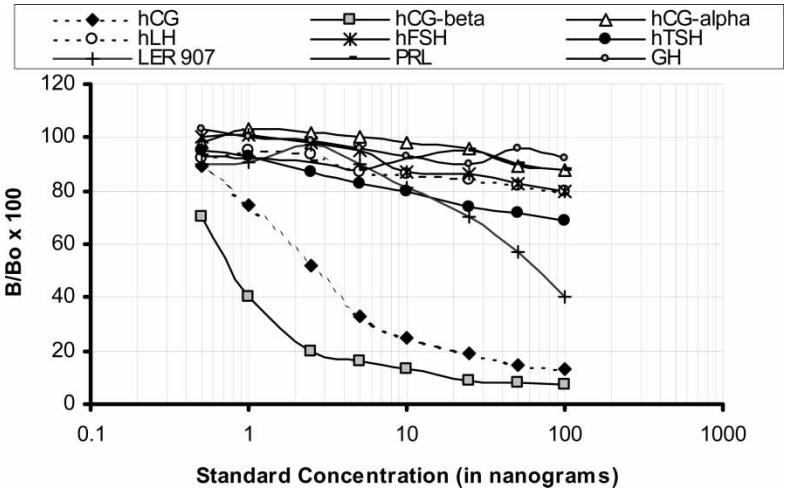

Figure 8. Cross-reactivity studies of hCG-β antisera with some of the protein hormones by RIA (displacement analysis).

Table 1. Cross-reactions of antisera raised against α- and β-subunits of hCG

Hormone tested	% Cross-reaction with anti-hCG-α antiserum	% Cross-reaction with anti-hCG-β antiserum
hCG-α	100.0	$<$0.001
hCG-β	$<$0.10	100.00
Native hCG (NIH)	55.00	44.13
hLH (NIH)	99.2	0.30
Prolactin (NIH ref. prep.)	5.00	$<$0.50
LER-907 (NIH)	7.30	0.95
hFSH (Iodination grade, NIH)	31.42	$<$0.50
hTSH (NIH)	47.80	$<$0.50
hGH (NIH)	$<$0.50	$<$0.50

The hCG-β used for the immunization in the present study is virtually free from contamination with the α-subunit of hCG; a negligible amount of cross-reactivity of the antisera against hCG-α (less than 0.001%) may be due to contamination of hCG-α with free β-subunit or intact hCG. Alternatively, this activity may be directed against the carbohydrate moiety of the β-subunit, since both subunits are known to contain asparagine linked carbohydrate units.[14,22]

Sophisticated maneuvers, such as multiple adsorptions or chromatographic sample-purification steps, are often required to render polyclonal antisera completely specific. Moreover, antisera to isolated subunits of hCG are of very limited availability.

A polyclonal antiserum developed against hCG-β subunit in the present study has been used to develop a specific ELISA for measuring hCG and/or hCG-β subunits in our laboratory.

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