Purification and Heterogeneity of Human Chorionic Gonadotropin

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A highly active preparation of human chorionic gonadotropin (HCG) has been obtained by simple methods of column chromatography on diethylaminoethyl Sephadex and Sephadex G-100. The preparation was seen by SDS-polyacrylamide gel electrophoresis to consist of three bands of apparent molecular weights about 14,000, 15,000 and 30,000. It assayed at immunologically 12,000 IU/mg by the Gonavislide test.

Key words : human chorionic gonadotropin (HCG), preparation, column chromatography

INTRODUCTION

Human chorionic gonadotropin is a glycoprotein hormone discovered by Aschheim and Zondek in 1927 [1]. It can be detected in the blood and urine of pregnant women and also, in much larger amounts, in the blood and urine of tumor patients with such trophoblastic tumors as choriocarcinoma, hydatid mole, chorioadenoma destruens, and syncytial endometritis [2]. The gonadotropin titer of these patients can be used as an index of tumor growth or regression. Whereas the hormone from pregnancy urine has been purified and characterized [3], it has not been concentrated or purified thus far from the urine of patients with malignant tumors. Several investigators have attempted to purify the hormone from pregnancy [4-6] and from the urine of patients with trophoblastic tumors [7,8]. Got and Bourrillon [4] obtained a highly active preparation with a biological activity of 12,000 IU/mg from pregnancy urine. This preparation was homogeneous by free boundary, starch, and immunoelectrophoresis and by ultracentrifugetion. Their procedure used adsorption on benzoic acid, extraction, and fractional precipitation with ethanol, adsorption on kaolin [9], and either chromatography on Decalso and Dowex 2 ion exchange resin or starch electrophoresis. Another preparation of HCG was obtained by Reisfeld and Hertz [7] from the urine of the patients with trophoblastic tumors. Although this preparation had biological potency higher than that of Got and Bourrillon [4], it showed heterogeneity on free boundary electrophoresis. The isolation procedure involved adsorption on kaolin, DEAE-cellulose chromatography, and adsorption on $BaCO_3$. More recently, Wilde and Bhagshawe [8] have reported the isolation of HCG from a similar source. Their preparation was immunologically homogeneous and also assayed biologically at 14,600 IU/mg and immunologically at 5,300 IU/mg. They extracted HCG from urine with benzoic acid and subjected the crude material

thus obtained to DEAE-cellulose chromatography, column electrophoresis, gel filtration, and, finally, DEAE-Sephadex chromatography. Van Hell et al. [5] have been able to obtain an electrophoretically heterogeneous and immunologically homogeneous preparation of HCG from normal pregnancy urine with a biological of 18,800 IU/mg. Their procedure involved ethanol fractionation, column chromatography on carboxymethyl Sephadex, and gel filtration on Sephadex G-200 or G-100.

Although some physicochemical properties of these preparations have been reported [4-8], no detailed structural investigations have been carried out. Present investigation was undertaken with the object of isolating, a preparation of HCG which would be suitable for studies on the primary structure of the hormone. A simple procedure has been devised yielding a highly active preparation of HCG. I wish to report here studies on the purification of the hormone from the crude material, and some properties of the product.

MATERIALS AND METHODS

Crude HCG of potency 1,500 international unite (IU) /mg, was purchased from China. Crystalline ovalbumin and Bovine serum albumin were purchased from Sigma Chemical Company. Gonavislide test of immunologecation was obtained from Mochida Pharmaceutical Co., Tokyo. DEAE-Sephadex A-50, and Sephadex G-100 were products of Pharmacia Fine Chemicals, Inc.

1. Purification of HCG

All steps of purification were carried out at 4℃ unless otherwise specified. Protein concentration of the effluent fractions was estimated by measuring their absorption at 280nm with a Hitachi 124 spectrophotometer.

Step 1 : Ion Exchange Chromatography —The column of DEAE (diethylaminoethyl) -Sephadex A-50 (2.4 × 50 cm) was equilibrated in a 0.04 M Tris-phosphate buffer, pH 8.7. The solution of 0.5 g of crude HCG, which gave an assay value of 1,500 IU/mg by Gonavislide test, was dissolved in 10ml of 0.04 M Tris-phosphate buffer, pH 8.7 and applied to the column which had been equilibrated with the same buffer. The elution of the column was initiated with 0.04 M Tris-phosphate buffer, pH 8.7, followed by a series of buffers of increasing pH and NaCl concentration in a discontinuous stepwise gradient. Fractions of 10 ml were collected. The elution diagram shown in Fig. 1 represents the fractions pooled and the location of various buffer changes. After pool of selected fractions, it was concentrated and desalted by Immersible CX (Amicon) . The selected fractions were assayed for protein content by the method of Lowry et al. [10] using bovine serum albumin as a standard and measured for Gonadotropic (HCG) activity by Gonavislide test in immunization.

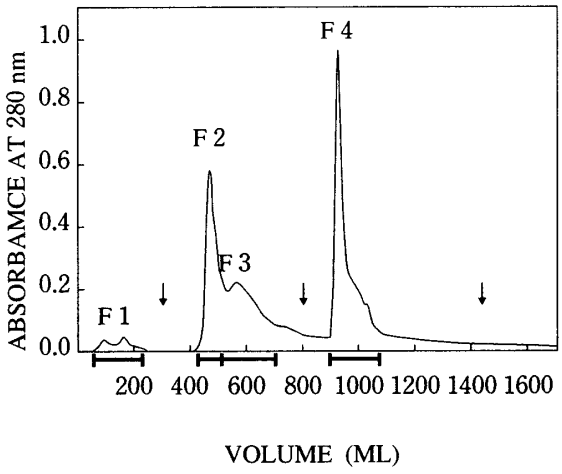

Fig. 1 . Chromatography of crude HCG on DEAE-Sephadex A-50

A 0.5 g sample of crude HCG in 10 ml of 0.04 M Tris-phosphate buffer, pH 8.7, was applied to a column (2.4 × 50 cm) of DEAE-Sephadex A-50 in 0.04 M Tris-phosphate buffer. The column was eluted in a stepwise discontinuous gradient. Elution was started with 0.04 M Tris-phosphate buffer, pH 8.7, and was changed to 0.1 M NaCl and 0.2 M NaCl-0.04 M Tris-phosphate buffer, pH 8.7, and finally 0.2 M NaCl -0.04 M Tris-phosphate buffer, pH 9.0. Fractions of 10ml were collected. Pooled fractions are indicated by solid bars.

↓, where buffer changes were made
—, fractions pooled
—, absorbance at 280nm

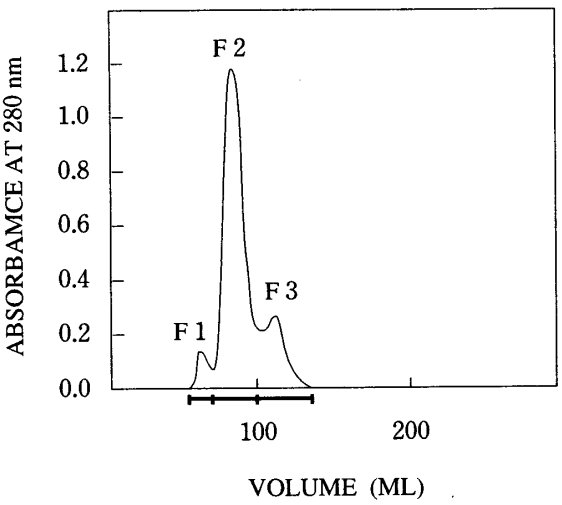

Fig. 2 . Gel filtration of HCG from Step 1 on Sephadex G-100

A sample of partially purified HCG in 1 ml of 0.04 M sodium phosphate buffer, pH 7.5, was applied to a column of Sephadex G-100 (2.4 × 50 cm) . The column was eluted with the same buffer and 5.0 ml fractions were collected.

—, absorbance at 280 nm ; —, fractions pooled.

Step 2 : Gel Filtration on Sephadex G-100— The major fraction between 430 and 700 milliters in the previous step was concentrated and desalted. Gonadotropic (HCG) activity and protein content were measured. The solution of the residue in 1 ml of 0.04 M sodium phosphate buffer, pH 7.5, was applied to a column of Sephadex G-100 (2.4 × 50 cm) previously equilibrated with the same buffer. The column was eluted with 0.04 M sodium phosphate buffer, pH 7.5. Fractions of 5.0 ml were collected. Fig. 2 indicates the fractions pooled. HCG was eluted from the column in three peaks. All fractions under the peak were pooled. The combined fractions were concentrated and desalted. Gonadotropic (HCG) activity and protein content were measured.

2. Slab gel electrophoresis

The slab electrophoresis technique was used in these studies with slight modification in the staining and destaining procedure. Analytical slab electrophoresis was conducted on the purified HCG as well as on all other fractions obtained from DEAE-Sephadex chromatography (Step 1) described above. Slab gel electrophoresis in 7.0% and 12.5% cross-linked polyacrylamide gels were carried out by the method of Davis [11] . Each sample (about 50-100 μ g of protein) was mixed with an equal volume of buffer [0.05 M Tris-HCl, pH 6.8, with 40% glycerol, and 0.01% BPB] and layered on polyacrylamide gels, a constant intensity (100V) was applied for about 2.5 h at ambient temperature, the gels were then stained for 1h in a 0.25% Coomassie brilliant blue R 250 solution in 45% methanol-10% acetic acid and destained in 7% acetic acid-5% methanol. HCG standard (Fuji Pharmaceutical Co., Tokyo) and protein molecular weight markers (Sigma) in sample buffer were also applied to gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on 12.5% cross-linked polyacrylamide gels in a Atto slab gel apparatus by the method of Leammli [12] . Each sample was mixed with an equal volume of buffer [0.05 M Tris-HCl, pH 6.8, with 40% glycerol, 1 % sodium dodecyl sulfate, 2.5% (vol/vol) β -mercaptoethanol, and 0.01% BPB]. The sample was then placed in a boiling water bath for 2 min, cooled, then applied to SDS-polyacrylamide gels. The gels were stained in 0.25% Coomassie brilliant blue R 250 solution and destained.

RESULTS

Purification of HCG— Table 1 shows distribution of total protein during purification of HCG in all steps. Chromatography on DEAE-Sephadex A-50 resulted in the isolation of several fractions designated as 1 to 4 in Fig. 1.

Fig. 1 illustrates that most of the activity can be eluted from the column at a NaCl concentration ranging from 0.1 to 0.2 M [13,14] . The bulk of inert proteins is thus separated from the active material. The effluent containing the hormone was practically colorless, whereas fractions eluted with 0.2 M NaCl and up showed a tan to dark-brown color. The appearance of color thus serves almost as a demarcation line. All

fractions were concentrated and desalted. Material with an activity of 5,300 IU/mg was obtained from 0.5 g of crude product (1,500 IU/mg). (Table 2) Thus inactive material was removed while most of the activity was retained. The effluent fractions containing most of the chorionic gonadotropin activity (Fraction 2 and 3) were subjected to gel filtration on Sephadex G-100 (Fig. 2).

Polyacrylamide gel electrophoresis of these fractions indicated that the Fraction 2 and 3 were consisted of one band of apparent molecular weight above 200,000 [14,15]. Also SDS-polyacrylamide gel electrophoresis showed to consist of three closely spaced bands of apparent molecular weight between 14,000 and 30,000.

Fig. 2 shows the gel filtration of HCG from Step 1 on Sephadex G-100. The bulk of activity was found between fractions 70 and 100 milliters. The potency of the product was enhanced about 8-fold (12,000 IU/mg) (Table 2). Preparation of step 2 was obtained from step 1. HCG activity, as determined by Gonavislide test, are summarized in Table 2. Pure HCG, as purified from crude material, is seen to consist of three bands of about 14,000, 15,000 and 30,000 by SDS-polyacrylamid gel electrophoresis.

DISCUSSION

The present investigation was aimed to isolate, a highly active and homogeneous preparation of HCG which would be suitable for structural studies. The results reported in this indication that the procedure of purification described above yields a product suitable for

Table 1. Distribution of total protein during purification of HCG

Crude HCG 0.5g (800 IU/mg)

Activity (IU/mg Protein)	1500
Weight (mg Protein)	268
Weight (%)	100

↓

Step 1. DEAE-Sephadex A-50 Chromatography

	F 1	F 2	F 3	F 4	etc
Activity (IU/mg Protein)	13	5400	5200	200	50
Weight (mg Protein)	14	44	39	51	58
Weight (%)	5	17	14	19	22

↓

Step 2. Sephadex G-100 Chromatography

	F 1	F 2	F 3
Activity (IU/mg Protein)	7700	12000	360
Weight (mg Protein)	1.8	23	6.0
Weight (%)	—	8.5	—

Table 2. Activity of chorionic gonadotropin preparations

Preparation		Activity IU/mg	Purification
Crude		1500	
Step 1	F 2	5400	×4.0
	F 3	5200	×4.0
Step 2	F 2	12000	×8.0

structural investigations. Although the preparation of HCG thus obtained is homogeneous by using various physicochemical criteria, it apparently shows less biological activity than that reported by Van Hell et al [5].

Applying this procedure to preparation of step 1, HCG was able to increase the activity from 5,300 IU/mg to 12,000 IU/mg. However, when this material was examined by SDS-polyacrylamide gel electrophoresis, it was exhibited three distinct components of heterogeneity. Preparation of step 2 was examined by SDS-polyacrylamide gel electrophoresis, applying a current of 203 mA and constant 100 V. The presented data shows the feasibility obtains highly purified chorionic gonadotropin from crude material by simple methods of DEAE-sephadex chromatography. Although this conclusion must remain tentative, it shows a condition of isolation of homogeneity from the crude material.

From this on, I will pursue this study on a comparison of some of the physical-chemical properties of the hormone obtained from the pregnancy source and from the trophoblastic tumor source.

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