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Isolation of Placental Protein with Immunological and Receptor Activities Similar to Growth Hormone

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Summary

It is well known that human placenta synthesizes and secretes into maternal circulation polypeptide hormones. Two hormones which have established clinical use are placental lactogen and chorionic gonadotropin. Recently, it has been discovered that human placenta also synthesizes and secretes a variant form of human growth hormone (hGH-N), now called hGH-V protein. The aim of this work was to isolate hGH-V hormone from human term placenta. The purification steps were monitored by measuring hGH as well as placental lactogen and chorionic gonadotropin immunoactivities. hGH-V immunoactivities were separated from placental lactogen and chorionic gonadotropin immunoactivities by three simple chromatographic steps. By ion-exchange chromatographies (anion- and subsequent cation-exchange chromatography) placental lactogen immunoactivities were separated from hGH-V immunoactivities. The contaminating, chorionic gonadotropin immunoactivity was separated from hGH-V immunoactivity by size-exclusion chromatography. When compared with the crude placenta homogenate, the isolated protein was purified 648-fold with an overall recovery of 0.06%. The isolated protein, M_r around 22,000, possessed immunological as well as receptor activities similar to human growth hormone (hGH-N), isolated from human pituitary.

Keywords: human placenta, human growth hormone, placental growth hormone, placental lactogen, chorionic gonadotropin

Introduction

Genes coding for human growth hormone(s) (hGH), as well as genes coding for human placental lactogen (hPL), are clustered on human chromosome 17 *(1)*. Two genes, that is, hGH-N and hGH-V code for hGH-N protein and for hGH-V protein, and three other genes code for hPL protein. Only two hPL genes are active and they express identical protein *(2)*. In 1981 it was demonstrated that both hGH-N and hGH-V genes can be expressed in monkey cells *(3)*, but it was not until 1985 that a Liege group *(4)* detected hGH immunoactivity in human placenta. Later, the same group described the expression of hGH-V gene in human placenta *(5)* as well as the isolation of placental growth hormone (now called hGH-V hormone) *(6)*.

hGH-N gene is specifically expressed in the pituitary *(7,8)*. The main product of the hGH-N gene is the protein with M_r around 22,000 (22K) built from 191 amino acids, generally known as 22K hGH *(9)*. A secondary product of the hGH-N gene is the variant with M_r around 20,000 (20K hGH), which arises from alternative pre-mRNA splicing *(10)*. It corresponds to 22K hGH except for an internal deletion of amino acids 32–46 *(11)*. The hGH-V gene is active in placenta during the second half of pregnancy; it is secreted into the maternal circulation and supplants pituitary hGH that is suppressed *(6)*. hGH-V is a 191-amino acid protein that differs from hGH-N in 13 amino acid positions dispersed throughout the sequence *(1,12)*. Unlike hGH-N, hGH-V does not exist as a 20K variant *(13)* but instead its primary transcript undergoes a different type of alternative splicing, leading to a 230-amino acid protein, M_r around 26,000, which because of a frameshift, diverges in sequence from hGH-V after residue 125 *(14)*. This protein termed hGH-V2, has a carboxy-terminal membrane anchor and is not secreted *(14,15)* and therefore does not strictly qualify as a hormone. hGH-V, unlike hGH-N, contains a

consensus sequence for N-linked glycosylation and exists both as a nonglycosylated and as a glycosylated protein at Asn^{140} *(16,17)*.

hGH-N hormone is necessary for normal linear growth, but it also affects many aspects of metabolism, so that it has been described as being anabolic, lypolytic and diabetogenic *(18)*. From pituitary, hGH-N is secreted episodically *(18)*. hGH-N secretion is regulated by several hormones including hypothalamic hormones, somatoliberin which is stimulatory and somatostatin which is inhibitory, and by insulin-like growth factor-I (IGF-I) *(19,20)*. hGH-N initiates its anabolic effect by binding to specific cell surface receptors. The receptor consists of three distinct domains: an extracellular ligand binding domain, a transmembrane domain and an intracellular effector domain (reviewed in *9)*. The exact molecular mechanisms that mediate the effects of growth hormone upon binding to its receptor are poorly understood. A truncated form of the hGH receptor, which approximates to the extracellular ligand binding domain, is found in serum *(21)*. This serum protein (growth hormone binding protein GHBP) is capable of binding hGH and may play an important role in its clearence from the body *(22)*. The binding complex consists of one molecule of hGH and two molecules of the receptor-like binding protein *(9)*. Growth hormone receptors have been detected in a number of tissues. The hormone exerts its primary influence on the liver, where it stimulates the synthesis of IGF-I. IGF-I directly mediates most of the growth promoting effects of hGH-N *(23)*.

In contrast to known biological effects of hGH-N, the biological role of hGH-V hormone is largely unknown. One study demonstrated that hGH-V is involved in autocrine mechanism in the syncitiotrophoblast *(24)*. It has been demonstrated that by this mechanism syncitiotrophoblast cells produce placental GH and express GH receptors *(25)*. hGH-V protein probably regulates maternal IGF-I during pregnancy *(26)*, and as demonstrated *(27)*, hGH-V protein has full potency compared with hGH-N as a ligand for the human high affinity GHBP/receptor.

Human term placenta is a rich source of different enzymes and peptide hormones *(28)*. Placental lactogen (hPL) is related to the functional mass of the placenta *(29)* and is considered as a major secretory product of the term placenta, with an output of about 1 g/day *(30)*. It has been shown that hPL is a single polypeptide of M_r around 22,000, with a 96% sequence homology with hGH-N gene (reviewed in *28)*, and with 85% amino acid sequence homology when compared to hGH-N hormone. The other common polypeptide hormone, produced by placenta and found in the maternal circulation is chorionic gonadotropin (hCG) *(28)*. Like the other gonadotropins, hCG is an α-β dimer with a common α-amino acid sequence and specificity confered on the β sub-unit *(31)*. The synthesized and secreted amount of these two peptide hormones vary during the pregnancy. Dimeric hCG in maternal circulation is one of the first signs of pregnancy. The amount of hCG found in maternal circulation rises during the first eight weeks of pregnancy, but sharply declines between the 12th and 18th weeks and stays low for the remainder of pregnancy. On the other hand, hPL is detected in maternal plasma at three weeks, then rises progressively until a week or so before term *(28)*. Therefore, in our study we analyzed the concentrations of these two peptide hormones in our pooled fractions, since we consider the presence of either of these hormones as a contaminant of a hGH-V hormone.

This study was undertaken with the aim to isolate hGH-V protein. The availability of larger amounts of hGH-V protein would help the study of different metabolic roles of this hormone and it would certainly help in studying hGH-V structure and its physicochemical properties. The knowledge about this hormone and its different forms would be one step forward in understanding the role of hGH proteins, which, as recently reviewed *(32)*, represent a family of about 100 different entities found in human pituitary, blood and placenta.

Materials and Methods

Materials

Iodination grade hGH (isolated from human pituitaries), and radioimmunoassay grade bovine serum albumin (BSA) were purchased from Calbiochem; protease inhibitors aprotinin and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma; $Na^{125}I$ was obtained from Amersham; Sephadex G-25, Sephadex G-100, DEAE-Sephacryl Fast Flow, SP-Sephacryl Fast Flow, Sephacryl S-100 HR, Low molecular weight calibration kit and kits for estimation hGH and hPL concentrations were obtained from Pharmacia; Fractogel TSK HW-55 (F) and polyethylenglycol 6000 were from Merck; a kit for estimating human chorionic gonadotropin (βhCG) was obtained from Mallincrodt. Goat anti-rabbit IgG serum was obtained from Zavod za transfuziju, Zagreb; female Balb/c mice, 2.5 month old were obtained from R. Bošković Institute, Zagreb. All other chemicals were analytical grade and were obtained from Kemika, Zagreb.

Anti hGH serum was prepared by immunizing rabbits with hGH (Serono preparation for human use). After 90 days of immunization, rabbits were bled and hGH antibodies were precipitated by ammonium sulfate and further purified by DEAE-cellulose chromatography *(33)*. When diluted 1:100,000, these antibodies precipitated 30% of hGH tracer, and as demonstrated *(34)* specific radioimmunoassay for hGH was developed. With this radioimmunoassay (RIA) it was possible to estimate hGH in the range from 0.5–30 μg/L. These antibodies crossreacted with hPL and prolactin (PRL) when these hormones were present in g/L amounts (hPL) or mg/L amounts (PRL) *(33)*.

Tissue source

Human term placentas were obtained at the Department of Obstetrics and Gynecology, University hospital »Sestre milosrdnice«, Zagreb, after non-complicated labor and delivery of healthy children. After the placenta had been examined by a pathologist, it was immersed into an ice-cold physiological saline and brought into our laboratory.

Methods

Purification of hGH-V hormone

1) Extraction and ammonium sulfate precipitation

Term placenta, after removal of amniotic membranes, was cut in small pieces and then homogenized in cold 0.1 M ammonium bicarbonate, pH = 9.5 buffer, containing 7.7 mM NaN_3, 50,000 IU/L aprotinin and 1.0 mg/L PMSF (4 mL buffer/g wet tissue) using Potter homogenizer with a Teflon pestle. The homogenate was stirred for 2 hours at 4 °C, rehomogenized, and centrifuged 1 hour at 3,500 g. The supernatant was brought to 1.05 M ammonium sulfate concentration, mixed for 8 hours at 4 °C and the precipitate was removed by centrifugation for 1 hour at 3,000 g. The ammonium sulfate concentration of the supernatant was brought to 2.31 M, mixed for 16 additional hours, and the suspension was centrifuged as described above. The pellet which precipitated between 1.05–2.31 M ammonium sulfate concentration was extensively dialyzed against 0.09 M ammonium bicarbonate, pH = 8.5 buffer, containing NaN_3 (0.03 M) and PMSF (1 mg/L) and freeze dried. This material was stored at 4 °C, until it was further purified by chromatographic techniques.

2) Chromatography procedures

All chromatography purification steps were performed at 25 °C. The flow of the buffer was obtained by using LKB-HPLC pump.

a) DEAE-Sephacryl ion-exchange chromatography

The lyophilized powder (1.05–2.31 M ammonium sulfate precipitate) was dissolved in water, dialyzed against 0.09 M ammonium bicarbonate buffer, pH = 8.5, and a fraction containing not more than 100 mg of proteins was applied to a column (2.5 × 27 cm) of DEAE--Sephacryl, previously equilibrated with 0.09 M ammonium bicarbonate, pH = 8.5. After eluting the column with the starting buffer, the column was eluted with 0.5 M ammonium bicarbonate pH = 8.5 buffer.

b) SP-Sephacryl ion-exchange chromatography

Fractions, obtained by DEAE-Sephacryl chromatography, which were immunoreactive to hGH antiserum, were pooled and freeze dried. Before cation-exchange chromatography the lyophilized powder was dissolved in water and dialyzed against 0.09 M ammonium bicarbonate buffer, pH = 8.5. The dialyzate was applied to a column (2.5 × 20 cm) of SP-Sephacryl exchanger, previously equilibrated with 0.09 ammonium bicarbonate buffer, pH = 8.5. After washing the column with the starting buffer, the column was processed with 0.5 M ammonium bicarbonate buffer, pH = 8.5.

c) Fractogel TSK HW-55 (F) size-exclusion chromatography

Fractions that reacted with hGH antiserum, after the cation-exchange chromatography, were pooled and lyophilized. This material was dissolved in 2.5 mL of water and applied to Fractogel TSK HW-55 (F) size-exclusion chromatography (column 2.5 × 90 cm). The column was eluted with 0.05 M ammonium bicarbonate buffer, pH = = 8.5, at the flow rate of 0.5 mL/min, and 4.0 mL fractions were collected.

d) Sephacryl S-100 size-exclusion chromatography

The fraction that was immunoreactive to anti-hGH was concentrated by lyophilization. It was dissolved in water and applied to the second size-exclusion column. The Sephacryl S-100 column (1.0 × 90 cm) was previously equilibrated with 0.025 M phosphate buffer, pH = 7.5 containing 0.2 M NaCl, and the column was also calibrated with Pharmacia »Low molecular weight« calibration proteins. The chromatography was performed at the flow rate of 0.05 mL/min and 10.0 minute fractions were collected.

Analytical procedures

1) Protein estimation

Protein concentrations in collected fractions obtained during different chromatographic procedures were estimated spectrophotometrically measuring absorbance at 280 nm *(35)*. The protein content of the original homogenate and of the pools obtained at different stages of fractionation was determined by the method of Lowry *et al. (36)* using bovine serum albumin (BSA) as standard.

2) Radioimmunoassays (RIA)

a) Determination of placental hormones in pooled fractions

hPL, βhCG and hGH in concentrated pooled fractions were assayed using commercial RIA (for hPL) and immunoradiometric (IRMA) assay (for hGH and βhCG) kits. The concentrations of hormones in pooled fractions were estimated from standard curves established with the standard hormone preparations, provided with the kits. The results were calculated using LKB-Wallac RIA-CALC program.

b) RIA for hGH in collected chromatographic fractions

Monitoring of hGH-V immunoreactivity (see below) in each collected chromatographic fraction was performed by utilizing ^{125}I-hGH and anti-hGH sera prepared in our laboratory. The preparation of hGH tracer and the assay procedure are briefly described below:

Radiolabeling of hGH – hGH was iodinated by Chloramine T method *(37)* using 1 mCi of $Na^{125}I$ and 5 μg of iodination grade hGH. Iodinated hormone was purified from unreacted ^{125}I by Sephadex G-25 chromatography (column: 1.0 · 10.0 cm) and final purification was carried out by Sephadex G-100 chromatography (column: 1.0 × 50 cm). Fractions containing ^{125}I-hGH monomer were pooled and diluted in RIA assay buffer (0.015 M phosphate buffer pH = 7.5, containing 0.137 M NaCl, 0.033 M EDTA, 0.015 M NaN_3 and 33 g/L BSA).

Assay procedure – The tubes containing ±2,000 Bq ^{125}I-hGH--N, the amount of anti-hGH sera *(34)* required for binding 30% of the tracer in the absence of unlabeled hGH and 100 μL sample (chromatographic fraction) in a total volume of 0.3 mL were incubated overnight at 25 °C. The bound antigen was precipitated by incubation for 30 minutes with 100 μL goat anti-rabbit IgG antiserum (diluted 1: 2 in RIA assay buffer) and 2 mL of 80 g/L polyethylene glycol 6000 dissolved in water. The tubes were centrifuged for 30 minutes at 3,000 g, supernatants were

discarded and the pellets were counted in a gamma counter. The relative amount of hGH immunoactivity in each collected chromatography fraction was expressed as (B-NSB)/(B_o-NSB) %, where B_o represents the measured radioactivity when only ^{125}I-hGH and hGH antiserum were incubated, B denotes measured radioactivity when ^{125}I- hGH and collected eluans competed for anti-hGH binding sites, while NSB represents »not specific radioactivity«, i. e, measured radioactivity when only ^{125}I-hGH and RIA assay buffer were incubated.

All RIA assays utilizing either commercial kits or the assay procedure for hGH estimation developed in our laboratory, were done in duplicate.

c) Radioreceptor assay *(38)*

Preparation of GH receptors – All membrane isolation procedures were performed at 4 °C in 0.3 M sucrose solution. Five Balb/c mice were sacrificed, the livers were removed, trimmed of connective tissue, cut into small pieces weighing 0.2–0.5 g and homogenized in 5 g/mL of 0.3 M sucrose solution in a Potter homogenizer. The crude homogenate was filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 1,500 g for 20 minutes and the resulting supernatants were centrifuged sequentially at 15,000 g for 20 minutes and at 100,000 g for 90 minutes. The 100,000 g pellet was suspended in 0.025 M Tris/HCl buffer, pH = 7.6, containing 10 mM $MgCl_2$ in a ratio of 1 mL buffer per g wet liver tissue. The microsomal suspension was homogenized with a glass homogenizer and then kept frozen at –20 °C. When required for assay the frozen receptor was thawed, homogenized and diluted with 0.025 M Tris/HCl pH = 7.6 buffer, containing 10 mM $MgCl_2$, such that the protein concentration of the suspension was 2.0 mg/mL.

Incubation procedure – The assays were carried out in a total volume of 0.5 mL containing 200 μL of 0.025 M Tris/HCl, pH = 7.6 buffer, containing 1.0 g/L BSA, 100 μL of receptor preparation, 100 μL of hGH-N standard or sample, and 100 μL of ^{125}I-hGH (0.2 ng/mL). During the 2 hour incubation period at room temperature, the tubes were shaken vigorously for 30 seconds every 30 minutes. The reaction was terminated by the addition of 3 mL of ice-cold RIA assay buffer. Under these conditions the hGH receptors were sedimented by centrifugation at 1,500 g for 20 minutes at 4 °C. The supernatant was decanted and the membrane-bound ^{125}I-hGH in the pellet was counted in a gamma counter.

Results and Discussion

Presented results were obtained from a single human term placenta tissue. The results obtained from two other human term placenta preparations were used to develop the purification scheme for hGH-V hormone described in this paper.

The hGH-V purification started with the extraction of the hormone in alkaline conditions. This step was identical to that described by Frankenne and co-workers in their published isolation procedure of hGH-V hormone *(6)*, and similar to the extraction scheme applied to isolate hPL from placenta tissues obtained from different species *(39–41)*. As can be seen from the results presented in Table 1, only about 30% of the immunoreactive material in 1.05–2.31 M ammonium sulfate precipitate from crude placenta homogenate was extracted. Although the extraction at different pH values, as well as re-extraction of the starting material was tried, the re-

Table 1. Protein and hormone contents detected in each hGH-V hormone purification step

		hGH				
	m(protein)/mg	*m*(hGH)/μg	purification factor	overall recovery/%	*m*(hPL)/mg	*m*(βhCG)/μg
crude homogenate[a)]	261,769	1,420.0	1.0	100	252.7	574.3
1.05–2.31 M precipitate[a)]	10,400	426.0	7.6	30	109.5	349.7
DEAE Sephacryl fraction[a)]:						
9–25	238.8	121.5	94.2	8.5	ND	69.2
49–56	1,311.5	62.4	–	–	102.3	7.1
57–69	5,322.0	107.0	–	–	ND	3.0
SP Sephacryl fraction[a)]:						
13–17	73.0	18.6	–	–	2.6	0.5
45–49	86.8	62.5	133.3	4.4	ND	34.7
50–56	26.2	43.2	–	–	ND	3.0
Fractogel TSK HW-55 (F) fraction[b)]:						
63–69	44.80	33.6	–	–	ND	20.8
70	0.38	1.3	633.3	0.09	ND	ND
Sephacryl S-100 fraction[c)]:	0.26	0.91	648.1	0.06	ND	ND

[a)] 10 mg of the lyophilized fraction were dissolved in 5.0 mL of water. The protein content was determined by serial dilutions of the fraction(s) with water, while for hormone assay(s) the fraction(s) were further diluted with RIA assay buffer.
[b)] The freeze-dried fractions were dissolved in 0.6 mL of water. 0.1 mL of this fraction was analyzed for protein, i. e. for hormone content as described in [a)].
[c)] 0.1 mL of the collected fractions were diluted either in 0.025 M phosphate buffer, pH = 7.5 (for protein determination) or in RIA assay buffer for hormone determination.
ND = the immunoactivity of the particular hormone was not detected by commercial hormone assays.

sults were similar. This is rather in contrast to the results obtained by Frankenne *et al. (6)*. The subsequent anion-exchange chromatography step differed from the scheme described by Frankenne *et al.*, since the chromatography at pH = 8.5 was performed, while the Liege group used ammonium bicarbonate buffer, pH = 9.5 to elute the proteins. This difference might be because the anion-exchanger from a different producer was used in this study. As shown in Fig. 1, purification of 1.05–2.31 M ammonium sulfate fraction by anion-exchange chromatography separated three immunoreactive fractions. The material eluted from the column with the starting buffer (fractions 9–25, Fig.1) was mainly hGH-V protein, while hPL eluted when higher ionic strength buffer was applied (Table 1). The hGH immunoactivity eluted after the hPL fraction (fractions 57–69, Fig.1), were proteins with M_r 10,000–15,000 as estimated by size exclusion chromatography (our unpublished results). The material (fractions 9–25) not bound to the anion-exchanger under the described conditions, collected from 10 subsequent anion-exchange chromatographies (about 100 mg of proteins were applied per one run) was pooled, and freeze-dried. A fraction of this freeze-dried material was dissolved in water and analyzed (Table 1). With this step the obtained overall purification of hGH-V hormone was 94-fold with about 8% recovery. As can be seen from the results presented in Table 1, the most of hPL was separated from hGH-V, but hCG immunoactivities co-eluted with hGH-V hormone.

The lyophlilized material with the highest hGH-V activity (eluted with the starting buffer from the anion-exchanger) collected after anion-exchange chromatography step was dissolved in water and dialyzed against 0.09 M ammonium bicarbonate buffer, pH = 8.5. About 100 mg of proteins were applied per one cation-exchange chromatography run and the chromatography was performed as described in Fig. 2. After three runs performed under identical conditions, three fractions with different immunoactivities (Fig. 2) were pooled and freeze dried. A small amount of each lyophilized fraction was analyzed (Table 1). As can be seen from the results presented in Table 1, the remaining contamination of hPL eluted when the column was processed with the starting buffer, while hGH-V and βhCG immunoactivities were eluted when 0.5 M ammonium bicarbonate, pH = 8.5 buffer was applied. As shown in Fig. 2, the majority of hGH immunoactivity eluted as a sharp peak (fractions 45–49) while some fractions (fractions 50–56) being also immmunoreactive to hGH-antiserum, eluted with higher ionic strength buffer. These basic fractions represent probably hGH-V degradation products since their M_r was in the range of 12,000 as determined by size-exclusion chromatography (data not shown). By cation-exchange chromatography step about 60 μg of hGH immunoactive material was obtained, which presents 133-fold overall purification with around 4% recovery from the crude placenta homogenate (Table 1).

The separation of βhCG immunoactivities from hGH immunoactivities was obtained by size-exclusion chro-

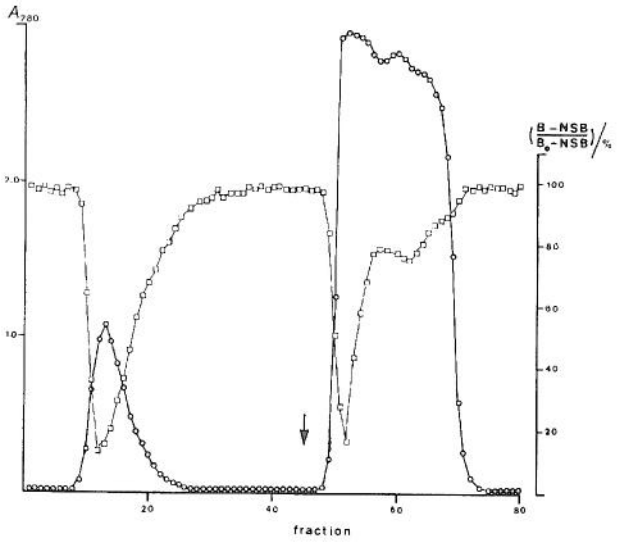

Fig. 1. Anion-exchange chromatography
100 mg of lyophilized 1.05–2.31 M ammonium sulfate precipitate was dissolved in 10 mL of water and dialyzed against 0.09 M ammonium bicarbonate buffer, pH = 8.5. This material was loaded onto DEAE-Sephacryl Fast Flow column. The DEAE-Sephacryl Fast Flow column (dimensions: 2.5 × 27 cm) was eluted at the rate 12 cm/h, first with 450 mL of the starting buffer (0.09 M ammonium bicarbonate, pH = 8.5) and thereafter (indicated by the arrow) with 0.5 M ammonium bicarbonate buffer, pH = = 8.5. During the run 10 mL fractions were collected. Absorbancies (A_{280}) as well as hGH immunoactivities, expressed as (B-NSB/B_o-NSB)%, were determined in each collected fraction. (A_{280} O–O–O, hGH immunoactivity □–□–□).

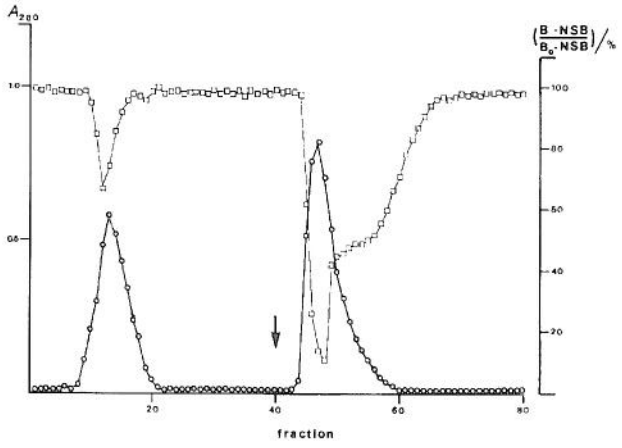

Fig. 2. Cation-exchange chromatography
After 10 consecutive anion-exchange chromatographies, fractions which showed the highest hGH immunoactivities (eluted with the starting buffer on anion-exchanger) were pooled and lyophilized. 100 mg of this freeze-dried material was dissolved in water and dialyzed against 0.09 M ammonium bicarbonate buffer, pH = 8.5. The dialyzate was loaded onto SP-Sephacryl Fast Flow column. The SP-Sephacryl column (dimensions: 2.5 × 20 cm) was eluted at the rate of 12 cm/h with 400 mL of the starting buffer (0.09 M ammonium bicarbonate, pH = 8.5) and thereafter (indicated by the arrow) with 400 mL of 0.5 M ammonium bicarbonate buffer, pH = 8.5. 10 mL fractions were collected and in each fraction A_{280} as well as hGH immunoactivity were determined. (A_{280} O–O–O, hGH immunoactivity □–□–□).

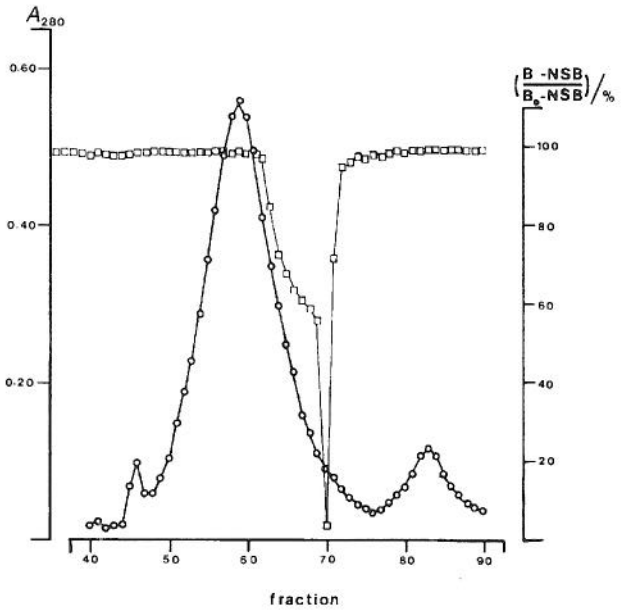

Fig. 3. TSK HW-55(F) size-exclusion chromatography
hGH immunoactivities (eluted with 0.5 M ammonium bicarbonate buffer, pH = 8.5) pooled and collected after three consecutive cation-exchange chromatographies, were lyophilized. The freeze-dried material was dissolved in 2.5 mL of water and loaded onto TSK HW-55(F) size-exclusion column (dimensions: 2.5 × 90 cm). The column was eluted at the rate of 6 cm/h with 0.025 M phosphate buffer, pH = 7.5, containing 0.2 M NaCl and 0.5 mL fractions were collected. In each collected fraction A_{280} and hGH immunoactivity were determined. (A_{280} O–O–O, hGH immunoactivity□–□–□).

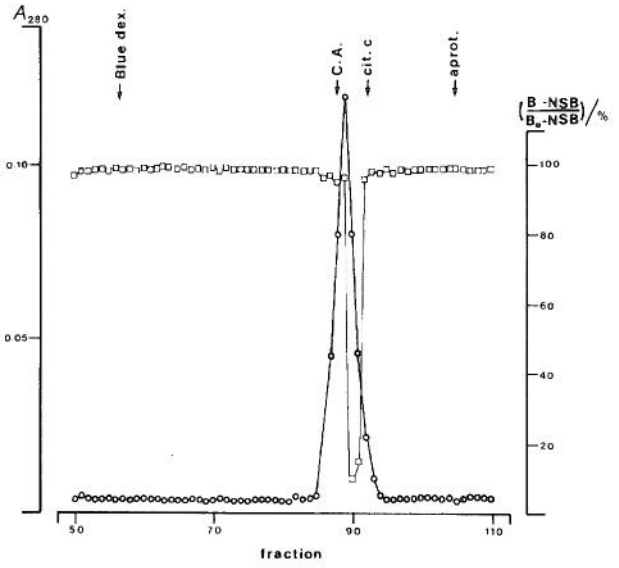

Fig. 4. Sephacryl S-100 HR size-exclusion chromatography
The fraction which possessed the highest hGH immunoactivity (fraction 70) after TSK-size-exclusion chromatography was concentrated by lyophilization. The freeze-dried material was dissolved in water, and 0.5 mL were loaded onto Sephacryl S-100 HR size-exclusion chromatography column (dimensions: 1 × 90 cm). The column was eluted at the flow rate of 3.82 cm/h with 0.025 M phosphate buffer, pH = 7.5, containing 0.2 M NaCl, and 0.5 mL fractions were collected. In each collected fraction A_{280} and hGH immunoactivity were determined. (A_{280} O–O–O, hGH immunoactivity □–□–□).
The Sephacryl S-100 column was calibrated by low molecular weight gel-filtration calibration proteins: aprotinin (aprot.) M_r = = 6,800; cytochrome c (cit.c) M_r = 12,400; carbonic anhydrase (C. A) M_r = 29,000; albumin (alb.) M_r = 66,000 and Blue Dextran 2000 (Blue dex.). The elution was performed as described above. The positions in which the calibration proteins were eletuted from the column are indicated by the arrow. These values were used for M_r estimation.

matography (Table 1). The freeze-dried material collected after cation-exchanger chromatography runs (fractions 45–49, Fig.2, see also data in Table 1) was dissolved in 2.5 mL water and applied to size-exclusion column (Fig. 3). The collected chromatographic fractions were analyzed for hGH immunoactivities. As shown in Fig. 3, hGH immunoactivities started to elute in fraction 63 and continued until fraction 70, where the majority of the hGH immunoreactivity was detected. Immunoreactive material found in fractions 63–69 was pooled and freeze dried separately from the immunoactivity found in fraction 70. The βhCG, as well as higher molecular forms of hGH were detected in freeze-dried fractions 63–69, while fraction 70 contained only hGH immunoactivity (Table 1). After analysis (determination of protein and hormone content), 0.5 mL of fraction 70 was further purified by Sephacryl S-100 HR size-exclusion chromatography (Fig. 4). The immunologicaly active material collected in two chromatographic fractions (volume 1.0 mL) was analyzed (Table 1). As can be seen from data presented in Table 1, the overall recovery after described purification steps was 0.06% and the material was purified 648-fold, compared with the crude placenta homogenate.

The mass of hGH after chromatography on Sephacryl S-100 matrix, determined with the commercial kit specific for hGH, was 0.91 μg. This material was diluted several fold and tested for binding to isolated GH-receptors. From the results presented in Fig. 5 it can be seen that the isolated material competed in an almost identical way for the receptor binding sites as hGH-N standards.

As shown in Fig. 4, the hGH-V protein with M_r of around 22,000 was isolated. During the development of the isolation scheme presented in this study, when each pooled fraction was studied by gel-filtration, immunoactivities in higher molecular weight range were observed. In some instances, especially after the anion-exchange step, high molecular weight forms of hGH immunoactivities eluting almost in void volume of the size-exclusion chromatography were detected. Similar results were found by Hizuka *et al. (42)* when they analyzed the expression of hGH-V gene in monkey kidney cells. The nature of this high molecular weight forms is mainly unknown, since after the cation-exchange chromatography the detected forms by gel-filtration were in the range of M_r 40,000–65,000 (Fig.3). These forms probably represent hGH-V oligomers. Upon repeated size-exclusion chromatography only low molecular form of hGH-V was detected. One explanation is, as stated by Hurley *et al. (41)*, that growth hormone and related hormones tend to form oligomers at higher pH values. The determination of protein M_r by size-exclusion chromatography is not very precise and it is not possible to exclude the possibility that the final isolate (Fig. 4) contained also traces of glycosilated hGH-V M_r 24,000–26,000 *(16,17)* or perhaps traces of hGH-V2 *(14)*.

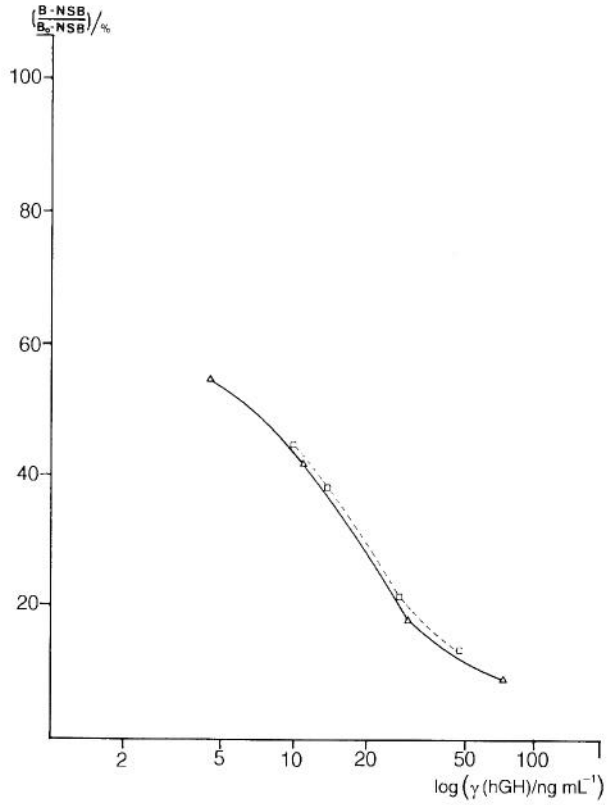

Fig. 5. Radioreceptor assay
The binding of ^{125}I-hGH to the prepared hGH receptors was competed either with hGH-N standards or with the isolated hGH-V hormone. Each point represents a mean of three parallel determinations. (hGH-N activity Δ–Δ–Δ, hGH-V activity □–□–□).

Two groups in the United States isolated hGH-V protein. One group isolated the hGH-V protein after the hGH-V gene was inserted into simian monkey kidney cells *(3,42)*, and the other group *(13–16)* purified the hormone when the gene was inserted into mouse fibroblast cells. Although very important properties of the hGH-V hormone were found, we cannot compare our isolation/purification scheme with purification steps of these groups, since they started the isolation from quite different material. It is more appropriate that we compare our results with the results of Frankenne *et al.* who isolated hGH-V hormone from human placenta *(6)*. As shown in Table 1, we isolated 0.91 μg of hGH-V protein from one human term placenta (1,789 g wet weight) with the »specific activity« (estimated hGH immunoactivity μg/mg protein) of $3.5 \cdot 10^{-3}$, while the Liege group, who purified hGH-V protein by immunoaffinity methods, obtained from 1,000 g (wet weight) term placenta 0.8 μg of hGH-V protein with the »specific activity« of $1.0 \cdot 10^{-3}$.

As already mentioned, the hGH immunoactivities eluted both in anion- as well as in cation-exchange chromatography steps, when higher ionic strength buffer was applied, represent probably proteolytically degraded forms of the native hGH-V molecule. This degradation occurred either when the material was dialyzed (after freeze drying), or during the ion-exchange chromatography steps that were performed at room temperature.

There are at least three points that make us belive that we isolated the true hGH-V hormone: (a) hGH-V is a basic protein *(13)* with an iso-electric point (pI) around 9, while hGH-N hormone has pI around 5.5 *(43)* and hPL protein has pI between 6.5–7.0 *(40,41)* – this observation is indirectly confirmed by our ion-exchange chromatographies (Figs. 1 and 2); (b) we isolated the protein with M_r around of 22,000 that reacted only with anti-hGH antibodies (no hPL immunoactivity was detected); (c) the isolated material reacted with GH-receptors in an almost identical way as the hGH-N standards.

From the data presented in Table 1, it can be seen that applying the same purification scheme, besides hGH-V hormone, two other placental hormones could easily be purified. As mentioned above, the bulk of hPL immunoactivity was successfully separated from hGH-V immunoactivity in a single anion-exchange chromatography step. By this step hPL was purified 80-fold with about 40% recovery. The other common placental hormone, hCG, co-eluted with hGH-V immunoactivities. By size-exclusion chromatography, we separated higher molecular weight forms of hGH-V »contaminated« with hCG, from monomeric hGH-V with M_r around of 22,000 (Table 1 and Fig. 4). As can be calculated from the data presented in Table 1, by size-exclusion chromatography step we purified βhCG immunoactivities 211-fold with the overall recovery of 3.6%.

In conclusion, it is possible to isolate hGH-V hormone from human term placenta. The applied purification scheme separated hGH-V from major contaminants (hPL and hCG) utilizing simple chromatographic steps. It is worth mentioning that hGH-V purification scheme is easily scaled up and that with slight modifications the other two placental hormones could easily be isolated.

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Izolacija proteina iz placente s imunološkim i receptorskim djelovanjem sličnim hormonu rasta

Sažetak

Poznato je da se polipeptidni hormoni koji se sintetiziraju u humanoj placenti izlučuju u krvotok trudnice. Dva polipeptidna hormona, placentarni laktogen (hPL) i korionski gonadotropin (hCG), pokazatelji su stadija trudnoće, a rutinski se određuju u majčinom serumu. Nedavno je dokazano da humana placenta sintetizira i izlučuje izo-hormon humanog hormona rasta u krvotok trudnice. Za razliku od hipofizarnog hormona rasta (hGH--N), placentarni se hormon naziva hGH-V. Svrha je ovog rada izolacija hGH-V hormona iz humane placente. Postupak izolacije praćen je radioimunokemijskim određivanjem hormona rasta, placentarnog laktogena i korionskoga gonadotropina. S pomoću tri kromatografska postupka razdvojena je frakcija koja je reagirala s protutijelima specifičnim na hormon rasta, ali ne i s protutijelima specifičnim na placentarni laktogen ili korionski gonadotropin. Kromatografijama na ionskim izmjenjivačima (anionski te nakon njega kationski izmjenjivač) odijeljena je frakcija koja je reagirala sa specifičnim protutijelima na placentarni laktogen od frakcije koja je reagirala sa specifičnim protutijelima na hormon rasta. Sljedećim kromatografskim postupkom, gel-filtracijom, razdvojena je frakcija koja je reagirala s protutijelima specifičnim na korionski gonadotropin od frakcije koja je reagirala s protutijelima na hormon rasta. Izolirani protein hGH-V imao je 648 puta veću specifičnu aktivnost od početnog materijala (homogenat placente), a iskoristivost postupka izolacije iznosila je 0,06%. Relativna je molekularna masa izoliranog proteina 22.000, a protein je imao imunokemijsko i receptorsko djelovanje slično humanom hormonu rasta izoliranom iz humane hipofize.