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ORIGINAL ARTICLE

Application of artificial gel antibodies for investigating molecular polymorphisms of human pituitary growth hormone

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Abstract Artificial gel antibodies were used to investigate human growth hormone (GH) activity in preparations purified from human pituitary glands. A partially purified fraction containing differently sized structural variants of GH was processed to yield monomeric and dimeric forms suitable for synthesizing artificial polyacrylamide gel antibodies. These two types of GH antibodies were used for investigating GH activity in experiments using HPLC gel-permeation and ion-exchange chromatography. In the size-exclusion experiments, both hormone fractions eluted as homogeneous peaks, whereas the ion exchanger resolved the hormones into several active components. The GH monomer antibodies exhibited a much higher affinity for monomeric GH than for dimeric GH, and the GH dimer antibodies exhibited a much higher affinity for dimeric GH than for monomeric GH. It was concluded that these two sets of antibodies might be useful for discriminating between dimeric and monomeric GH in clinical samples.

Keywords Assay · Electrophoresis · Growth hormone (GH) · HPLC · Human · Pituitary · Polyacrylamide gel antibodies · Purification · Quantification

Abbreviations

GH	Growth hormone
TEMED	*N*,*N*,*N*′,*N*′-tetramethylethylenediamine
T-unit	Tiselius-unit (10^{-5} cm^2/V s)
Tris	Tris(hydroxymethyl)aminomethane
RIA	Radioimmunoassay
HPLC	High performance liquid chromatography
CSF	Human cerebrospinal fluid
ALS	Amyotrophic lateral sclerosis

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Introduction

Access to highly specific, sensitive, reproducible techniques for the detection of peptides and proteins is essential for most areas of biochemical and biomedical research. This is of particular importance for studies of these molecules in body fluids retrieved from patients in clinical studies. In some diseases it is essential to be able to discriminate between different variants of circulating peptides or proteins. For instance, the ratios of the various sizes of growth hormone (GH) and prolactin molecules differ in patients with pituitary adenoma from those in healthy humans (Oosterom and Lamberts 1985). It has also been shown that the occurrence of larger forms of the GH molecule may differ between acromegalic subjects and normal individuals (De Palo et al. 1990). Since, in clinical studies, most polypeptide or protein hormones present in the circulatory system are investigated using radioimmunoassay (RIA), enzyme-linked immunoassay or, more recently, chromatographic or electrophoretic immunoassays, their detection depends on the properties of the antibodies. As most antibodies used in routine clinical analysis do not discriminate between hormones of different size, an estimated RIA value will usually reflect the concentration of all immunoreactive components regardless of size (De Palo et al. 2006). With this difficulty in mind, we

used a recently developed technique based on artificial antibodies prepared from polyacrylamide gel to produce an assay discriminating between differently sized hormone components.

The idea of preparing artificial antibodies using a polyacrylamide gel as matrix was first introduced in 1996 (Liao et al. 1996). In sharp contrast to earlier attempts at "fishing out" proteins using molecular imprinting, Hjertén et al. did not use ligands to attach the protein. The individual bonds between the protein molecule and the gel are weak (e.g. hydrogen bond and van der Waals forces) but there are many of them because of the close fit between the protein and the matrix, as a result of the gel polymerization occurring in the presence of the whole protein. We have recently prepared artificial gel antibodies against various blood proteins, such as albumin, transferrin, hemoglobin and γ-globulin (Ghasemzadeh et al. 2008a). These antibodies, which appear to be highly selective, were used to develop a method for quantitative analysis of the corresponding antigenic protein. We then established suitable standard curves based on conventional spectrophotometry for measuring these proteins in body fluids. In a subsequent study, we used gel antibodies against human albumin to quantify this constituent in human cerebrospinal fluid (CSF) and plasma collected from patients with amyotrophic lateral sclerosis (ALS). A standard curve was developed and levels of albumin in the two fluids could be determined (Ghasemzadeh et al. 2008b).

In the present study, we used gel antibodies to detect GH activity in fractions recovered and purified from frozen human pituitaries. The hormone was recovered as two separate entities of different molecular size (22 and 45 kDa) and was imprinted in polyacrylamide gels to obtain artificial antibodies which recognized the different sizes. The selective recognition of the template entity was studied using a very recently developed method based on free-zone electrophoresis of the granular artificial gel antibody (Ghasemzadeh et al. 2010). Using this approach, the complex gel antibody/antigen only has the charge of the template (e.g. for a protein, at a pH which differs from the pI of the protein). When the antigen is completely removed from the gel, it becomes non-charged again. The procedure can distinguish very small differences in the structure of various proteins and bio-particles. In this study, we combined artificial gel antibodies and electrophoretic analysis to study the selectivity of artificial gel antibodies for the two recovered forms of GH, designated monomeric and dimeric GH. We found that the generated antibodies exhibit much higher affinity for the hormone form used as "antigen" in the production procedure than for the other hormone form.

Experimental

Materials and methods

The chromatographic materials Sephadex G-100 and DEAE–Sepharose CL-6B were purchased from GE Healthcare (Uppsala, Sweden). Acrylamide, *N*,*N*′-methylenebisacrylamide, ammonium persulfate (APS), tris(hydroxy methyl) aminomethane (Tris) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, USA). TCPK-treated trypsin from bovine pancreas was purchased from Sigma (Stockholm, Sweden). Calcium chloride, ammonium bicarbonate, sodium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). The Cation Resource Q column was purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Preparation of growth hormone fractions

The starting material was a side fraction provided by a previously developed process for the purification of GH from whole frozen pituitary glands (Roos et al. 1963). Briefly, fresh frozen human pituitary glands were homogenized and extracted at pH 5.5. The extract was centrifuged to yield a supernatant which was then subjected to ammonium sulfate precipitation. The precipitate was dissolved in 20 mM Tris buffer (pH 7.5) and poured onto a gel filtration column (Sephadex G-100). This step yielded GH activity in two peak fractions, one of which contained proteins of molecular size ranging from approximately 25–50 kDa (Roos et al. 1963). This fraction was kept frozen pending further purification. In the present study this frozen fraction was thawed and dialyzed overnight in 20 mM Tris–HCl buffer (pH 7.5) before further purification by successive chromatography on DEAE–Sepharose CL-6B and Sephadex G-100. For the DEAE–Sepharose CL-6B separation, the dialyzed material was diluted with water (1:1) and adsorbed to the column (5 × 15 cm), equilibrated with 50 mM Tris–HCl buffer (pH 7.5). After washing the column with one volume of this buffer, the GH-containing material was eluted with 50 mM Tris buffer containing 0.5 M NaCl. Fractions of 5 ml were collected at a flow rate of 60 ml/h. The protein fractions 40–48 (see Fig. 1) was taken for further purification on a Sephadex G-100 column (2.5 × 80 cm), equilibrated with 0.04 M ammonium bicarbonate and operating at a flow rate of 50 ml/h. Fractions of 2 ml were collected, yielding GH activity in two separate fractions, in areas corresponding to those for

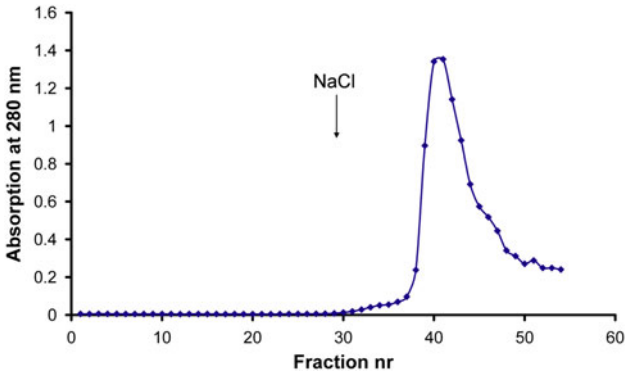

Fig. 1 Enrichment of hGH isoforms from partially purified human pituitary extract by DEAE anion-exchange chromatography. Partially purified human pituitary extract containing hGH isoforms was separated on a 5 × 15 cm DEAE–Sepharose CL-6B column equilibrated with 20 mM Tris–HCl buffer (pH 7.5) at 4°C and a 5-ml fraction was collected at a flow rate of 60 ml/h and a detection wavelength of 280 nm. Proteins were adsorbed to the DEAE column in loading buffer A (20 mM Tris–HCl pH 7.5). GH-containing material was eluted with 20 mM Tris buffer containing 0.5 M NaCl. DEAE fractions 40–48 were pooled for further fractionation

proteins of molecular weight 22 and 45 kDa, respectively. These two fractions, designated dimeric (fractions 50–58) and monomeric (fractions 66–75), were collected for further purification using HPLC gel filtration and subsequent analysis using HPLC ion-exchange chromatography. The protein content in each fraction was determined by measuring the UV absorbance in a cuvette with a 1-cm light path length at 280 nm, assuming that one unit was equal to a protein concentration of 1 mg/ml. HPLC gel filtration was carried out using the ÄKTA System equipped with a pre-packed Superdex 75 HR10/30 column (Pharmacia, Uppsala, Sweden). About 3 mg of lyophilized monomeric or dimeric GH was applied for each run. The column was eluted with 0.05 M NH_4HCO_3 buffer and fractions of 1.0 ml were collected at a flow rate of 0.75 ml/min. The fractions collected were assayed for GH activity using artificial antibody techniques combined with zone electrophoresis, as described below. In a next step, ion-exchange chromatography (IEC) was conducted on the ÄKTA-purifier using a 6-ml pre-packed cation exchange column (Resource Q, Pharmacia, Uppsala, Sweden). 1 ml of the fraction obtained from the gel permeation experiment was diluted with an equal volume of 20 mM Tris–HCl (pH 8.8) and loaded onto the IEC column. Unbound sample was washed out before starting the elution of the bound sample compounds by a linear gradient of 0–0.5 M potassium chloride in 20 mM Tris–HCl (pH 8.8). The length of the gradient was set to 14 column volumes and fractions of 1 ml were collected at a flow rate of 4 ml/min.

Preparation of artificial gel antibodies selective for GH

The artificial gel antibodies against the monomeric and dimeric GH fractions were synthesized using a procedure described previously (Ghasemzadeh et al. 2008a, b). Briefly, this method includes mixing of the template proteins (in this case, the purified growth hormone fractions) with the acrylamide monomer solution, polymerization, gel-granulation and removal of template molecules. The purified proteins were thus added to a solution of the monomers acrylamide and bis-acrylamide. Following polymerization the gel formed was granulated. Imprints of the proteins are created as they are selectively adsorbed to the gel granules and subsequently degraded by trypsin, leaving protein molecule-shaped cavities in the gel granules. Non-charged granules were also prepared in the absence of any template substance to be used as control *blanks*.

Preparation of a standard curve for determination of the concentration of GH in chromatographic fractions

The standard curve for determination of the concentration of the monomer was prepared using a procedure developed previously for somatropin and glycosylated GH (Ghasemzadeh et al. 2010). The measured migration velocity of the monomer selectively adsorbed to the gel granules was inserted into the calibration curve (Fig. 3a), and the *x*-axis then provided the concentration of the monomer in the sample solution.

For the dimeric form of GH, the procedure was identical to that above with the exception that the dimeric form of the hormone was used for preparation of the gel antibodies. The migration velocity of the GH dimer in the gel granules, determined by free-zone electrophoresis, was plotted against the concentrations of dimer in the sample solutions for development of the calibration curve (Fig. 3b).

Analysis by free-zone electrophoresis

The calibration curves were then used to determine the concentrations of monomer and dimer GH in the eluates from the gel filtration experiments (Figs. 4, 5). To 450 µl from each fraction obtained from gel-permeation chromatography was added 450 µl of a settled suspension of the gel antibodies and 450 µl of 50 mM Tris–HCl buffer, pH 8.5. Following incubation for 2 h, the supernatant was removed by washing and decantation using the Tris–HCl buffer. The analysis of the GH concentrations was

performed as described in a previous study (Ghasemzadeh et al. 2010).

Results

The fractionation of the dialyzed material by chromatography on a column packed with DEAE–Sepharose CL-6B yielded the hormone-containing material in a concentrated volume suitable for the subsequent chromatographic step (Fig. 1). The protein material eluted after inclusion of 0.5 M NaCl in the Tris buffer (fractions 40–48) was collected (40 ml) and purified further. The distribution of proteins, as recorded by UV absorbance at 280 nm following gel filtration chromatography on Sephadex G-100, is shown in Fig. 2. The protein material resolved into at least three separate peaks, one eluting close to the void volume and two eluting as proteins with molecular sizes of about 45 and 20 kDa, respectively. The UV absorption associated with the 45-kDa protein was greater than that for the 20-kDa protein. The fractions 50–58 (dimeric fraction) and 66–75 (monomeric fraction) were collected separately. Aliquots of each fraction were lyophilized and then served as antigen material for preparation of the artificial gel antibodies. A part of the remaining material from each fraction was further separated by HPLC gel-permeation chromatography.

The artificial gel antibodies raised against the dimeric and monomeric fractions were used for the preparation of standard curves which were subsequently applied to the hormone fractions separated on the HPLC gel-permeation column (Fig. 3). The standard curves were obtained after free-zone electrophoresis of hormone-containing fractions in sample tubes containing artificial antibodies selective for the dimeric or monomeric fractions, as described in the "Materials and methods".

As shown in Fig. 4, gel-permeation chromatography resulted in the UV-absorbing material present in the monomeric fraction appearing as a major peak, eluting as homogeneous protein of molecular size about 20 kDa. However, some UV-absorbing material consistent with a 45-kDa protein and some material close to the void volume were also observed. Analysis using antibodies directed against the monomeric fraction gave an activity profile in good agreement with the monomeric UV peak, while the assay using antibodies against the dimeric material gave an

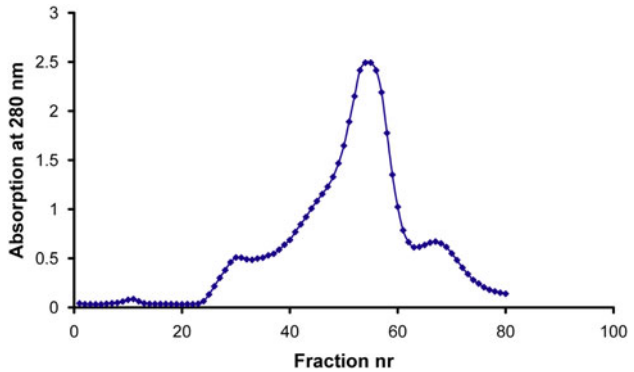

Fig. 2 Separation of hGH isoforms from partially purified human pituitary extract by gel-filtration chromatography. The DEAE fractions 40–48, containing dimeric and monomeric hGH, were pooled for further separation on a Sephadex G-100 column, equilibrated with 0.04 M ammonium bicarbonate. Fractions of 2 ml were collected at a flow rate of 50 ml/h and a detection wavelength of 280 nm. The growth hormone activity yielded two separate fractions, designated dimeric (fractions 50–58) and monomeric GH (fractions 66–75), eluting in areas corresponding to those of proteins of molecular weight 45 and 22 kDa, respectively. These two fractions were collected for further purification using HPLC gel filtration and subsequent analysis using HPLC ion-exchange chromatography

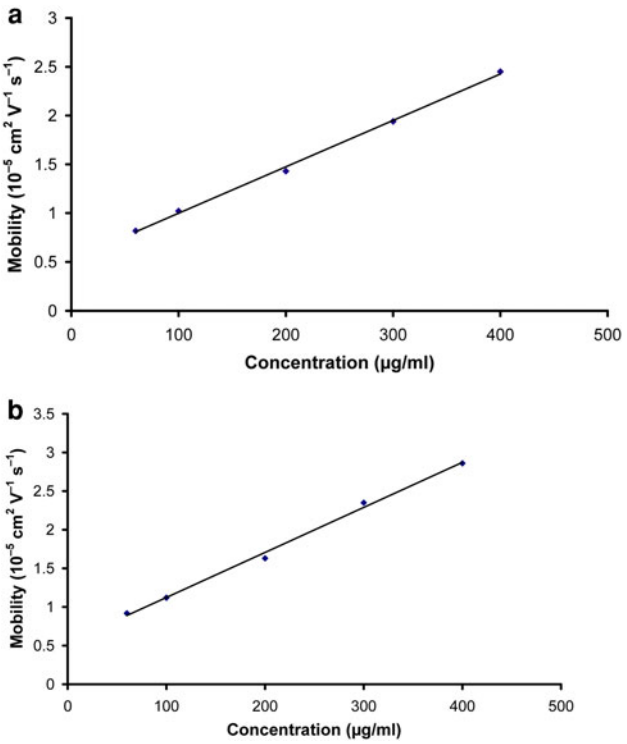

Fig. 3 **a** Standard curve for the determination of the concentration of GH monomer (antigen) based on free-zone electrophoresis experiments. The test tubes contained artificial gel antibodies selective for the monomer, prepared as described in the "Materials and methods" and resaturated with different concentrations of the GH monomer. The control tube contained a suspension of monomer-selective gel granules (control gel). The measured migration velocity (on the *y*-axis) gives the concentration of the antigen in the sample solution. **b** Standard curve for the determination of the concentration of GH dimer (antigen), based on free-zone electrophoresis experiments. The test tubes contained artificial gel antibodies selective for the dimer, prepared as described in the "Materials and methods" and resaturated with different concentrations of the GH dimer. The control tube contained a suspension of dimer-selective gel granules (control gel). The measured migration velocity (on the *y*-axis) gives the concentration of the antigen dimer in the sample solution

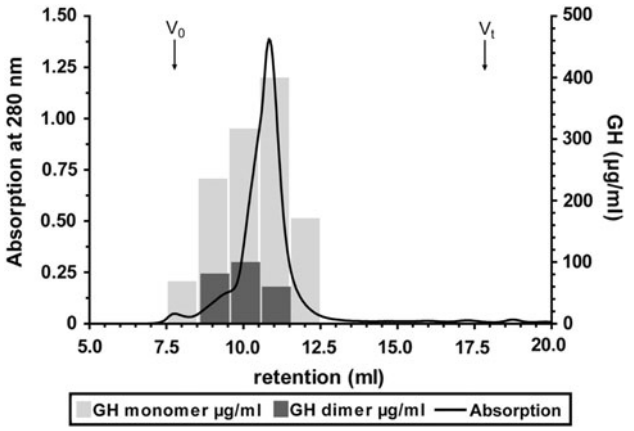

Fig. 4 Analysis of the concentration of monomeric GH by HPLC gel filtration. About 3 mg of lyophilized monomeric hGH dissolved in 250 µl of 0.05 M NH_4HCO_3 was applied for each run. Free-zone electrophoresis was carried out in a rotating capillary of monomeric-selective gel granules, incubated with monomeric (8–12 ml) or dimeric fractions (8–11 ml) obtained from HPLC gel filtration prior to the run. The standard curves in Fig. 3**a**–**b** were used to determine the concentrations of monomeric and dimeric GH. The two forms of GH were easily differentiated using artificial gel antibodies, since the complex monomeric-selective gel granules/monomer was more electrophoretically mobile than the complex monomeric-selective gel granules/dimer

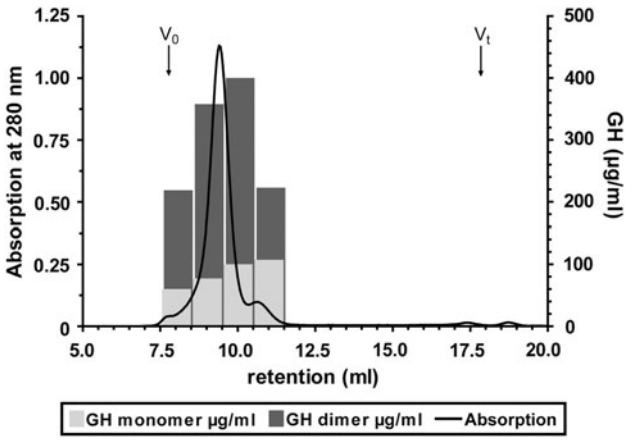

Fig. 5 Analysis of the GH activity of the dimeric form of GH using HPLC gel filtration. About 3 mg of lyophilized dimeric hGH dissolved in 250 µl of 0.05 M NH_4HCO_3 was applied for each run. Free-zone electrophoresis was carried out in a rotating capillary of dimeric-selective gel granules, incubated with monomeric f (8–12 ml) or dimeric fractions (8–11 ml) obtained from HPLC gel filtration. The standard curves in Fig. 3**a**–**b** were used to determine the concentrations of monomeric and dimeric GH selectively adsorbed to artificial gel antibodies. The two forms of GH were easily differentiated using artificial gel antibodies, since the complex dimeric-selective gel granules/dimer was more electrophoretically mobile than the complex dimeric-selective gel granules/monomer

activity peak in an area close to the dimeric UV peak. A similar situation was observed after HPLC gel-permeation chromatography of the dimeric fraction (Fig. 5). Here, the major UV peak was consistent with the dimeric hormone (MW = 45 kDa). The assay using antibodies raised against the dimeric fraction gave an activity profile in agreement with the major UV peak, while the assay with the monomer-directed antibodies indicated maximum activity in the area of the monomeric hormone fraction. It appeared that the artificial monomeric antibodies recognized the monomeric material better, while the antibodies directed against the dimeric fraction were more effective for measuring the dimeric hormone fraction than the monomeric hormone material. In the experiments applying IEC it was found that the ion exchanger resolved each forms of the hormone into several active components.

Discussion

Previous studies have shown that human pituitary GH can exist in differently sized forms. For instance, Brostedt and Roos (1988, 1989) found that, in addition to monomeric forms (molecular sizes of about 20 kDa), the hormone can exist in covalently and noncovalently linked dimeric forms. These researchers also observed that the dimeric form of the hormone was composed of monomeric forms of various sizes, from 20 to 22 and 24 kDa. It has also been shown that dimeric and monomeric forms differ in their affinity to the antibodies used for their detection (Bidlingmaier 2008). For example, although one monoclonal antibody did not discriminate between monomeric and dimeric GH, its potency for polymeric GH was significantly reduced (Ochoa et al. 1993). When a polyclonal antibody was used, a significant lower affinity was also found for dimeric GH. The proportion of circulating monomeric and dimeric GH has been reported to be altered in some pathological conditions, such as galactorrhea (Wallis et al. 1982).

Several studies have confirmed the existence of differently charged monomeric GH variants, as reflected by differing migration paths in electrophoretic experiments with both rodents (Roos et al. 1987) and humans (Silberring et al. 1991). The charge difference is suggested to reflect differences in deamidated glutamine and asparagine residues (Silberring et al. 1991). Electrophoretic heterogeneity was also observed in dimeric forms of GH (Brostedt et al. 1990). Glycosylation of GH could also be a reason for charge or size heterogeneity. Previous research has revealed the presence of N-glycosylated variants of GH in human pituitary extracts (García-Barros et al. 2000) and in studies using lectin-binding techniques (Sinha and Lewis 1986). The heterogeneity of GH is one important reason for the well-known disparity among immunoassay results (Baumann 2009).

In our study, we observed that the affinity of GH for the artificial antibodies seems to depend upon the antigen used

for their production; i.e., gel antibodies raised against monomeric GH exhibit much higher affinity for the monomeric hormone than for the dimeric form. Conversely, the artificial antibodies raised against dimeric GH were much more likely to recognize the dimeric hormone than the monomeric hormone. This observation suggests that the gel antibodies could be used to discriminate between GH monomer and dimeric forms in assays of circulating GH content.

The comparatively strong ability of the artificial antibodies to discriminate between the monomeric and dimeric GH forms compared with antibodies raised in animals may be because most of the protein molecule is recognized in the gel material by the gel antibodies, whereas when antibodies are raised in, for example, rabbits, only a specific part of the surface of the protein, the antigenic determinant, is recognized. Also, when antibodies raised in animals against monomeric GH are used to detect dimeric forms of the hormone, the antigenic area of the hormone could be hidden in the dimeric complex, i.e., the site for the immunological interaction between the antibody and the hormone could more or less coincide with the site for the interaction between the hormone units forming the dimeric entity. In contrast, with artificial gel antibodies, there are enough interaction sites for both monomers and dimers of GH to be recognized.

One important question which should be addressed concerns the purity of the antigens used in the present study. The artificial antibodies were raised against two hormone fractions, which were analyzed by HPLC gel permeation chromatography, where both fractions eluted as apparently homogeneous fractions (see Figs. 4, 5). However, when analyzed on SDS/polyacrylamide gel, the dimeric fraction resolved into several components (data not shown). Nonetheless, the fact that the dimeric fraction also contains noncovalently linked monomeric entities of sizes 20, 22 and 24 kDa raises the possibility that the dimers of GH could be split into these entities in the presence of SDS. In addition, even if some impurities not related to GH were present in the material, the GH fractions were pure enough to generate antibodies suitable for establishing practical and useful standard curves. Earlier attempts to raise antibodies in animals have also shown that partially purified proteins can also serve as antigens for the production of antibodies appropriate for radioimmunoassays (Bagdasarian et al. 1974).

The great challenge for future studies is to further refine the procedure, including purification, to reach detection levels in the range of those that are relevant for routine analysis in the clinic. The standard curve established in our study allows detection of GH concentrations of about 60 μg/ml. The true levels of the hormone in plasma are in the range of a few ng/ml to 100 ng/ml. Also, future studies need to take into consideration that the pattern of GH polymorphism in plasma may be more complicated than indicated by only monomeric and dimeric forms of the hormone. It has been suggested that there may be more than 100 GH forms in plasma (Baumann 1991). It is also noteworthy that the free monomeric 22 kDa entity accounts for only 21% of the total immunoreactivity in plasma.

In conclusion, this study shows that it is possible to raise artificial polyacrylamide gel antibodies against differently sized forms of GH purified from human pituitary glands. It also indicates that the artificial antibodies can discriminate between monomeric and dimeric GH forms. Although the antigens did not represent 100% pure protein hormones, it was possible to use the generated antibodies to establish standard curves for assays of the hormone in biological samples. Access to assays discriminating between different sizes of GH could well be of importance in clinical diagnostics as the proportions of monomeric and polymeric GH seem to differ in several pathological conditions.

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