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Purification and Characterisation of Polyclonal Anti-hGH Antibodies

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Summary

Antisera to human growth hormone (hGH) were obtained by immunising rabbits with hGH preparations. These antisera were further purified by ammonium sulphate precipitation (»unpurified« antibodies) and by DEAE-cellulose chromatography. Antibodies which were eluted with the starting buffer, 10 mM phosphate buffer, pH = 8.0 (antibody preparation I) and antibodies eluted with rising phosphate concentrations, i.e. in the concentration range of 15–40 mM (antibody II preparation) and in the concentration range 45–60 mM of phosphate buffer (antibody III preparation) were further characterised. By gel-filtration it was found that all purified anti-hGH antibodies belong to the IgG class, since the majority of »immunoactivity« was eluted in M_r range 165–158 000. That anti-hGH antibodies belong to IgG was further confirmed by precipitation with ***Staphylococcus aureus*** *cells (Pansorbin cells). pH optima, influence of various salts, as well as affinity constants were determined for each antibody preparation. Further, each antibody preparation was tested for specificity to various hormones. It was found that different antibody preparations react differently with hGH related hormones such as prolactin (hPRL) and placental lactogen (hPL). In further experiments it was found that antibody I preparation reacted with different epitopes of hGH than antibody II and antibody III preparations. From these studies it can be concluded that by purification of antisera, by simple ion-exchange chromatography, antibodies with different specificity could be isolated.*

Keywords: human growth hormone (hGH), purification of antisera, antibody preparation, anti-hGH antibodies

Introduction

Today, specific antigen-antibody reactions are used in different very sensitive and precise immunochemical techniques in order to determine antigen concentrations (ELISA, RIA, IRMA, etc. (*1*)), to isolate specific antigens (immunoaffinity chromatography, immunoprecipitation techniques (*2,3*)), to characterise antigens (*4*) (e.g. enzymes (*5*)) or to detect and visualise antigens (Western blots (*6*), or histological preparations (*7,8*)).

The key component in all these techniques is the antibody. On its characteristics, i.e. avidity and specificity, depend the sensitivity and the precision of a particular immunochemical technique. In RIA or ELISA techniques, often the unpurified primary antibodies (antisera) are used (*1,9*). Although sensitive and precise assays were developed when unpurified primary antibodies were used in e.g. RIA assays (*9–11*), these assays are usually inferior to assays in which purified and defined antibodies are used (IRMA, »sandwich« ELISA)(*1,11,12*).

The well defined antibody is necessary to prepare so called »sandwich« assays, where one antibody is immobilised, and reacts with one (or some) epitope(s) of the antigen, while the other antibody, which is labelled but soluble, reacts with other antigen epitope(s)(*1*). For both of these modifications, especially for antibody labelling (iodination, attachment of enzymes, fluorescent dyes, etc.) but also for more sophisticated immobilisation techniques, the antibody must be pure and its immunological specificity well defined (*1*).

The aim of this study was to purify and characterise antibodies obtained by immunising rabbits with hGH (*10*). hGH is a polypeptide hormone, which under different physiological conditions in serum exists as mono-

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mer (20 K, 22 K, hGH-V), dimer, oligomer, bound to different binding proteins, in short, in more than 100 different isoforms (*13*). Also, there are very similar hormones to hGH, i.e. prolactin (hPRL) and placental lactogen (hPL)(*14*). Therefore, there is a need for a very precise assay for hGH in biological fluids. Besides the improvement of hGH assay, with purified antibodies it would also be possible to prepare immunoaffinity sorbents and to isolate particular hGH isoforms either in serum or in different tissues, such as pituitary(*15*) or placenta (*16*).

Materials and Methods

Materials

Somatotropin (hGH) >9 000 IU/g WHO 1st IRP standard 75/537, sodium metabisulphite, chloramine T, Pansorbin cells (100 g/L solution of Pansorbin *Staphylococcus aureus* cells), bovine serum albumin (BSA) RIA grade, as well as Aquacide III, were obtained from Calbiochem, Switzerland. Human prolactin (hPRL), human placental lactogen (hPL), human lutropin (hLH), human folitropin (hFSH), human thyrotropin (hTSH) and human chorionic gonadotropin (hCG) were obtained from Bioproducts, Belgium. Sephadex G–25, coarse, Sephadex G-100, fine, high and low calibration kits for chromatography and Pharmacia hGH RIA 100 kit, were obtained from Pharmacia, Sweden. Hepes (N-2-hidroxyethyl-piperizine-N'-(2-ethansulfonic acid)) and triethanolamine were obtained from Sigma, Germany. DE-52 pre-swollen microgranular anion exchanger was obtained from Whatman, UK. Sodium ^{125}iodide, specific activity 610.5 MBq/μg of iodine was obtained from Amersham International, UK. Polyethyleneglycol (PEG) 8000 was obtained from Merck, Germany and Visking dialysis tubes were obtained from Serva, Germany. Reactigel 6× (1,1'-carbonyl diimidazol activated 6% agarose matrix) was obtained from Pierce, USA. Goat anti-rabbit antiserum was obtained from Zavod za transfuziju, Zagreb. All other chemicals were p.a. grade and were obtained from Kemika, Zagreb.

Antisera to hGH

Antisera to hGH were obtained by immunising rabbits with outdated hGH preparations for human use (Creshormone) Serono, Italy. The antisera were specific to hGH, and when diluted 1 : 100,000 (volume ratio) it was routinely used for RIA hGH determination in serum by double antibody assay (*10*).

Methods

Purification of anti-hGH antibodies from rabbit serum

a) Ammonium-sulphate precipitation step

Lyophilised hGH antisera were stored at 4 °C and no loss of immunological activity was observed upon prolonged storage. Lyophilised antiserum was dissolved in double distilled water (1 mg/10 mL) and antibodies were precipitated by addition of saturated ammonium sulphate solution (1.94 M final concentration) in order to precipitate IgG (17). The precipitate was collected by centrifugation (20 min, 10 000 × g), dissolved, and dialysed against 0.01 M phosphate buffer, pH = 8.0. Assuming A_{280} = 1.0 equals 0.65 mg/mL protein in the antibody solution (17), the protein concentration of dialysed antibodies was 8.0 mg/mL. When tested for hGH RIA determination, it was necessary to dilute the antibodies 1:120 000 (volume ratio).

b) DEAE chromatography

Dialysed antibodies were further purified by DEAE--cellulose chromatography. The ion exchanger, DE-52 was prepared as recommended by manufacturer (Whattman), and the column (1.2 × 20 cm) was equilibrated with 0.01 M phosphate buffer, pH = 8.0, at the flow rate of 60 mL/h. The volume of 2 mL of dialysed antibodies, after ammonium sulphate precipitation step, was applied to the column. The column eluent was collected in 5 mL fractions. The column was first eluted with equilibration buffer (10 column volumes) and thereafter by applying 10 column volumes of linear phosphate buffer (pH = 8.0) gradient. The linear phosphate buffer gradient was formed by LKB Ultrograd gradient former and LKB HPLC pump. For each fraction obtained after DEAE-chromatography, A_{280}, phosphate buffer concentration (by conductometry), and ^{125}I-hGH bindability were determined. Peak fractions of separated antibodies were pooled, concentrated by dialysis against Aquacide III, dialysed against 0.01 M phosphate buffer pH = 8.0 and re-chromatographed on DE-52 column as described above. The finally separated peaks were concentrated by lyophilisation. To characterise further separated antibodies, lyophilised fractions were dissolved in water and dialysed against PBS buffer. The aliquots of 1 mL of separated antibodies were stored at –20 °C.

HPLC gel-filtration chromatography

LKB HPLC gel-filtration column (TSK-SW 3 000, 7.5 × 600 mm) was equilibrated with 0.2 M phosphate buffer pH = 7.2, flow rate 0.5 mL/min using LKB HPLC appliances. In each chromatographic step, 0.5 mL fractions were collected and corresponding A_{280} was monitored. The column was calibrated with proteins (»high« and »low molecular weight« kits for column chromatography M_r determination). M_r's of separated antibodies were determined from the plot K_{av} *vs.* log M_r, where $K_{av} = (V_e - V_t)/(V_o - V_t)$, V_e = elution volume of corresponding protein, V_t = elution volume of thyramine and V_o = elution volume of blue dextran. In each run, the column was loaded with 50 μL of protein solution, i.e. with 10–25 μg of calibration protein or 25 μg of corresponding antibody. ^{125}I-hGH bindability was determined for each fraction, and fractions showing the greatest binding were considered as antibody.

^{125}I-hGH preparation

hGH was labelled with Na^{125}I using modified Hunter & Greenwood chloramine T technique as described (*18*). The ^{125}I-hGH was purified by Sephadex G-100 gel-filtration chromatography. Finally purified ^{125}I- hGH was diluted with the assay buffer and stored at –20 °C. Specific activity of so prepared ^{125}I-hGH was 1.8 MBq/μg protein.

Immobilisation of antibodies

Purified antibodies were coupled to Reactigel 6× according to the method of Hissey (*19*). Briefly, three 5 mL aliquots of acetone slurry of Reactigel 6× were filtered on the sintered glass funnel and washed with 0.1 M borate buffer pH = 8.5 which contained 0.1 M NaCl. Purified antibodies were dialysed against the same borate buffer and 10 mL of each purified antibody was mixed with 5 mL of the washed Reactigel 6x. Antibody–Reactigel 6× mixtures were incubated 24 h at 4 °C under constant shaking. Antibodies bound to the matrix were precipitated by centrifugation (1 000 × g, 10 min) and remaining imidazolyl reactive groups on the matrix were saturated with 3 M triethanolamine (3 × 2 h incubations at 4 °C with constant shaking). Immobilised antibodies were washed with PBS buffer, and stored at 4 °C as a slurry in PBS buffer containing 0.4 g/L NaN_3 .

^{125}I-hGH binding to »free« antibodies

The binding of labelled hGH to various antibodies was performed in 7.5 × 120 mm polystyrene tubes and the reaction mixture, typical volume of 300 μL, contained ^{125}I-hGH, appropriate buffer and particular antibody. After 20–24 h incubation period at room temperature, the reaction was usually stopped by adding 100 μL goat anti-rabbit antiserum (1 : 3 dilution in assay buffer) and 1 mL of 80 g/L PEG in 0.1 M phosphate buffer pH = 7.4. The contents of the tubes were vigorously shaken and incubated for additional 30 min. The tubes were centrifuged for 20 min, 1 500 × g, supernatants were decanted and pellets, remaining in the tubes, were counted in LKB 1271 Clinigama counter.

In cases when precipitation of ^{125}I-hGH – antibody complex was performed with the Pansorbin cells, 400 μL of the 100g/L cell suspension was added to the 1.0 mL of the diluted reaction mixture (for more details see legends to Figs. 3 and 4), the mixture was incubated for 30 min at 4 °C and finally centrifuged for 20 min, 2 000 × g, at 4 °C. The tubes were decanted, and the remaining cell-pellets, with the bound ^{125}I-hGH-antibody complex, were counted in the gamma counter. Results are expressed as (B/T)/% or (B/B_o)/%, where B = amount of ^{125}I-hGH bound to appropriate antibody, T = amount of radioactivity (^{125}I-hGH) added to each tube in particular assay, B_o = amount of radioactivity bound to antibodies when no unlabelled hGH or competitor were added. All results were corrected for non-specifically bound ^{125}I-hGH to the test tube (NSB), i.e. when antibodies were not present in the reaction mixture.

Scatchard analysis (20)

Scatchard analysis was performed according to the equation:

$$r/A = nK_a - rK_a$$

where r = bound antigen concentration

A = free (not bound) antigen concentration

n = number of antigen binding sites

K_a = association constant

When r/A values are plotted against r, K_a and n could be calculated. The concentration of not bound

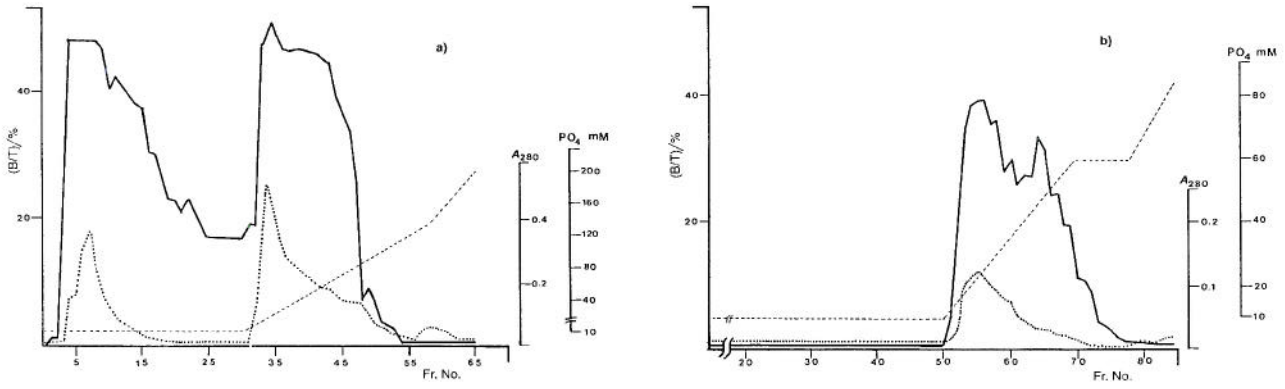

Fig. 1 Purification of anti-hGH antibodies by DEAE-chromatography.

Fig. 1a 2.0 mL (8g/L) of dialysed antibodies, after the ammonium sulphate purification, were loaded onto the DE-52 (1.2 × 20 cm) column equilibrated with the 0.01 M phosphate buffer, pH = 8.0. The column was first eluted with 150 mL of 0.01 M phosphate buffer, pH = 8.0, and thereafter with 150 mL of linear phosphate gradient obtained by mixing 0.01 M and 0.2 M phosphate buffers, pH = 8.0. Finally, the fractions were eluted with 30 mL of 0.2 M phosphate buffer, pH = 8.0. Fractions 1–25 and fractions 33–47 were pooled. The pooled fractions (1–25) were concentrated, dialysed against 0.01 M phosphate buffer, pH = 8.0 and again applied to the DE-52 column, run under the same conditions as described. Almost identical elution pattern was observed as shown in Fig. 1a. After concentration, this fraction was termed »antibody I«.

Fig. 1b Pooled fractions 33–47 were concentrated, dialysed against 0.01 M phosphate buffer, pH = 8.0 and 10 mL of this preparation was loaded onto the DE-52 column, prepared in the same way as described above (Fig. 1a). The elution conditions were the same as described, except the column was first eluted with 250 mL of 0.01 M phosphate buffer, pH = 8.0, and thereafter with linear gradient obtained by mixing 0.01 M and 0.06 M phosphate buffers, pH = 8.0, followed by elution with 40 mL of 0.06 M phosphate buffer, pH = 8.0. Fractions forming the first peak (Fr. 51–58) and fractions which formed the second peak (Fr. 65–70) were pooled. After concentration, pooled fractions 51–58 were termed »antibody II«, while the concentrated fractions 65–70 were termed »antibody III«. In each fraction presented in Fig. 1, A_{280} ····, phosphate concentration (mM/L) – – –, and ^{125}I-hGH-bindability to eluted antibodies (each fraction was diluted 1 : 100) expressed as (B/T)/%, (T = 200 Bq/reaction mixture) ——, was determined.

(free) antigens (A) as well as r could be calculated from the measured radioactivity and the known total (A_o) amount of antigen (hGH) present in a particular reaction mixture. In our experiments, the bound or free concentrations of antigens were corrected for the concentration (g/L) of anti-hGH antibodies present in each reaction mixture.

^{125}I-hGH binding to immobilised antibodies

The standard reaction mixture contained immobilised antibodies of appropriate dilution, buffer and ^{125}I--hGH in a volume of 0.3 mL. After 20–24 h incubation period at room temperature the reaction was stopped by adding 1 mL »Pharmacia decanting solution«, the tubes were vigorously shaken and centrifuged for 10 min, 1 500 × g. The supernatants were decanted and the radioactivity remaining in the pellets was counted in LKB gamma counter. The results, corrected for non-specific binding, were expressed as (B/T)/% or (B/B_o)/%.

Results and Discussion

Purification of anti-hGH antibodies

Lyophilised rabbit serum, with high anti-hGH avidity, was first purified by ammonium sulphate precipitation. Ammonium sulphate purified antibodies, were further purified by DEAE-cellulose chromatography. As shown in Fig. 1a, two peaks were eluted. The bulk of antibodies (fractions 1–25) were not bound to the ion-exchanger, since they were eluted with the equilibration buffer. Pooled fractions 1–25 (data not shown), were again applied onto the similar DEAE-column, and anti-hGH antibody elution profile from the second column was almost identical to the elution profile obtained by the equilibration buffer (peak I) presented in Fig. 1a. The fractions with anti-hGH activity were pooled, concentrated by lyophilisation and termed »antibody I«. The fractions corresponding to the second peak (Fig. 1a), eluted with the rising phosphate concentration, i.e. fractions 33–47, were concentrated, dialysed against the 0.01 M phosphate buffer, pH = 8.0, and applied onto the third DEAE-colum equilibrated with the same buffer. As shown in Fig. 1b, when a shallower phosphate gradient was applied, anti-hGH antibodies were eluted as two separate peaks, i.e. one peak was observed when phosphate concentration was in the range of 15–40 mM (»antibody II«), and the other peak eluted when the phosphate concentration was in the range of 45–60 mM (»antibody III«). After this separation step, fractions containing either antibody II or antibody III activities, were pooled, dialysed and concentrated by lyophilisation.

The purity of DEAE-column separated peaks (antibodies I, II and III) was checked by gel-filtration. As shown in Fig. 2a, antibodies purified only by ammonium sulphate precipitation contained IgG molecules as well as truncated IgG fragments, which are probably the result of proteolytical degradation. Also, elution of low molecular mass species (peptides or amino acids) without immunological activity, was observed. As it can be seen (Fig. 2b) antibodies I presented the most homogenous population of the isolated antibodies, since they were eluted as a single symmetrical peak with the immunological activity. Antibodies II, were eluted from the gel-filtration column in two peaks which possessed the immunological activity (Fig. 2c). It can be concluded that some aggregation between the IgG molecules occurred, since a high molecular mass peak, besides the IgG peak, with the anti-hGH activity was eluted. Also, the detected immunological activity in the IgG peak was not symmetrical, which is probably the result of traces of the truncated IgG. From the gel-filtration analysis of antibody III (Fig. 2d), it can be seen that this fraction besides IgG, contained truncated IgG molecules. Although, each DEAE-purified anti-hGH fraction contained some impurities without immunological activity (Figs. 2b–2d), antibody III fraction contained the most of the impurities.

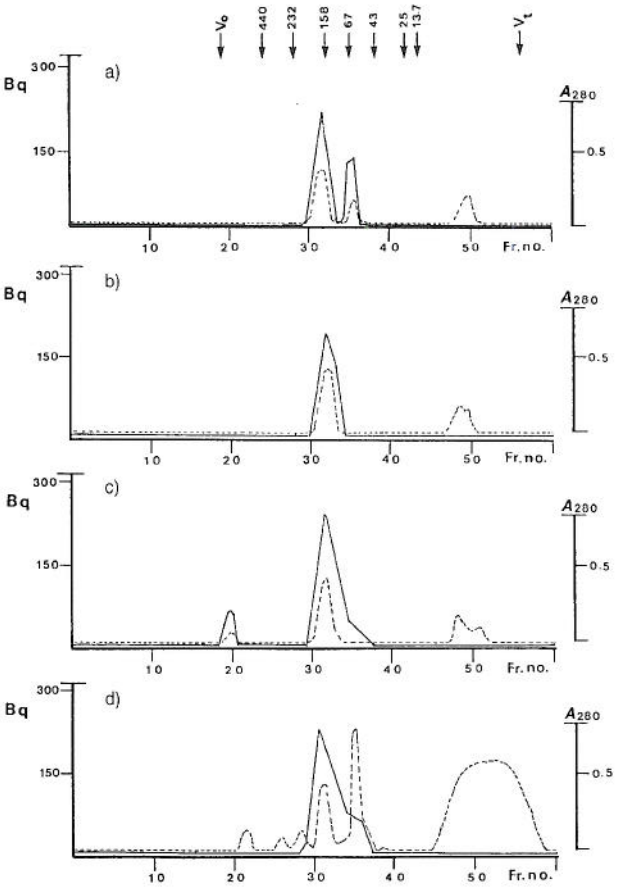

Fig. 2 Gel-filtration of purified anti-hGH antibodies
Concentrated fractions of anti-hGH antibodies were analysed by gel-filtration. Each antibody preparation was loaded separately onto 7.5 × 600 mm TSK-SW 3000 HPLC column and the column was eluted as described in Methods: ammonium sulphate purified antibodies, termed »unpurified antibodies« (Fig. 2a), antibodies I (Fig. 2b), antibodies II (Fig. 2c) and antibodies III (Fig. 2d). A_{280} (sensitivity of the monitor was set to AU = 0.01) was continuously monitored – – –, and the amount of ^{125}I-hGH bound to eluted antibodies (expressed in Bq) ——, in each collected fraction was determined. (To 0.1 mL of each eluted fraction 300 Bq of ^{125}I-hGH was added and the assay was performed under standard conditions). K_{av} values of »high« and »low molecular weight markers« and the corresponding M_r values are indicated by arrows on top of Fig. 2.

Physico-chemical properties of anti-hGH antibodies

The antibodies not purified by DEAE-chromatography step (»unpurified antibodies«), as well as antibodies I, II and III, were diluted with the appropriate buffer and ^{125}I-hGH bindability was tested in buffers of different pH, as well as in buffers with different amounts of various salts. As described in Methods, after the antigen – antibody reaction, the antibodies, as well as ^{125}I-hGH-antibody complexes, were precipitated by Pansorbin cells.

As it can be seen from Fig. 3a, at low pH values, i.e. below pH = 4.0, antibodies were inactive. The immunological activity increased significantly after pH = 5 was reached, and declined sharply at high pH values (above pH = 9.0). As shown in Fig. 3b, the optimal pH i.e. pH ranges with the highest ^{125}I-hGH binding were: »unpurified antibody« pH range 7.0–7.8; antibody I, pH = 7.0–7.4; antibody II, pH = 7.8–8.2; antibody III, pH = 7.4–7.8.

The influence of different salts on the bindability of ^{125}I-hGH to different antibody preparations was tested in Hepes buffer, pH = 7.8, containing 330 mg/L BSA. As shown in Fig. 4, the activity of antibodies was inhibited with increasing amounts of salts in a reaction mixture. The lowest inhibition was observed with the addition of KCl, while the highest inhibition was observed when $CaCl_2$ was present. It is worth mentioning that the immunological activity of antibody I resisted increased salt concentrations to the grater extent (except $CaCl_2$ amounts higher than 100 μmol) than the immunological activities of other tested antibodies.

Since the precipitation of antibody-^{125}I-hGH complex was performed with *Staphylococcus aureus* cells, it was confirmed that all antibody preparations belong to IgG class.

From the above results, and in order to simplify the assay buffer, experiments shown in Fig. 5, were per-

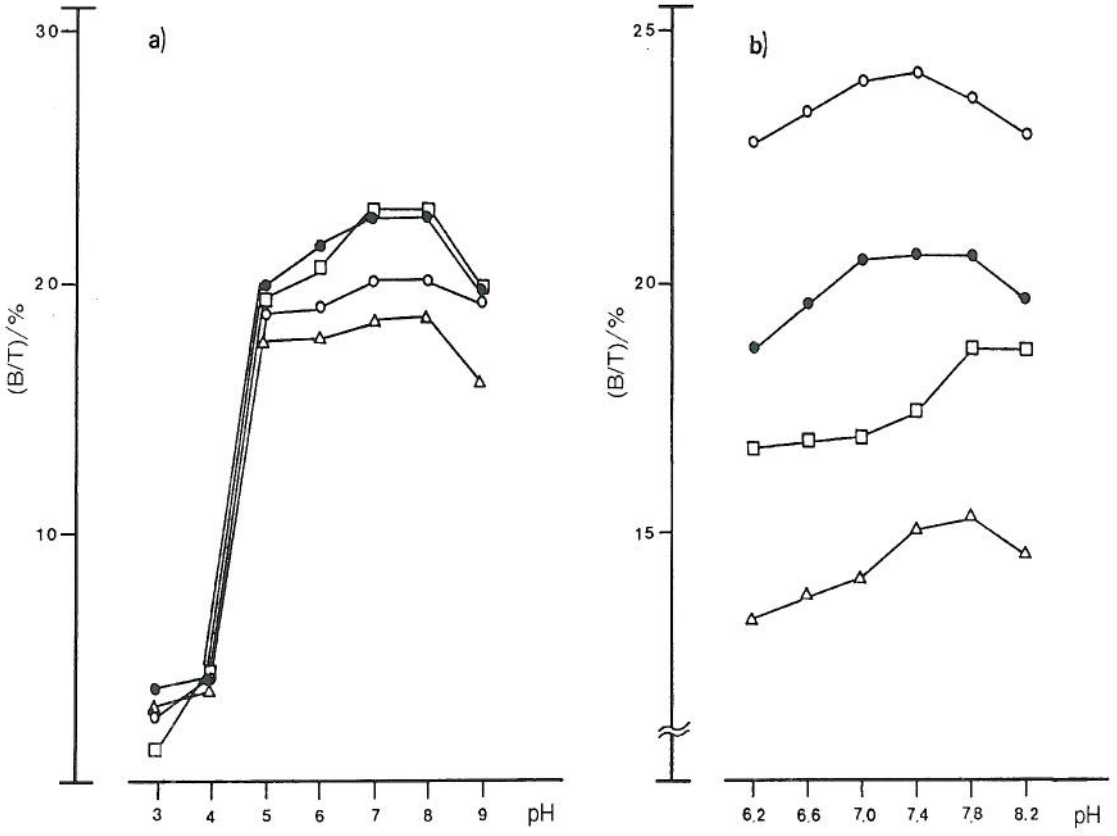

Fig. 3 Influence of pH on ^{125}I-hGH binding to anti-hGH antibodies.
The solutions of antibodies, which in standard assay bound 30% of ^{125}I-hGH, as well as a solution of ^{125}I-hGH, were prepared in 0.015 M Hepes/NaOH buffer (or 0.015 M Tris/HCl buffer) of different pH values, containing 330 mg/L BSA. The reaction mixture consisted of 0.1 mL ^{125}I-hGH, 0.1 mL antibody, and 0.1 mL of buffer of particular pH value. Each reaction mixture was incubated for 20 h at 25 °C, and stopped by addition of ice-cold 0.7 mL 0.2 M Tris/HCl buffer, pH = 7.4, which brought the pH to pH = 7.4. After this neutralisation step, 0.4 mL of 100 g/L Pansorbin cells, resuspended in 0.1 M Tris/HCl buffer, pH = 7.4 were added and the reaction mixtures were additionally incubated for 30 min at 4 °C. Thereafter, the tubes were centrifuged (2 000 × g, 15 min), supernatants were decanted and the pellets counted in the gamma counter.
The results are presented as (B/T)/% *versus* pH (The amount of ^{125}I-hGH present in each tube, i.e. T = 533 Bq). In Fig. 3a the results of testing broad pH values in Hepes buffer, are presented, and in Fig. 3b the results of testing a narrow pH range in Tris buffer, are shown. Each point is the mean of duplicate determinations.Unpurified antibodies ●——●, antibody I preparation O——O, antibody II preparation □—□, antibody III preparation Δ—Δ.

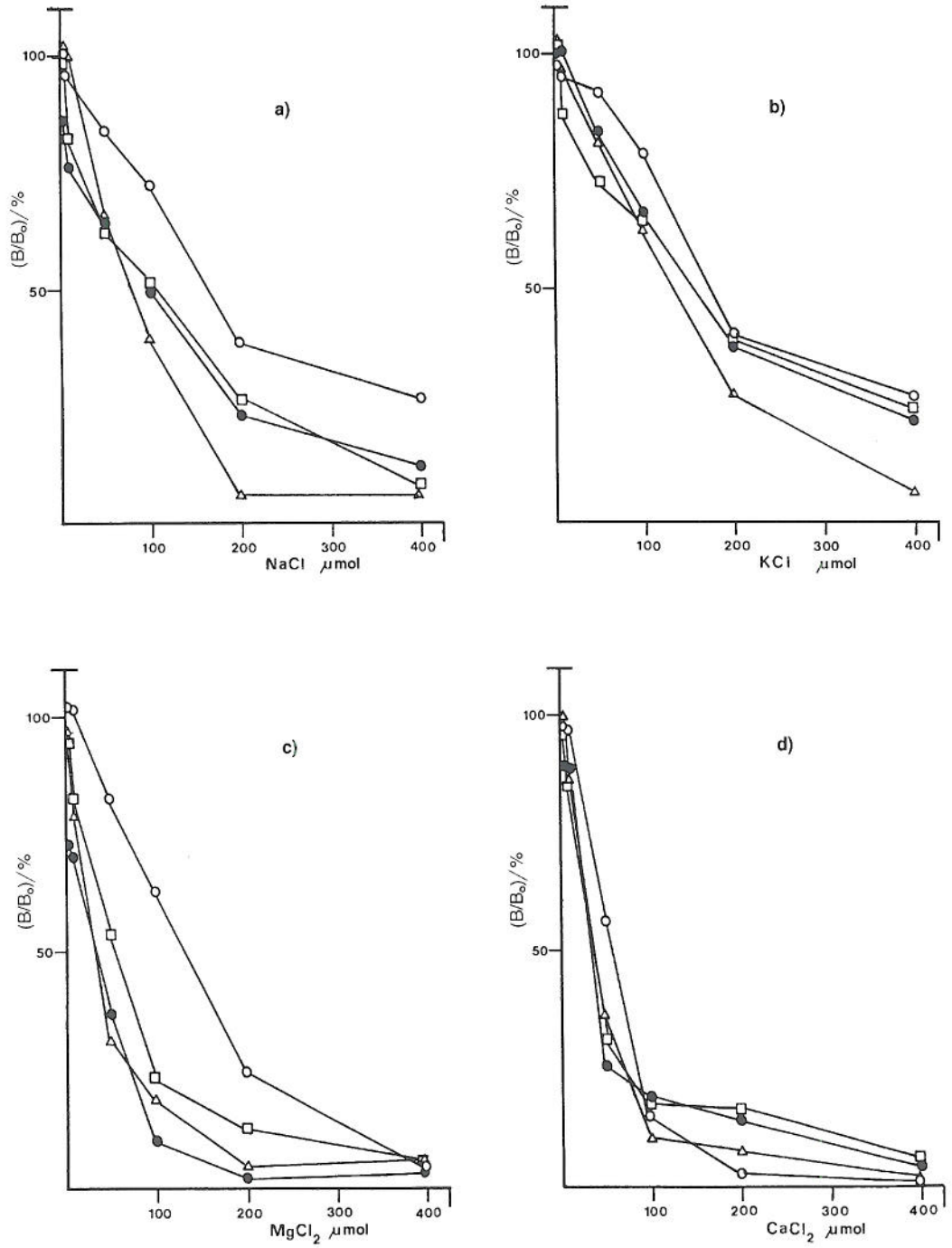

Fig. 4 Influence of salts on ^{125}I-hGH binding to anti-hGH antibodies

The solutions of antibodies which in standard assay bound 30% of added ^{125}I-hGH, and the ^{125}I-hGH solution, were prepared in 0.015 M Hepes/NaOH buffer, pH = 7.8, containing 330 mg/L BSA. Various salts, in the concentration range 0.01–4.0 M were prepared in the same buffer. The reaction mixture consisted of 0.1 mL of ^{125}I-hGH, 0.1 mL of particular antibody preparation and 0.1 mL of buffer or particular salt concentration. The tubes with specific mixtures were incubated for 20 h at 25 °C. The reaction was stopped by addition of 0.7 mL of ice-cold Hepes/NaOH, pH = 7.4 buffer, which contained 0.5 M EDTA. Thereafter, tubes were further processed as described in Fig. 3.

The results are presented as (B/B$_o$)/% *versus* amount of particular salt (μmol) present in the reaction mixture. B$_o$ is the amount of radioactivity when no salt was added into the reaction mixture; for unpurified antibodies B$_o$ was 170 Bq, for antibody I preparation B$_o$ = 185 Bq, for antibody II preparation B$_o$ = 183 Bq, and for antibody III preparation B$_o$ = 186 Bq. The amount of ^{125}I-hGH present in each tube was T = 550 Bq. In Fig. 4a the influence of NaCl, in Fig. 4b the influence of KCl, in Fig. 4c the influence of $MgCl_2$ and in Fig. 4d the influence of $CaCl_2$ on ^{125}I-hGH binding to different antibody preparations are presented. Each point is the mean of duplicate determinations. Unpurified antibodies ●——● , antibody I preparation O——O , antibody II preparation □——□, antibody III preparation Δ—Λ.

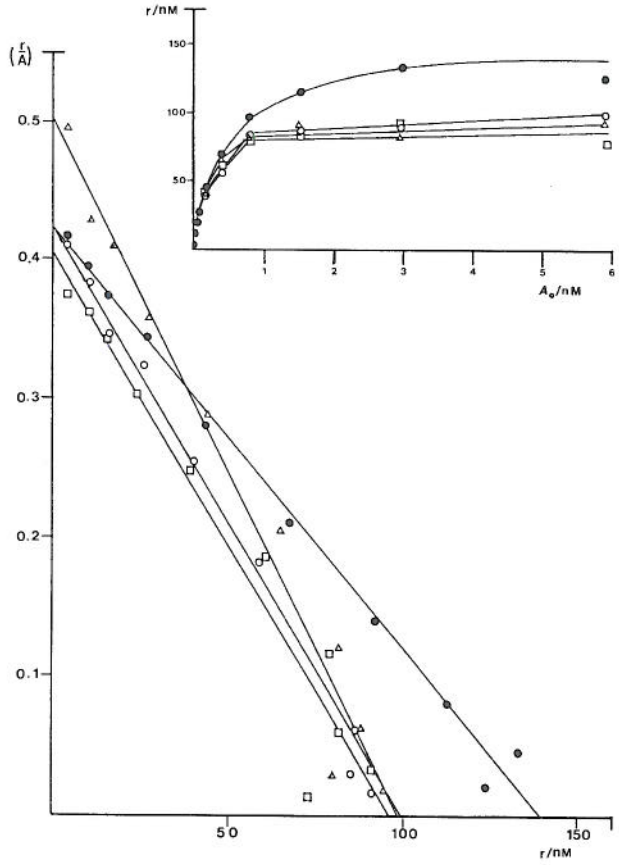

Fig. 5 Scatchard plots for different anti-hGH preparations.
Aliquots of 1,000 Bq of ^{125}I-hGH were mixed with different amounts of unlabeled hGH (concentration range 0.25–128 μg/L) and these different concentrations of hGH were incubated with prepared anti-hGH antibodies. The concentrations of antibodies in particular reaction mixtures which bound 30% of ^{125}I-hGH (see also Figs. 3 and 4) were: unpurified antibodies 22.23 μg/L, antibody I preparation 1.07 μg/L, antibody II preparation 1.14 μg/L, antibody III preparation 1.86 μg/L. The binding reaction was performed in 0.3 mL in 0.015 Tris/HCl buffer pH = 7.8, containing 330 mg/L BSA for 20 h at 25 °C, and it was stopped by addition of goat anti-rabbit antibodies and PEG, as described in Methods. Each point is the mean of triplicate determinations. Unpurified antibodies ●——●, antibody I preparation O——O, antibody II preparation □——□, antibody III preparation Δ—Δ.

formed in 0.015 M Hepes buffer, pH = 7.8, containing 330 mg/L BSA. When the binding curves, shown in the intercept of Fig. 5, were corrected for the amount of antibody present in the reaction mixture and analysed as Scatchard plots (*20*), it could be seen that antibody III preparation possessed the highest binding constant (5.15×10^9 L/mol), while the lowest binding constant was determined for unpurified antibody (2.93×10^9 L/mol). The binding constant for antibody I (4.29×10^9 L/mol) was very similar to the binding constant of antibody II (4.22×10^9 L/mol). The number of binding sites (n), expressed in nM, determined for each antibody preparation were: n = 138 for unpurifed antibody, n = 99 for antibody I preparation, n = 96 for antibody II preparation and n = 98 for antibody III preparation.

Specificity of isolated anti-hGH antibodies

The isolated antibodies were tested for their specificity to polypeptide hormones. As shown in Fig. 6, all antibodies showed high specificity to hGH, and, as already found earlier (*10,11*), reliable hGH calibration curves and precise hGH determinations in sera samples could be obtained. As it can be seen (Fig. 6), antibodies reacted with non-physiological concentrations of hPRL, although much less specifically than to hGH. It is interesting to mention that unpurified antibodies and antibodies II and III reacted more strongly to hPRL than fraction of antibody I. Also, all tested antibodies reacted to high concentrations of hPL. In this case, purified antibodies I and unpurified antibodies reacted very strongly to hPL, while this was not observed to such extent for antibodies II and III. None of the tested antibodies reacted with hTSH, hLH, hFSH or hCG.

From these results it was concluded that purified antibodies probably react with different epitopes present on the hGH molecule, since pronounced differences were observed in reactivity of different antibody isolates to hGH related proteins, i.e. hPRL and hPL. Therefore, further experiments with purified antibodies immobilised onto the Reacti-gel 6× were performed. As it can be seen

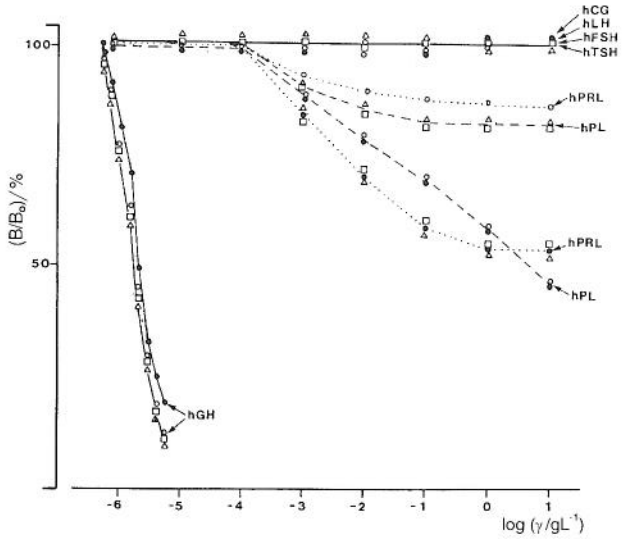

Fig. 6. Competition of ^{125}I-hGH and unlabeled hormones for binding to anti-hGH antibody preparations.
Antibodies, ^{125}I-hGH and different concentrations of unlabeled hormones were prepared in 0.015 M Tris/HCl buffer, pH = 7.8, containing 330 mg/L BSA. The reaction in 0.3 mL proceeded for 20 h at 25 °C, and it was stopped by addition of goat anti-rabbit antibodies and PEG. hGH competition with ^{125}I-hGH to different antibody preparations is shown only in the range of 0.25–32 μg/L. All points are the mean of duplicate determinations. The total amount of ^{125}I-hGH in each tube was 500 Bq, and the corresponding B_o values for each antibody preparation were: unpurified antibodies B_o = 170 Bq, antibody I preparation B_o = 168 Bq, antibody II preparation B_o = 165 Bq, antibody III preparation B_o = 162 Bq. The ^{125}I-hGH bindability to particular antibody is presented as: unpurified antibodies ●——●, antibody I preparation O——O, antibody II preparation □——□, antibody III preparation Δ—Δ.

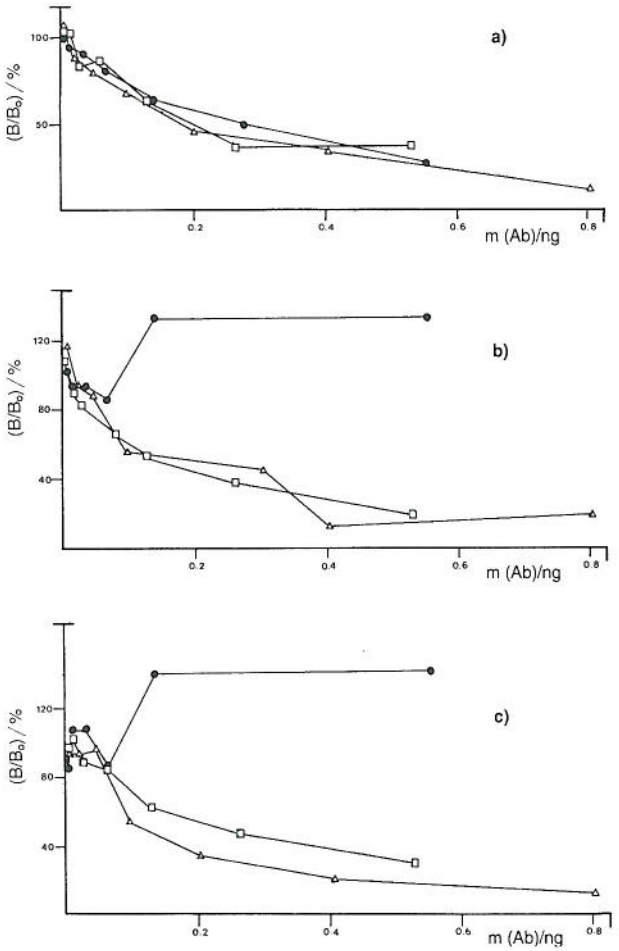

Fig. 7. Inhibition of ^{125}I-hGH to immobilised anti-hGH antibodies by soluble anti-hGH preparations.
22.4 μg of antibody I preparation, 17.12 μg of antibody II preparation and 11.7 μg of antibody III preparation were bound to Reactigel 6× as described in Methods. Appropriate dilution of these immobilised antibodies which bound 30% of added ^{125}I-hGH into the reaction mixture were used in further experiments. The total amount of ^{125}I-hGH in each reaction mixture was 550 Bq, and the corresponding B_o values were: antibody I B_o = 160 Bq, antibody II B_o = 163 Bq, antibody III B_o = 170 Bq. Each point is the mean of triplicate determinations. Inhibition of ^{125}I-hGH to immobilised antibody I (Fig. 7a), immobilised antibody II (Fig. 7b) and to immobilised antibody III (Fig. 7c) by addition of soluble antibodies: antibody I preparation ●—●, antibody II preparation □—□, antibody III preparation Δ—Δ.

from Fig. 7a, in which competition of binding of ^{125}I-hGH to soluble antibodies (antibodies I, II or III) or to the immobilised antibody I was studied, the addition of any soluble antibody inhibited the ^{125}I-hGH binding to the immobilised antibody I. In contrast, marked differences were observed when ^{125}I-hGH competed for binding to soluble antibody preparations (antibodies I, II and III) and to the immobilised antibody II (Fig. 7b) or immobilised antibody III (Fig. 7c). In neither of these studies, the addition of soluble antibody I inhibited the ^{125}I-hGH binding to either of the immobilised antibodies, while the inhibition of binding was observed when either soluble antibody II or antibody III were present in the reaction mixture(s).

The experiments presented in Fig. 8. confirmed the existence of the differences in the hGH epitope that purified antibodies react to. In this study, each immobilised antibody was first incubated for 20 h with different concentrations of unlabeled hGH, and thereafter the same amount of ^{125}I-anti-hGH antibody (Pharmacia hGH RIA 100 kit) was added into each reaction mixture, and the incubation proceeded for three additional hours. After the last incubation, the reaction in each tube was stopped by addition of 1.0 mL of decanting solution, tubes were centrifuged, supernatants decanted, and the radioactivity remaining in the pellets was counted in a gamma counter. As it can be seen from Fig. 8, unlabeled hGH which was bound onto immobilised antibody I, did not bind any ^{125}I-anti-hGH antibodies, while hGH bound to immobilised antibodies II and III bound also the labelled hGH-antibodies. The highest amount of ^{125}I-anti-hGH antibodies bound onto saturated immobilised antibodies was observed for hGH-saturated immobilised antibody II. It can be concluded that the hGH epitopes which reacted with antibody II differed the most from the hGH epitopes which reacted with antibody I. Antibody I recognised probably the same hGH epitopes as the ^{125}I-anti-hGH, and therefore practically no binding was observed.

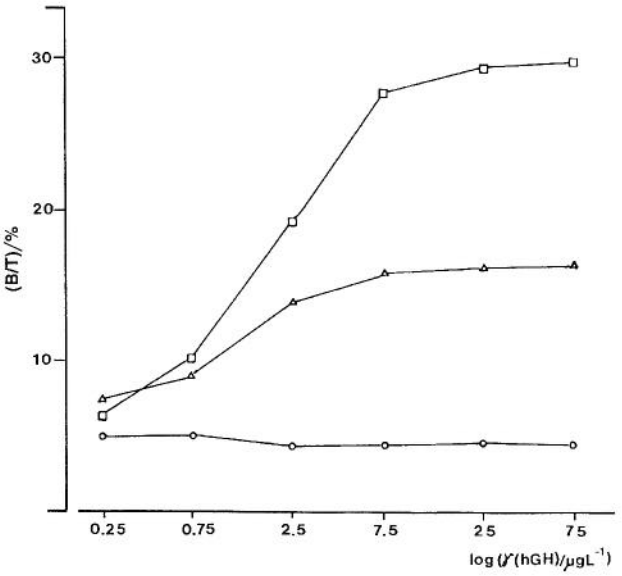

Fig. 8 ^{125}I-anti-hGH antibody binding to immobilised anti-hGH antibodies saturated with different amounts of hGH.
Immobilised anti-hGH preparations (the amount which bound 30% of ^{125}I-hGH, as described in Fig. 7) were incubated with indicated amounts of hGH for 20 h at 25 °C in a volume of 0.3 mL. All reactants were in Tris/HCl buffer, pH = 7.8, containing 330 mg/L BSA. Thereafter, 0.05 mL of ^{125}I-anti-hGH antibodies (Pharmacia hGH RIA 100 kit) were added into the reaction mixture and incubation proceeded for three additional hours. The reaction was stopped by addition of »Pharmacia decanting solution«, and the pellets were counted in a gamma counter. The amount of ^{125}I-anti-hGH antibodies added into each tube was 1226 Bq. The results are presented as (B/T)/% values *versus* concentration of hGH in the reaction mixture. Each point is the mean of duplicate determinations. Immobilised antibody I O—O, immobilised antibody II □—□, immobilised antibody III Δ—Δ.

From all these experiments it can be concluded that by simple purification step on an ion-exchanger, antibodies with different pI possessed different physico-chemical properties, as well as specificity towards different hGH epitopes. Although no information was obtained for which particular amino acid sequence these antibodies are specific (for such an information peptide mapping studies should be performed (*21–24*)), it is obvious that our isolates of anti-hGH antibodies reacted differently with various hGH epitopes. These purified antibodies could be used in the future for preparation of specific immuno-sorbents for isolation of hGH iso-forms, or to develop specific IRMA methods.

In conclusion, it is important to purify and characterise polyclonal antibodies from hyper-immune sera. In this way one can obtain highly specific antibodies for a very specific epitope, which is characteristic for a particular antigen. Certainly, much better defined antibodies, and better immunochemical techniques could be developed by using purified monoclonal antibodies.

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Čišćenje i karakterizacija poliklonskih anti-hGH protutijela

Sažetak

Imunizacijom kunića pripravcima humanog hormona rasta (hGH) dobiven je specifičan antiserum. Antiserum je prvo pročišćavan taloženjem s amonijevim sulfatom (»nepročišćena protutijela«), a zatim kromatografijom na kationskom izmjenjivaču (DEAE-celulozi). Frakcija protutijela koja je s kolone eluirana s ekvilibracijskim puferom, 10 mM fosfatni pufer, pH = 8,0, nazvana je »protutijela I«, a frakcije eluirane s kolone u rasponu koncentracija fosfatnog pufera 15–40 mM, odnosno 45–60 mM nazvane su »protutijela II«, odnosno »protutijela III«. Analizom pripravaka gel-filtracijom, te mjerenjem imunološke aktivnosti eluata, utvrđeno je da su M_r protutijela 165–158000. Da izolirana protutijela pripadaju IgG klasi, potvrđeno je testovima taloženja kompleksa ^{125}I-hGH-protutijelo s pomoću bakterija ***Staphylococcus aureus****. Ispitivan je utjecaj pH i različitih soli u reakcijama vezivanja ^{125}I-h-GH na različita protutijela, a svim pripravcima protutijela određene su i konstante vezivanja. Imunološka specifičnost protutijela ispitana je u reakcijama s različitim polipeptidnim hormonima. Utvrđeno je da različiti pripravci protutijela reagiraju različitim intenzitetom s hormonima srodnim humanom hormonu rasta, tj. s humanim prolaktinom (hPRL) i humanim placentarnim laktogenom (hPL). Iscrpnim ispitivanjima potvrđeno je da su epitopi hGH koje veže protutijelo I različiti od epitopa koje vežu protutijela II i III. Zaključeno je da se pročišćavanjem antiseruma s pomoću ionskog izmjenjivača mogu izolirati protutijela koja se razlikuju po svojim imunološkim značajkama.*