

Chapter 8

A specific immunoprecipitation
method for isolating isoforms of
insulin-like growth factor binding
protein-3 from serum

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Abstract

Background

This report describes an in-house developed immunoprecipitation method to isolate insulin-like growth factor binding protein-3 (IGFBP-3) and its isoforms from serum. The method was compared to other existing immunoprecipitation methods. The study of IGFBP-3 isoforms is relevant for further studies on congenital defects in glycosylation (CDG), galactosemia, and alcoholic liver cirrhosis.

Methods

Monoclonal and/or polyclonal anti-human IGFBP-3 antibodies were covalently immobilised on protein-A Sepharose beads using dimethyl pimelimidate as cross-linker. By incubation with these immobilised antibodies, intact IGFBP-3 and fragments of IGFBP-3 were isolated from serum. Enzyme-linked immunosorbent assay (ELISA) and one-dimensional gel electrophoresis (1-DE) experiments were performed to define the optimal immunoprecipitation method. Isolated proteins were separated by 1-DE and two-dimensional gel electrophoresis (2-DE) and visualised by Western blotting.

Results

ELISA and 1-DE results illustrated that an optimal isolation was performed using PBS for the incubation with serum. Laemmli sample buffer, containing 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride and sodium dodecyl sulphate, or urea/CHAPS was optimal for the elution. Clinical validation was performed using CDG-Ia serum samples. The 2-DE experiments showed characteristic isoform patterns for CDG-Ia.

Conclusions

The optimized in-house developed immunoprecipitation method resulted in specific detection of IGFBP-3 isoforms and is suitable for further studies on glycosylation defects.

Introduction

Immunoprecipitation methods are very useful for the purification and identification of low-abundance proteins from complex matrices, like serum or plasma. In most studies, the immunoprecipitation technique was developed for extracting and concentrating proteins from cell lysates^{1,2} and not from serum. An immunoprecipitation method for extracting and concentrating low-abundance proteins from cell lysates using dimethyl pimelimidate (DMP) cross-linked antibodies to protein-A Sepharose beads was described by Schneider et al.³. Sisson et al.⁴ modified the procedure of Schneider et al. in two ways to improve the cross-linking efficiency. First, the number of coupling reactions was increased and secondly the cross-linker solution was prepared immediately prior to matrix construction. Michielsen et al.⁵ used the previously described method of Sisson et al. for extracting and concentrating low-abundance proteins from human serum. The cross-linking method using DMP as a cross-linker is rarely used.

Conventional methods of immobilizing antibodies on solid matrices usually employ cyanogen bromide-activated Sepharose although other matrices have also been used, like brome acetylated cellulose or other activated matrices⁶. These conventional methods do not use cross-linkers and tend to be low-efficiency methods due to the fact that there are numerous reactive sites on an antibody for covalent binding to the matrix. Thus the orientation of the antibody relative to the matrix is often such that the combining site participates in the cross-linking or is sterically blocked to its orientation⁷. Protein-A Sepharose beads overcome these difficulties, because protein-A from *Staphylococcus aureus* binds specifically to the fragment crystallizable region (Fc region) of an antibody⁴. The Fc region is the constant region of an antibody composed of two heavy chains that each contribute three or four constant domains, depending on the class of the antibody. This results in an optimal surface orientation of the antibodies, leaving the antigen-binding sites free from antigen capture. Antibodies are covalently cross-linked to solid surfaces or micro particles to prevent loss of antigen binding capacity due to unwanted desorption, caused by weak non-covalent binding, competitive inhibition with antibodies present in samples, or harsh elution conditions. A typical cross-link protocol using imido-esters starts with the non-covalent capture of antibodies by immobilised protein-A or -G. The choice of either protein-A or protein-G depends on the subclass of the antibody and should be determined individually for each antibody. In this study, protein-A Sepharose beads were used. After antibody capture, DMP was added at a pH between 8 and 9 for covalent cross-linking of the antibody to protein-A Sepharose. Because DMP was susceptible to hydrolysis even at basic pH, it was added in two or more portions with regular intervals to ensure complete reaction, which was also described by Sisson et al.⁴. Remaining free reactive groups are quenched with ethanolamine and all non-bound antibodies were eluted.

In this study a specific immunoprecipitation method developed by our group⁵ was optimized. Western blotting and enzyme-linked immunosorbent assay (ELISA) experiments were performed to define the optimal condition to purify and isolate insulin-like growth factor binding protein-3 (IGFBP-3) from serum. In blood insulin-like growth factors (IGFs) are bound by IGF-binding proteins, which serve as carriers of circulating IGFs and modulators of IGF action⁸. IGFBP-3, a ~ 43/45 kDa glycoprotein, is most abundant in serum and sequesters insulin-like growth factor I and II into a ternary 150 kDa complex with the 85 kDa acid-labile subunit⁹. Because IGFBP-3 levels are in the range of 1-9 mg/l (dependent on age and assay), a specific immunoprecipitation method is needed to isolate and purify IGFBP-3. The aim of the present study was to optimize our immunoprecipitation assay for the isolation and purification of IGFBP-3 from serum to study the isoforms of IGFBP-3. The study of IGFBP-3 isoforms in different disease entities such as congenital defects in glycosylation, galactosemia⁸, and alcoholic liver cirrhosis¹⁰ is important to unravel pathogenetic mechanisms. The method described in this study was clinically validated by comparing serum samples of CDG-Ia children with serum samples of healthy children. The CDG-Ia samples were used as positive controls to clinically validate our method because CDG-Ia patients have a genetic defect in N-glycan synthesis. This causes hypoglycosylation of serum transferrin¹¹ and many other glycoproteins. Serum IGFBP-3 contains three N-glycosylation sites¹², and should thus be hypoglycosylated in CDG-Ia.

Materials and methods

Patients and samples

The serum samples of CDG-Ia children were collected at the University Hospital Gasthuisberg, Leuven, Belgium. The CDG-Ia children were 11 years (female) and 8 years (male) old, and showed a typical clinical presentation. Two serum samples of healthy children were collected at the University Hospital Maastricht and were matched by sex and age.

Antibodies covalently bound to beads using DMP

One hundred mg protein-A Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, USA) beads were blocked with 10 ml of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA) for 10 min at room temperature. This resulted in 400 µl of diluted protein-A Sepharose beads which can be used for antibody coupling. Seventy µg mouse monoclonal anti-human IGFBP-3 antibody (R&D Systems, Inc. Minneapolis, MN, USA) and 70 µg rabbit polyclonal anti-human IGFBP-3 antibody (RDI Division of Fitzgerald Industries, Concord, MA, USA) in 10 ml of PBS containing 0.1% bovine

serum albumin were mixed with the 400 μ l of diluted Sepharose beads and rotated for one hour at room temperature. After binding of the antibodies, the beads were washed twice with excessive PBS. Antibodies were cross-linked to protein-A Sepharose by the addition of 200 mmol/l triethanolamine in PBS to which 20 mmol/l DMP (Sigma-Aldrich) was added directly before use (pH 8-9). After rotation for 30 min at room temperature, the beads were washed with 200 mmol/l triethanolamine in PBS at room temperature. The cross-linking and washing steps were repeated twice to improve cross-linking efficiency. The remaining reactive amino groups were quenched by addition of 50 mmol/l ethanolamine in PBS for one hour at room temperature. Antibodies not cross-linked to beads were removed by incubation twice (20 min each time) with 1.0 mol/l glycine-HCl (pH 3.0) at 56°C. The antibodies cross-linked to beads were stored at 4°C in PBS containing 0.2 ml/l Tween-20 (PBST) and 0.2 g/l sodium azide until use. Batches, containing antibodies cross-linked to beads, were divided in tubes. Each tube contained 200 μ l of the stored batch, which corresponded with 2.5 mg (dry weight) of antibody-coupled Sepharose beads. This in-house developed immunoprecipitation was compared to other immunoprecipitation methods where no DMP was used as cross-linker.

Antibodies non-covalently bound to beads without using DMP

Antibodies were non-covalently bound to beads without using DMP as cross-linker. For this method, batches were prepared as described in detail above with the exception that no DMP was used.

Antibodies not bound to beads

Antibody-serum complexes were generated by first adding the antibodies to the serum. Subsequently protein-A Sepharose beads were added to the antibody-serum mixtures without using DMP as cross-linker.

Incubation and elution conditions for immunoprecipitation

To investigate the most efficient conditions, the immunoprecipitation of IGFBP-3 was performed with different amounts of diluted antibody-coupled Sepharose beads (200-800 μ l). To each tube, we added 75–500 μ l of serum, with 200 μ l of PBST and 100 μ l of PBS or 6 M urea. Beads were rotated for 90 min and then washed twice with PBST. Three different elution solutions were used; A) glycine solution: 1 M glycine-HCl (pH 3), B) Laemmli solution: 62.5 mmol/l 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (TRIS-HCl), 2% sodium dodecyl sulphate (SDS), 25% glycerol, 0.01% bromophenol blue (Bio-Rad Laboratories, Inc., Hercules, CA, USA), C) urea/CHAPS solution: 8 M urea, 2% ([3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate]) (CHAPS). Elution was

performed by heating (56°C) for 20 min in 20-50 µl of glycine, Laemmli, or urea/CHAPS solutions. The samples were centrifuged and the supernatant was kept for analysis.

One- and two-dimensional gel electrophoresis

The samples were prepared for one-dimensional gel electrophoresis (1-DE) by adding 5 or 10 µl of 10% SDS sample buffer (312.5 mmol/l TRIS-HCl, 10% SDS, 33% glycerol, 0.01% bromophenol blue) to 20 or 40 µl of eluate, except for the Laemmli eluates. We applied 20 µl of this mixture to a 4-15% linear gradient TRIS-HCl polyacrylamide precast gel (Bio-Rad). The SeeBlue Plus2 Pre-Stained Standard (Invitrogen Corp., San Diego, CA, USA) was used as the molecular mass marker. Electrophoresis was conducted for 15 min at 100 V for stacking followed by 40 min at 150 V. For the two-dimensional gel electrophoresis (2-DE), 20 µl of eluate were loaded for the first dimension. Isoelectric focusing was performed on an IPGphor electrophoresis unit (GE Healthcare Bio-Sciences) at 20°C. ReadyStrip IPG strips (pH 3-10 not linear, 11 cm long) (Bio-Rad) were rehydrated overnight in 180 µl of 8 M urea, 2% CHAPS, 0.5% Bio-lite 3-10 (Bio-Rad) at 30 V. Isoelectric focusing was performed using the following program: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, increased to 8000 V with a one hour linear gradient, and a final step at 8000 V for 2.5 h. After focusing, IPG strips were equilibrated for 15 min in 50 mM TRIS-HCl, pH 6.8, 6 M urea 30% glycerol, 2% SDS and were placed onto a slab gel and sealed with a 0.5% agarose solution in Laemmli buffer with a trace of bromophenol blue. The second dimension run was carried out on 12.5% SDS polyacrylamide gels (SDS-PAGE). Electrophoresis was conducted at a constant voltage of 200 V for one hour in an 11 cm Criterion cell (Bio-Rad).

Western Blotting

The gel was blotted on a nitrocellulose membrane (Bio-Rad) at 4°C at 100 V for 60 min. The membrane was blocked for 60 min in PBS containing 33 g/l non-fat dry milk. The primary mouse monoclonal anti-human IGFBP-3 antibody was added at a 1:1250 dilution in wash buffer. The membrane was incubated overnight at 4°C. The membrane was washed three times in wash buffer. The secondary antibody (peroxidase-labeled goat-anti-mouse, Dako, Glostrup Denmark) was then added at a 1:5000 dilution in wash buffer and incubated for 60 min at 4°C. To reduce non-specific binding, 250 µl of human serum were added to the wash buffer. The additional serum in the wash buffer resulted in less background signal. The membrane was washed four times with wash buffer and finally once with PBS. Membranes were developed with enhanced chemiluminescence buffer and captured on Kodak X-Omat Blue film (both from Perkin-Elmer Life Sciences, Waltham, MA, USA). Quantitative analysis of the negative images of the 1-DE Western blots was performed using the imaging and analyzing software program

Quantity One (Bio-Rad). The relative quantity is the trace quantity of a band expressed as a percentage of the total quantity of the bands in the lanes.

Protease inhibitors

Protease inhibitor cocktail (mixture of 4-(2-aminoethyl)benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and sodium ethylenediamine tetraacetic acid (EDTA) with broad specificity for the inhibition of serine, cysteine, aspartic, and metalloproteases (Sigma-Aldrich) was added to control serum of a healthy person directly after venipuncture to ensure that the detected fragments of IGFBP-3 were no in-vitro artefacts. This was also verified by applying an immunoprecipitated and non-immunoprecipitated human recombinant IGFBP-3 standard from Sigma-Aldrich (~41 kDa) on 4-15% linear gradient TRIS-HCl polyacrylamide precast gel (Bio-Rad).

ELISA

The IGFBP-3 ELISA kit (Diagnostic Systems Laboratories Inc., Webster, TX, USA) was performed according the recommendations of the manufacturer. The absorbance measured was directly proportional to the concentration of IGFBP-3 present. A set of IGFBP-3 standards was used, in adapted matrices when relevant, to plot a standard curve of absorbance versus IGFBP-3 concentration from which the IGFBP-3 concentrations in the samples can be calculated. Because the elution was performed in three different solutions; glycine, Laemmli, and urea/CHAPS solutions, the standards and controls were diluted two times in 2 M glycine-HCl (pH 3), concentrated Laemmli buffer (125 mmol/l TRIS-HCl, 4% SDS, 50% glycerol, 0.01% bromophenol blue), and 10 M urea/ 2% CHAPS for plotting curves in adapted matrices. The initial IGFBP-3 concentrations of the serum samples were 5, 7, and 6 mg/l for the incubation and elution solution experiment, the serum experiment, and the beads experiment, respectively.

Results

Generation of antibody-serum complexes

To check the influence of cross-linking with DMP three different types of immunoprecipitation were compared. We compared a batch of mouse monoclonal anti-human IGFBP-3 antibodies covalently bound to beads using DMP as cross-linker with a separate batch of antibodies non-covalently bound to beads without using DMP as cross-linker. This second batch of antibodies non-covalently bound to beads resulted in very low density signals of the intact IGFBP-3. The fragments were not visible on the negative image of the 1-DE Western blot, probably because of the low densities of these fragment bands. In another method, antibody-serum

complexes were generated by first adding the antibodies to the serum. Subsequently protein-A Sepharose beads were added to the antibody-serum mixtures without using DMP as cross-linker. With this last method, the intact IGFBP-3 including the fragments were detected, but the densities of these bands were low. The method with cross-linking resulted in the most clear and highest density bands (intact and fragments). These results demonstrate that the method described in this study, with DMP as cross-linker is optimal for purifying and isolating IGFBP-3 from serum. The results are illustrated in Figure 8.1.



Figure 8.1 Influence of DMP as cross-linker.

Three different types of immunoprecipitation were compared using mouse monoclonal anti-human IGFBP-3 antibodies. Type I: the antibodies were covalently bound to beads using DMP as cross-linker (lane 1). Type II: the antibodies were non-covalently bound to beads without using DMP as cross-linker (lane 2). Type III: antibody-serum complexes were generated by first adding the antibodies to the serum. Subsequently the protein-A Sepharose beads were added to the antibody-serum mixtures without using DMP as cross-linker (lane 3).

Protease inhibitors

Protease inhibitor cocktail was added to serum to demonstrate that the IGFBP-3 fragments were formed *in vivo* and not *in vitro*. With protease inhibitors, intact IGFBP-3 and fragments were still seen, although with less density due to dilution. Even with an excess of protease inhibitors, fragments of IGFBP-3 remain unchanged. To confirm that the fragments were not the result of the immunoprecipitation itself, the IGFBP-3 standard from Sigma-Aldrich (~41 kDa) was immunoprecipitated. The 41 kDa IGFBP-3 standard from Sigma-Aldrich did not show any additional fragmentation after immunoprecipitation.

ELISA

To investigate the optimal incubation and elution conditions, 200 μ l of diluted beads and 250 μ l of serum were used. These amounts of beads and serum were used by Michielsen et al.⁵ to isolate cardiac troponin T (cTnT) from serum. Because cTnT is also a very low-abundance protein (reference value: 0.01–0.10 μ g/l), the same conditions were used in the first instance. In previous experiments was also found that 90 min incubation resulted in the most optimal elution (data not

shown). To determine the optimal conditions to isolate IGFBP-3 from serum with immunoprecipitation, different incubation solutions (PBS and 6 M urea, both in combination with a PBST solution), and elution solutions (glycine, Laemmli, and urea/CHAPS solutions) were compared. Subsequently, different amounts of serum (75-500 μ l) and protein-A Sepharose beads (200-800 μ l) were compared.

Incubation and elution solution experiment

In this study batches with monoclonal and polyclonal antibodies were used for the isolation. Immunoprecipitation of IGFBP-3 from serum in the presence of 100 μ l of 6 M urea and 200 μ l of PBST was compared with immunoprecipitation in the presence of 100 μ l of PBS and 200 μ l of PBST. Both incubation conditions were combined with glycine, Laemmli, and urea/CHAPS elution solutions. All three elution solutions showed a higher amount of IGFBP-3 using PBS instead of 6 M urea to dilute the serum for immunoprecipitation using the monoclonal batch, except for the elution with Laemmli solution using the polyclonal batch (Figure 8.2). Elution with Laemmli or urea/CHAPS solutions resulted in a clearly higher absolute amounts of IGFBP-3 compared to elution with glycine solution. This was in agreement with the negative images of the 1-DE Western blots. These negative images also illustrated that the elution with Laemmli and urea/CHAPS solutions resulted in a more intense signal compared to elution with glycine solution (data not shown).

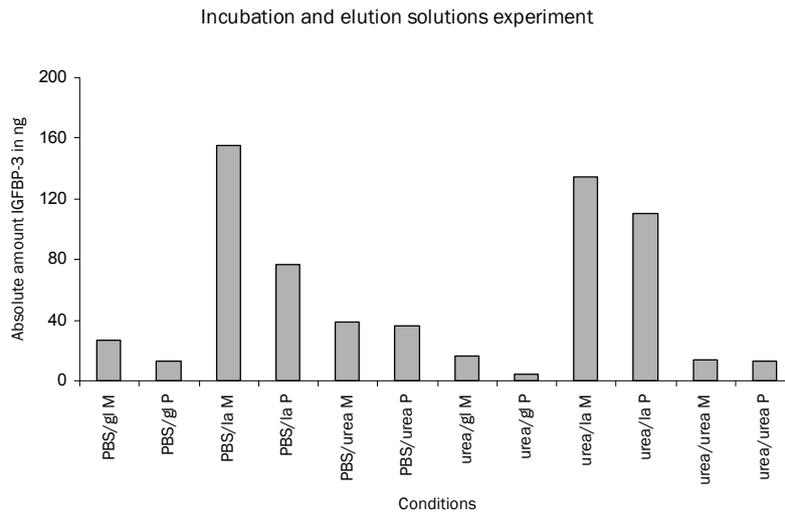


Figure 8.2 The absolute amount of IGFBP-3 (in ng) after immunoprecipitation of IGFBP-3 from serum in the presence of PBS or 6 M urea (both in combination with a PBST solution). Both incubation conditions were combined with glycine (gl), Laemmli (la) or urea/CHAPS (urea) elution solutions. The monoclonal batch (mouse monoclonal anti-human IGFBP-3 antibody) (M) and the polyclonal batch (rabbit polyclonal anti-human IGFBP-3 antibody) (P) were used. Two hundred μ l of protein-A Sepharose beads and 250 μ l of serum were used for the immunoprecipitation.

Serum experiment

Secondly, different amounts of serum (75 / 125 / 250 / 500 μ l) were added to 100 μ l of PBS and 200 μ l of PBST. A constant incubation time of 90 min and 200 μ l of protein-A Sepharose beads (coupled with monoclonal antibody), which corresponds to 2.5 mg (dry weight) beads, were used for the immunoprecipitation. Figure 8.3 clearly illustrates that the highest absolute amount of IGFBP-3 was eluated using 250 μ l of serum. By using 250 μ l of serum, 8 ng IGFBP-3 could be eluated, while the absolute amounts for the other conditions were lower, namely 3.5, 5, and 6 ng IGFBP-3 when using 75, 125 or 500 μ l of serum, respectively. The elution-binding ratios were also determined by dividing the percentage of elution by that of binding for the four different conditions. The highest elution-binding ratio was also reached when 250 μ l of serum were used.

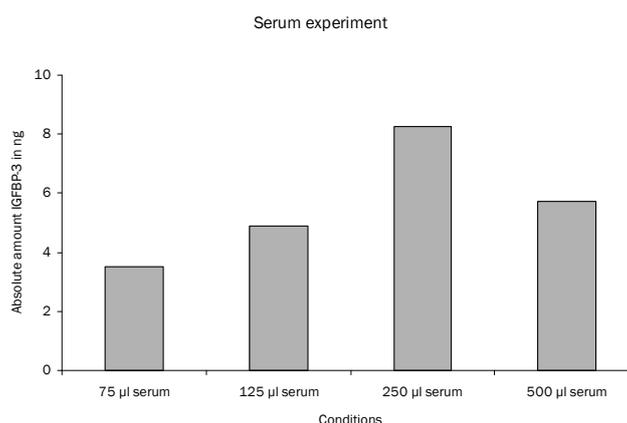


Figure 8.3 The absolute amount of IGFBP-3 (in ng) after elution with urea/CHAPS solution using different serum amounts.

Different amounts of serum: 75 / 125 / 250 / 500 μ l were used with 200 μ l of protein-A Sepharose beads (coupled with mouse monoclonal anti-human IGFBP-3 antibody) for the immunoprecipitation.

Beads experiment

In the third experiment, different amounts of protein-A Sepharose beads, 200 / 300 / 400 / 800 μ l of beads (coupled with monoclonal antibody), which correspond to 2.5 / 3.75 / 5.0 / 10.0 mg (dry weight) beads, and 250 μ l of serum were used for the immunoprecipitation. Figure 8.4 illustrates that the highest amount of IGFBP-3 was eluated when using 800 μ l of protein-A Sepharose beads. By using 800 μ l of protein-A Sepharose beads, 25 ng IGFBP-3 could be eluated, while the absolute amounts for the other conditions were lower, namely 8, 11, and 19 ng IGFBP-3 when using 200, 300 or 400 μ l of protein-A Sepharose beads, respectively. The highest elution-binding ratio was also reached when 800 μ l of protein-A Sepharose beads were used.

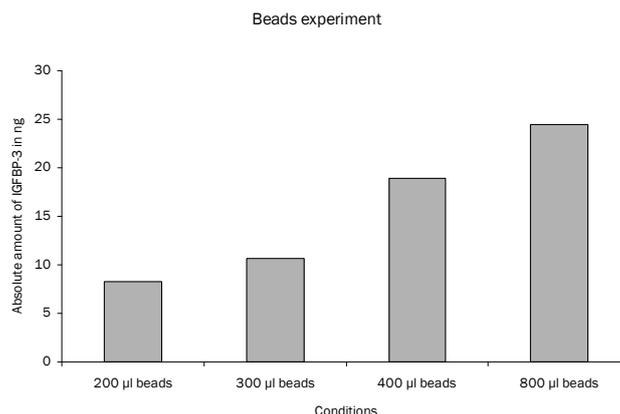


Figure 8.4 The absolute amount of IGFBP-3 (in ng) after elution with urea/CHAPS solution using different protein-A Sepharose beads amounts. Different amounts of protein-A Sepharose beads (coupled with mouse monoclonal anti-human IGFBP-3 antibody): 200 / 300 / 400 / 800 µl , which correspond to 2.5 / 3.75 / 5.0 / 10.0 mg (dry weight) beads were used with 250 µl of serum for the immunoprecipitation.

1-DE

Different isolation batches were used to compare the signals on the negative images of the 1-DE Western blot. In Figure 8.5 is illustrated that isolation with a batch with monoclonal antibodies, polyclonal antibodies, or a mixture of monoclonal and polyclonal antibodies resulted in a different pattern when visualized with mouse monoclonal anti-human IGFBP-3 antibody as primary antibody and peroxidase-labeled goat-anti-mouse as secondary antibody. The third and fifth band clearly show more density using the monoclonal batch compared to the other batches.

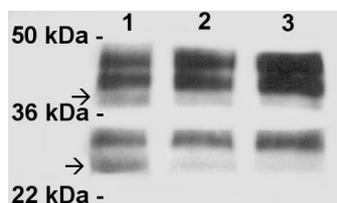


Figure 8.5 Negative image of a 1-DE Western blot from a control serum sample of a healthy person. Different batches were used for the isolation. The monoclonal batch (mouse monoclonal anti-human IGFBP-3 antibody) (lane 1), mixture batch of monoclonal and polyclonal batch (rabbit polyclonal anti-human IGFBP-3 antibody) (lane 2), and polyclonal batch (lane 3). Mouse monoclonal anti-human IGFBP-3 antibody was used as primary antibody and peroxidase-labeled goat-anti-mouse as secondary antibody. The third and fifth band are indicated with a vector.

The eluates from the serum experiment with 75 / 125 / 250 / 500 μ l of serum were also applied on 1-DE using mouse monoclonal anti-human IGFBP-3 antibody as primary antibody and peroxidase-labeled goat-anti-mouse as secondary antibody for the Western blotting (Figure 8.6). The band densities were quantified and are illustrated in Table 8.1. Four of the five bands gave the highest relative quantity using 250 μ l of serum. These results were comparable with the ELISA results.

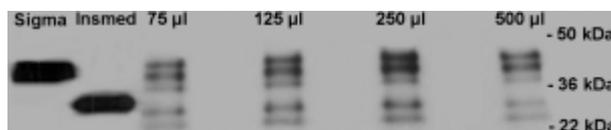


Figure 8.6 Negative image of a 1-DE Western blot from the serum experiment with 75 / 125 / 250 / 500 μ l of serum.

Mouse monoclonal anti-human IGFBP-3 antibody was used as primary antibody and peroxidase-labeled goat-anti-mouse as secondary antibody. In the first lane the IGFBP-3 standard with a molecular weight of 41 kDa from Sigma-Aldrich was added and in the second lane the IGFBP-3 standard with a molecular weight of 28 kDa from Insmed (Insmed, Inc. Richmond, VA, USA) was added as positive control.

Table 8.1 The results of imaging and quantifying analysis of the negative image of the 1-DE Western blot from the serum experiment with 75 / 125 / 250 / 500 μ l of serum.

	75 μ l serum	125 μ l serum	250 μ l serum	500 μ l serum
Band 1	9.7	20.2	23.6	21.5
Band 2	25.2	22.6	26.8	25.8
Band 3	12.3	12.3	10.3	9.6
Band 4	12.8	14.3	16.8	11.5
Band 5	5.9	10.6	11.5	9.3

Quantitative analysis of the negative image of the 1-DE Western blot was performed using the software program Quantity One. The relative quantities of the 5 bands which were present in all different conditions (75 / 125 / 250 / 500 μ l of serum) are illustrated. The relative quantity is the trace quantity of a band expressed as a percentage of the total quantity of the bands in the lanes.

2-DE

To investigate the isoforms of IGFBP-3, 2-DE Western blotting was used. The immunoprecipitation method described in this study was validated by using different serum samples; two serum samples of CDG-Ia children and two control serum samples of healthy children, matched by sex and age. Three areas of interest were identified, at which significant differences were observed. More acidic intact IGFBP-3 isoforms and more acidic fragment isoforms were detected in the serum samples of the healthy children compared to the CDG-Ia children samples. Although there were less acidic intact and fragment isoforms detected in the CDG-Ia samples, there were more basic fragment isoforms detected in the CDG-Ia samples. Thus, a shift of the fragment isoforms from the acidic side to the basic side was found for the CDG-Ia children samples (Figure 8.7).

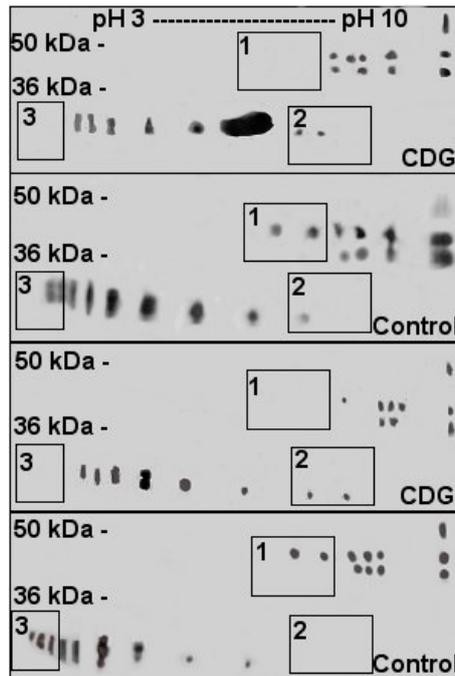


Figure 8.7 Negative images of 2-DE Western blots from two serum samples of CDG-Ia children (CDG) and two control serum samples of healthy children (Control).

The isoforms of intact IGFBP-3 (~ 41-45 kDa) and the isoforms of the fragments (~ 24-30 kDa) of IGFBP-3 are visible on these negative images of Western blots using rabbit polyclonal anti-human IGFBP-3 antibody as primary antibody and peroxidase-labeled goat-anti-rabbit as secondary antibody. ReadyStrip IPG strips (pH 3-10 not linear, 11 cm long) were used. The acidic isoforms are illustrated on the left side and the basic isoforms are illustrated on the right side. The areas with number 1 and 3 contain more spots in healthy control serum of healthy children compared to serum of CDG-Ia children. In contrast to this, the areas with number 2 contain more spots in the serum of CDG-Ia children.

Discussion

This report describes the optimization of an in-house developed immunoprecipitation method which can be used to isolate low-abundance proteins from serum. In our case, we have chosen for the low-abundant protein IGFBP-3, because we are interested in IGFBP-3 isoforms. Information about the IGFBP-3 isoforms can be useful to understand pathogenetic mechanisms in different disease entities such as congenital defects of glycosylation, galactosemia⁸, and alcoholic liver cirrhosis¹⁰. Our immunoprecipitation method was validated by

performing 2-DE Western blotting with serum samples of CDG-Ia children and serum samples of healthy children.

In this study was demonstrated that our immunoprecipitation resulted in the highest density bands on the negative image of the 1-DE Western blot compared to two other methods without using DMP as cross-linker. By using the protease inhibitor cocktail and by immunoprecipitating the human recombinant IGFBP-3 standard from Sigma-Aldrich we could confirm that the fragments originate in vivo and not in vitro.

To check which condition resulted in the optimal elution of IGFBP-3, ELISA and 1-DE experiments were performed. Both the ELISA and the 1-DE experiments illustrated that dilution of serum in PBS was superior to dilution in 6 M urea for the immunoprecipitation and the elutions with Laemmli or urea/CHAPS solutions were superior to elution with glycine solution. Optimal elution was reached when 250 μ l of serum and 800 μ l of protein-A Sepharose beads were used. The highest absolute amounts of IGFBP-3 and the highest elution-binding ratios were reached using 250 μ l of serum and 800 μ l of protein-A Sepharose beads. Imaging and quantifying analysis of the negative image of the 1-DE Western blot also illustrated that the highest relative quantities of both intact protein and fragments were reached using 250 μ l of serum.

More studies have been focused on IGFBP proteins. The aim of the study described by Nedic et al.¹³ was to investigate possible changes in the serum IGF-IGFBP system among patients with alcoholic liver cirrhosis. The serum samples were not immunoprecipitated, but after SDS-PAGE of the serum samples and Western blotting, autoradiography with ¹²⁵I-labelled IGF-I or IGF-II was used. In the study of Nedic et al. was shown that all control subjects exhibited the characteristic IGFBP doublet in the region of 40-45 kDa, while some of them contained a faint band at approximately 34 kDa. Zapf et al.¹⁴ also detected a 42-45 kDa doublet and an extra band at approximately 31 kDa using autoradiography with ¹²⁵I-labelled IGF-I or IGF-II. They described that a glycosylated degradation product of IGFBP-3 presumably lacking the carboxylic terminus can be assigned to the 31 kDa band.

Weber et al.⁸ performed 2-DE and Western ligand blot analysis using autoradiography with ¹²⁵I-labelled IGF-I or IGF-II to characterise the human IGFBP proteins. They found glycosylated IGFBP-3 as a broad band of spots with molecular masses of 41 and 45 kDa. The identity of IGFBP-3 was also confirmed by immunoblotting. The immunoblot showed some additional spots in the range of 29–31 kDa. These immunoreactive spots lacked binding capacity for labeled IGF-ligands and most likely represent proteolytic fragments of IGFBP-3. In our study we also detected a doublet in the range of 40-45 kDa, an extra band at approximately 36 kDa, and two lower fragments between approximately 24 kDa and 30 kDa were seen. The extra band in our study at 36 kDa can also be a glycosylated degradation product as described above by Zapf et al. The two lower fragments are probably proteolytic fragments, which were also detected by Weber et al.. The band patterns

on the negative images of the Western blots depend on the specificity of the antibodies, the molecular mass markers and the kind of polyacrylamide gels used. The advantage of our method in comparison to other methods^{8,13,14} is that we do not have to use radioactive isotopes to detect IGFBP-3.

To detect IGFBP-3 isoforms, the isolated proteins were separated according to charge and mass using 2-DE. The optimal immunoprecipitation conditions described in this study were used for the 2-DE experiments. Elution with urea/CHAPS is preferable for 2-DE followed by Western blotting. By comparing the serum samples of two paediatric CDG type Ia patients and 2 paediatric healthy persons, we could detect differences in IGFBP-3 isoform patterns. CDG-Ia is due to phosphomannomutase deficiency¹⁵, a key enzyme in the synthesis of guanosine 5'-diphosphate-D-mannose which is required for N-glycan assembly. Deficient synthesis of N-glycans results in a deficient incorporation of sialic acid, the terminal negatively charged sugar, so that glycoprotein molecules acquire a more positive charge, which causes a shift in the IEF patterns from more acidic to more basic¹⁶. Abnormal isoelectric focusing of transferrin in CDG with a shift towards the cathode was described earlier by Jaeken et al.. They found these features in CDG¹⁷ and in untreated classic galactosemia¹⁸. IGFBP-3 has three N-glycosylation sites and disturbed glycosylation results in a cathodal shift¹². In conclusion, the in-house developed immunoprecipitation method described in this study is suitable for studying IGFBP-3 isoforms. To confirm our results, the groups will be enlarged and we will also study IGFBP-3 isoforms in other patient groups such as other CDG types and galactosemia.

References

1. Karlsson GB, Platt FM. Analysis and isolation of human transferrin receptor using the OKT-9 monoclonal antibody covalently crosslinked to magnetic beads. *Anal Biochem* 1991;199: 219-222.
2. Veldschoote J, Berrevoets CA, Zegers ND, van der Kwast TH, Grootegoed JA, Mulder E. Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* 1992;31:7422-7430.
3. Schneider C, Newman RA, Sutherland DR, Asser U, Greaves MF. A one-step purification of membrane proteins using a high efficiency immunomatrix. *J Biol Chem* 1982;257:10766-10769.
4. Sisson TH, Castor CW. An improved method for immobilizing IgG antibodies on protein A-agarose. *J Immunol Methods* 1990;127:215-220.
5. Michielsen EC, Diris JH, Hackeng CM, Wodzig WK, Van Dieijen-Visser MP. Highly sensitive immunoprecipitation method for extracting and concentrating low-abundance proteins from human serum. *Clin Chem* 2005;51:222-224.
6. Wilchek M, Jakoby WB. The literature on affinity chromatography. *Methods Enzymol* 1974;34: 3-10.
7. Gersten DM, Marchalonis JJ. A rapid, novel method for the solid-phase derivatization of IgG antibodies for immune-affinity chromatography. *J Immunol Methods* 1978;24:305-359.
8. Weber MM, Spottl G, Gossel C, Engelhardt D. Characterization of human insulin-like growth factor-binding proteins by two-dimensional polyacrylamide gel electrophoresis and Western ligand blot analysis. *J Clin Endocrinol Metab* 1999;84:1679-1684.
9. Storch S, Kubler B, Honing S, Ackmann M, Zapf J, Blum W, et al. Transferrin binds insulin-like growth factors and affects binding properties of insulin-like growth factor binding protein-3. *FEBS Lett* 2001;509:395-398.
10. Nedic O, Nikolic JA, Prusic S, Acimovic J, hajdukovic-Dragojlovic L. Reactivity of IGF binding protein-3 isoforms towards concanavalin A in healthy adults and subjects with cirrhosis. *Addict Biol* 2003;8:81-88.
11. Jaeken J, Matthijs G. Congenital Disorders of Glycosylation: A Rapidly Expanding Disease Family. *Annu Rev Genomics Hum Genet* 2007;8:261-278.
12. Firth SM, Baxter RC. Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. *J Endocrinol* 1999;160:379-387.
13. Nedic O, Nikolic JA, Hajdukovic-Dragojlovic L, Todorovic V, Masnikosa R. Alterations of IGF-binding proteins in patients with alcoholic liver cirrhosis. *Alcohol* 2000;21:223-229.
14. Zapf J, Kiefer M, Merryweather J, Musiarz F, Bauer D, Born W, et al. Isolation from adult human serum of four insulin-like growth factor (IGF) binding proteins and molecular cloning of one of them that is increased by IGF I administration and in extrapancreatic tumor hypoglycemia. *J Biol Chem* 1990;265:14892-14898.
15. Van Schaftingen E, Jaeken J. Phosphomannomutase deficiency is a cause of carbohydrate-deficient glycoprotein syndrome type I. *FEBS Lett* 1995;377:318-320.
16. Grunewald S, Matthijs G, Jaeken J. Congenital disorders of glycosylation: a review. *Pediatr Res* 2002;52:618-624.
17. Jaeken J, van Eijk HG, van der Heul C, Corbeel L, Eeckels R, Eggermont E. Sialic acid-deficient serum and cerebrospinal fluid transferrin in a newly recognized genetic syndrome. *Clin Chim Acta* 1984;144:245-247.
18. Jaeken J, Kint J, Spaapen L. Serum lysosomal enzyme abnormalities in galactosaemia. *Lancet* 1992;340:1472-1473.