Isolation and characterization of circulating 13-kDa C-terminal fragments of human insulin-like growth factor binding protein-5

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Abstract The insulin-like growth factor binding proteins (IGFBPs) are responsible for regulation of the effects and the bioavailability of the insulin-like growth factors (IGFs). We screened for circulating fragments of human IGFBP-5 in human hemofiltrate. Identification of IGFBP-5 peptides in the fractions of our peptide bank generated from hemofiltrate was performed by their immunoreactivity and their capacity to bind IGF-I. Different fragments of IGFBP-5 with molecular sizes from 12 to 25 kDa were identified. C-terminal peptides of IGFBP-5 with molecular masses of 13.3 and 13.5 kDa were purified by consecutive chromatographic steps and sequenced. Sequence analysis of the peptides revealed the (double) sequences (K)FVGGAENXAHPRII and MVPRAVYLPNXDRKG. In addition, a smaller fragment with M_r 2722 of the central IGFBP-5 region was purified and showed the sequence HTRISELKAEAVKKDRRKKLTQS (residues 121–143) indicating plasma proteolysis of IGFBP-5 C-terminal to amino acids Lys-120, Ser-143, Lys-144, and Arg-188. According to mass spectrometric and sequence analysis, Thr-152 was shown to be *O*-glycosylated. Fractions containing C-terminal IGFBP-5 fragments revealed significant IGF-I binding properties. Our results indicate that plasma proteolysis of IGFBP-5 preferentially occurs C-terminally to basic residues and generates different C-terminal fragments, possibly acting in an IGF-dependent manner and bearing intrinsic biological functions.

Key words: Insulin-like growth factor-binding protein-5; Hemofiltrate; Plasma proteolysis; Glycosylation

1. Introduction

The insulin-like growth factor-binding proteins (IGFBPs) are a family of at least six related proteins involved in regulating the effects and the bioavailability of the insulin-like growth factors, IGF-I and IGF-II, which are responsible for growth and differentiation of cells and tissues [1,2].

IGFBPs are capable of either potentiating or inhibiting the effects of the IGFs, although they have also been shown to exhibit intrinsic activities independent of their binding to IGFs that modulate cell proliferation [3,4].

IGFBP-5 in particular was found to act via an IGF-independent mechanism on osteoblast proliferation [5]. In this context, the C-terminal amino acid residues of IGFBP-5 are supposed to be responsible for its biological action by interaction with a putative IGFBP-5 receptor [6] or by intracellular translocation and nuclear interaction [7].

The structure of the IGFBPs can be described as consisting of two cysteine-rich, structure-stable domains, the N- and C-terminal domains, and a non-conserved central region connecting these domains. Limited proteolysis of the IGFBPs into these two main fragments was demonstrated in vitro [3,8] and in vivo [9].

Proteolysis of IGFBPs is caused by a variety of extracellular proteases, a mechanism that is recognized as essential in the regulation of IGF bioavailability [10–12].

Since all six IGFBPs were detectable in human plasma, mostly occurring as high molecular weight complexes of 40–50 or 150 kDa, little is known about exact in vivo degradation and presence of low molecular weight IGFBP fragments [1]. Therefore, the aim of this study was to isolate circulating fragments of IGFBP-5 from human hemofiltrate (HF) [13] to determine cleavage sites and characterize the structural and functional properties of the fragments themselves.

Human HF is generated during blood filtration of patients with chronic renal failure and was shown to be an excellent source for the isolation of regulatory peptides with $M_r < 20\,000$ [14,15].

For the isolation of novel IGFBP-5 fragments, we started screening in the fractions of the HF peptide bank generated according to our standard procedure [13]. The screening included Western immunoblot, IGF-I ligand-blot analysis, and determination of the molecular size of the detected peptides by matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS). We identified several different IGFBP-5 fragments in the peptide bank and purified novel C-terminal fragments of IGFBP-5 retaining IGF binding capacity. The peptides reveal *O*-glycosylation and indicate that plasma proteolysis of IGFBP-5 occurs preferentially C-terminally to basic amino acid residues.

2. Materials and methods

2.1. Preparation of peptide extracts from human hemofiltrate

Peptides of human blood ultrafiltrate are prepared according to our concept and IPF laboratory standards [13,14]. In brief, the crude peptide extract is obtained from 10 000 l hemofiltrate (HF). Immediately after blood filtration, the filtrate is cooled to 4°C and conditioned to a pH of 2.7. After binding the peptides to an ion-exchange column, a batch elution follows. For the first separation step, stepwise batch elution is performed using a 10-l cation-exchange column and seven different buffers increasing the pH from 3.6 to 9.0. The resulting pools (15–25 l) are immediately fractionated by reverse-phase chromatography. Each pool is applied to a Source RP-C column (15 μm, 10×12.5 cm; Pharmacia, Freiburg, Germany) and separation is performed at a flow rate of 200 ml/min by an 8-l gradient from 100% A to 60% B (A: water, 10 mM HCl; B: 80% acetonitrile, 10 mM HCl). Fractions of 200 ml are collected, monitoring the absorbance at 280 nm. Aliquots of these peptide bank fractions corresponding to a 2-l

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Abbreviations: ESI-MS, electrospray mass spectrometry; HF, hemofiltrate; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein

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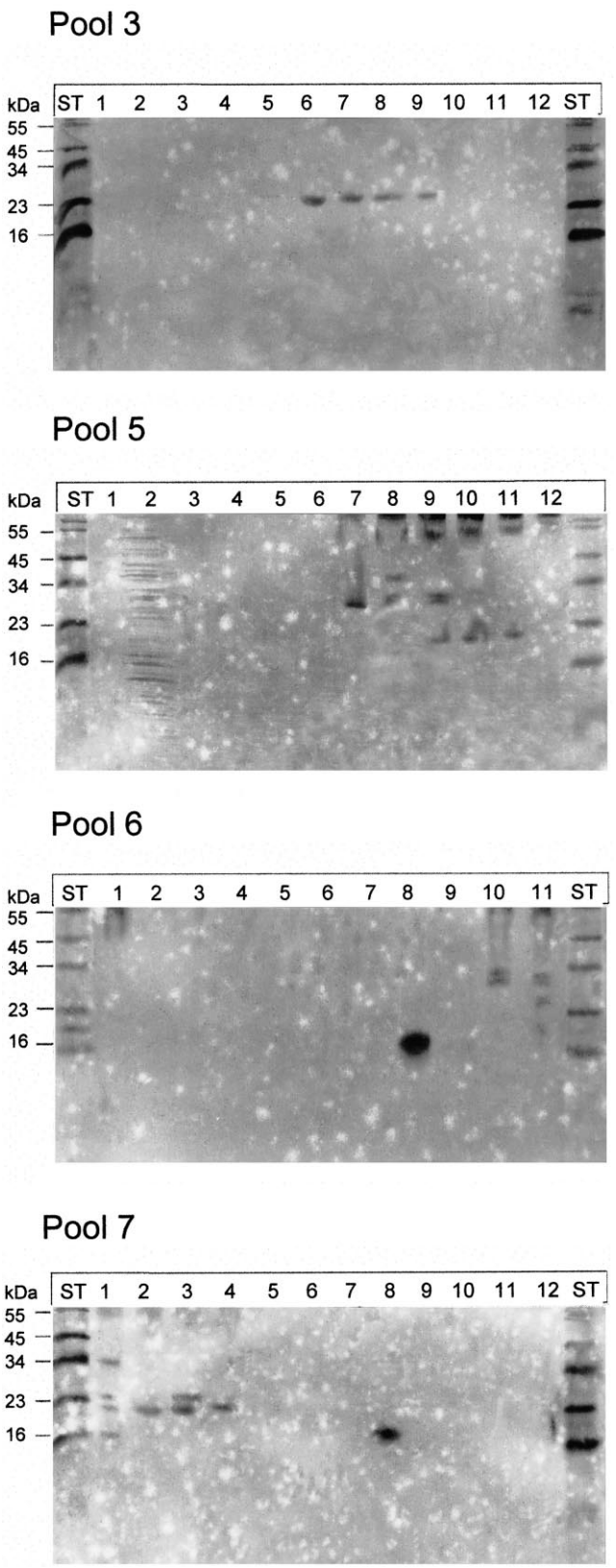

Fig. 1. IGFBP-5 immunoblotting. Fractions of the hemofiltrate peptide bank were separated by PAGE, electroblotted to a PVDF membrane, and incubated with rhIGFBP-5 antiserum. After development of the blot, IGFBP-5 immunoreactivity was detected in fractions of pH pool 3, pH pool 5, pH pool 6, and pH pool 7. The purified C-terminal IGFBP-5 fragments originated from fractions of pH pool 6 (lane 8) containing a strong immunoreactivity in the range of 16 kDa.

equivalent of hemofiltrate are lyophilized and used for the experiments as indicated below.

2.2. Further liquid chromatography

Aliquots of the peptide bank fractions corresponding to 100 ml equivalent of HF were analyzed by Western immunoblot and Western ligand-blot as described below.

The fractions showing both immunoreactivity and IGF-binding capacity were selected for further purification and analyzed by MALDI-MS.

To achieve further purification the selected fractions were applied to a preparative C18-column (15–20 μm, 30 nm, 4.7×30 cm; Vydac, Hesperia, USA). 0.1% trifluoroacetic acid and 80% acetonitrile in water and 0.1% trifluoroacetic acid were the solvents in gradient elution. Selected single fractions were loaded onto a cation exchange column (5 μm, 300 Å, 10×50 mm Pepkat; Biotek, Östringen, Germany) and separated by a gradient elution using 20 mM sodium-phosphate buffer, pH 3.0, or 20 mM sodium phosphate buffer, pH 3.0, containing 1 M NaCl. Further purification of selected fractions was performed using an analytical C18-column (5 μm, 30 nm, 1×25 cm; Vydac) with the mentioned RP-solvents in gradient elution.

2.3. Peptide analysis

Mass determination of the purified peptides was carried out on a Sciex API *III* quadrupole mass spectrometer (Sciex; Perkin-Elmer, Langen, Germany) with an electrospray interface (ESI-MS), mass determination in the fractions on a LaserTec RBT II MALDI-MS (PerSeptive Biosystems, Freiburg, Germany) as described previously [16]. Peptide sequencing was performed on a 473 A gas-phase sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin-amino acids using the standard protocol recommended by the manufacturer. To separate the disulfide-bridged 13-kDa IGFBP-5 isoforms, the native peptides were reduced with 2-mercaptoethanol and alkylated at cysteine residues with iodoacetamide using standard conditions. The carboxyamidomethyl-alkylated peptides were purified by RP-HPLC, analyzed by mass spectrometry, and sequenced.

2.4. SDS-PAGE and electrotransfer

Aliquots of the HPLC fractions were lyophilized, reconstituted in sample buffer and subjected to an SDS-PAGE according to Schägger and Jagow [17] in Mini-Protean systems (Bio-Rad, Munich, Germany) at 150 V for 2 h. Molecular mass standards of 4–210 kDa (SeeBlue; NOVEX, Offenbach/Main, Germany) were used. The separated peptides were transferred to a hydrophilic polyvinylidene difluoride (PVDF) membrane at 7 V for 2 h using a semi-dry electrophoretic transfer unit (Bio-Rad). The membrane was blocked with 5% powdered skimmed milk in Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 8.0) containing 0.05% Triton X-100 at 4°C overnight or for 1 h at room temperature.

2.5. IGFBP-5 immunoblotting

Electroblotted and blocked PVDF membrane was incubated with rabbit rhIGFBP-5 antiserum (Upstate Biotechnology, Lake Placid, NY, USA) diluted to 1:1000 in TBS containing 0.05% Triton X-100 at 4°C overnight (or for 2 h at room temperature). The membrane was washed in TBS containing 0.05% Triton X-100 and then incubated with anti-rabbit IgG coupled to alkaline phosphatase (Sigma, Deisenhofen, Germany) in the same buffer. Afterwards, the nitrocellulose

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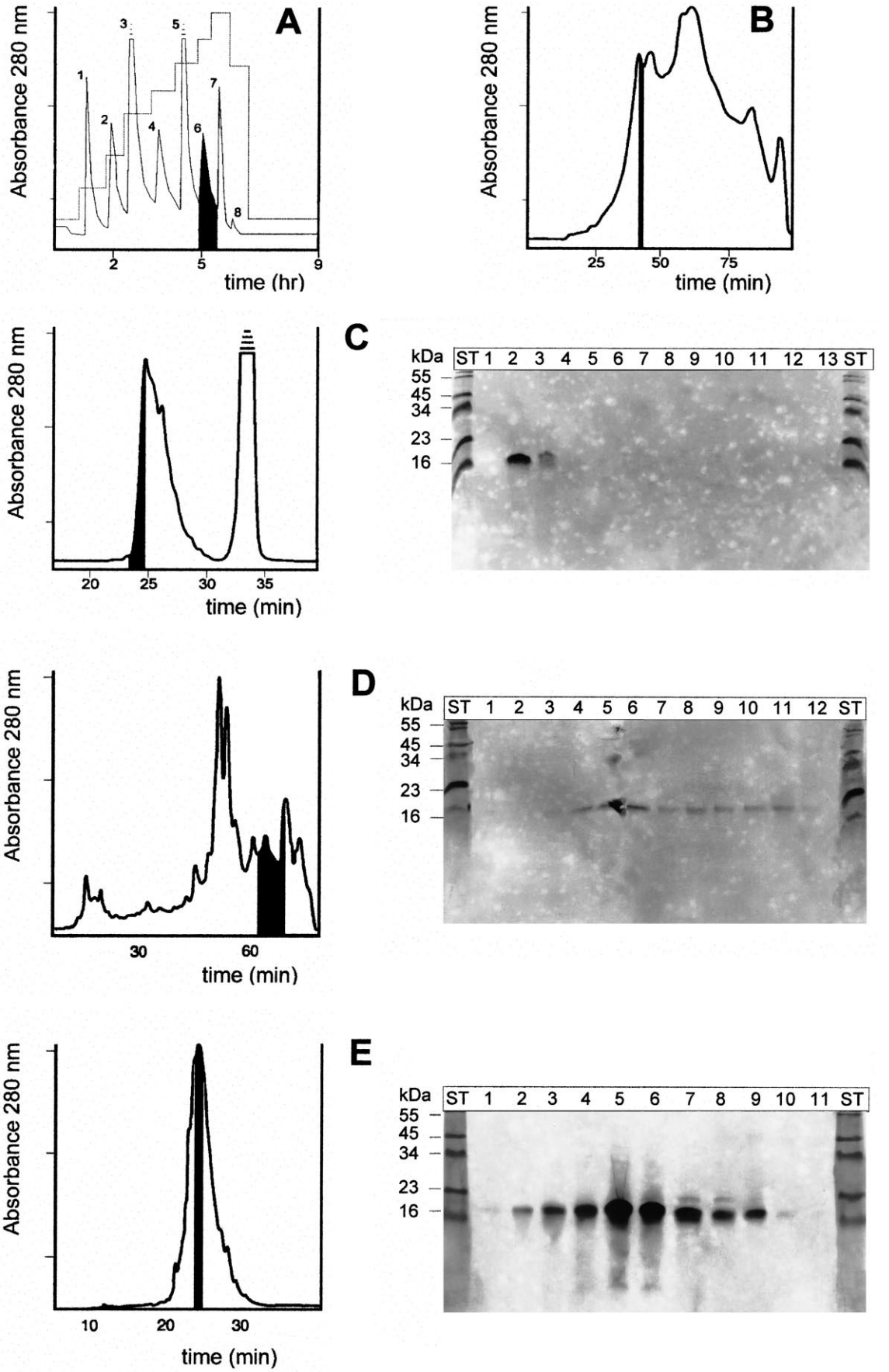
Fig. 2. Purification of the 16-kDa IGFBP-5 immunoreactivity from pH pool 6 chromatograms and pertinent Western immunoblot analysis. A: Preparative cation exchange chromatography and pH pool fractionation of 10 000 l human hemofiltrate. In this first separation step, peptides were eluted batchwise by means of a pH gradient. B: RP-HPLC fractionation of the peptides derived from pH pool 6 (marked in A). Aliquots of this chromatography were analyzed by immunoblot and ligand-blot as described in Section 2. C: Semi-preparative RP-HPLC of the active fraction from B. D: Analytical cation exchange chromatography of the marked fraction from C. E: Analytical RP-HPLC of the marked fraction from D. The immunoreactive fractions shown in E were analyzed by MALDI- and ESI-MS, and sequenced.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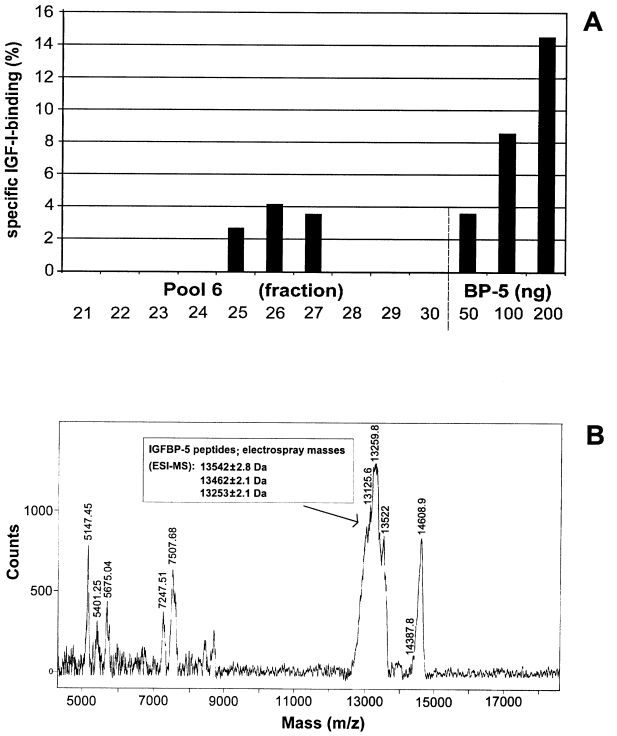


Fig. 3. A: Ligand-blot analysis of the immunoreactive fractions (Fig. 2B) from pH pool 6 using radiolabeled IGF-I. IGF-I binding to fractions 25–27 from pH pool 6 each containing 100 ml equivalent of hemofiltrate was comparable to IGF-I binding to 50 ng complete rhIGFBP-5 (BP-5). B: MALDI-MS spectra of the fraction (marked in Fig. 2E) containing C-terminal IGFBP-5 peptides and corresponding molecular masses of the IGFBP-5 peptides determined by ESI-MS. Note contaminant peptide with M_r 14.5 kDa (human pancreatic ribonuclease).

was washed first in TBS containing 0.05% Triton X-100, then in TBS, and finally with 10 mM Tris, 10 mM NaCl, and 10 mM $MgCl_2$, pH 9.5.

Bands were developed using a bromochloroindolyl phosphate/nitroblue tetrazolium substrate system as recommended by the manufacturer (Sigma).

2.6. Ligand blotting

Ligand blots were performed according to Hossenlopp et al. [18] using recombinant human IGFBP-5 (Boehringer Mannheim, Penzberg, Germany) as a control. IGFs (PeproTech, London, UK) were iodinated by the chloramine T method using standard conditions. Briefly, aliquots of the HPLC fractions and different amounts of the purified fragments and complete rh IGFBP-5 were placed directly onto nitrocellulose sheets (5×5 mm). The membrane sheets were dried for 10 min at room temperature, and then incubated in blot buffer (50 mM Tris, 0.9% NaCl and 0.5% gelatine, pH 7.4) at 4°C overnight, followed by incubation in blot buffer containing 0.05% Triton X-100 and ^{125}I-IGFs in a final concentration of 300 000 cpm/ml at 4°C overnight. Afterwards, the nitrocellulose was washed three times in blot buffer containing 0.05% Triton X-100, dried at room temperature, and the amount of bound radioactivity was determined using a γ counter (1470 Wallac Wizard; Pharmacia, Turku, Finland).

3. Results and discussion

3.1. Screening for fragments of IGFBP-5

Isolation of bioactive peptides from crude biological sources depends preliminarily on the structural and biological features of the selected peptide and therefore has to be optimized in each particular case. We attempted isolation of circulating fragments of the insulin-like growth factor-binding proteins (IGFBPs) and developed a strategy by characterizing different biochemical and immunochemical parameters before starting the purification. Screening for IGFBP-5 fragments was started in the 400 fractions of the peptide bank prepared from human hemofiltrate (HF) [13]. These fractions were generated by stepwise batch elution of 10 000 l hemofiltrate with seven buffers with each increasing pH (pH pools; Fig. 2A) and subsequent RP-HPLC fractionation of each pH pool (Fig. 2B). First, Western immunoblot analysis was carried out and gave a general overview of the IGFBP-5 immunoreactive substances in our peptide bank. Several immunoreactive fractions were detectable (Fig. 1). For example, significant IGFBP-5 immunoreactivity in the range of 25 kDa was detected in fractions of pH pool 3, and a prominent 16-kDa band was visualized in pH pool 6. Furthermore, strong IGFBP-5 immunoreactivity in the range of 16 kDa was monitored in pH pool 7 where minor bands with molecular sizes of 20–25 kDa also occurred. In addition, weak IGFBP-5 immunoreactivity in the range of 30 kDa was detected in pH pool 5. The mature IGFBPs strongly bind both IGF-I and IGF-II with dissociation constants (K_d) of 10^{-9} to 10^{-10} M. The mechanism of IGF binding is proposed to depend on two binding sites within the IGFBPs. Both the N-terminal and the C-terminal IGFBP regions participate in IGF binding and corresponding IGFBP fragments have been shown to bind the IGFs with reduced affinity [9,19,20]. To prevent the purification of non-IGFBP peptides, which have shown cross-reactivity to the IGFBP antisera used, we examined the IGF-I binding capacity of selected immunoreactive fractions. Using ligand-blot analysis with radiolabeled IGF-I, some immunoreactive fractions showed significant IGF binding capacity (Fig. 3A). On the other hand, other immunoreactive fractions did not possess any significant affinity for IGF-I. The results of the initial immunoscreening and ligand-blot analysis in the blood filtrate are summarized in Table 1.

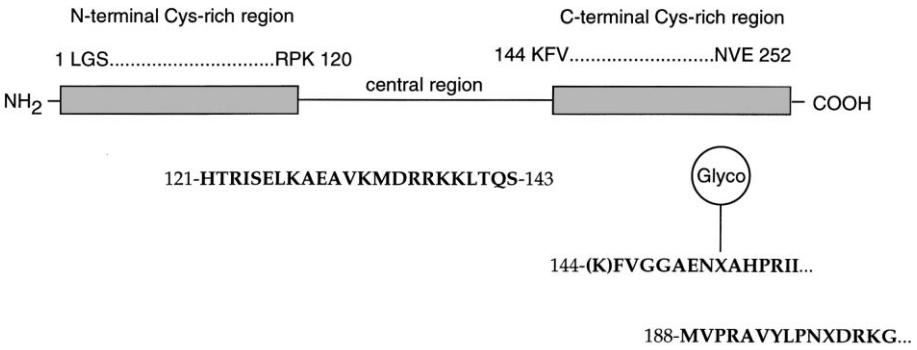
3.2. Isolation of C-terminal IGFBP-5 immunoreactive peptides

For further biochemical characterization, we performed mass spectrometrical analysis of selected fractions which showed both IGF binding and IGFBP-5 immunoreactivity. Therefore we used MALDI-MS, which was recently shown to be a valuable method of guiding the purification of bioactive peptides from complex biological mixtures [16].

Table 1
IGFBP-5 immunoreactive fractions and pertinent ligand-blot analysis in the hemofiltrate peptide bank

pH pool	Lane	IGFBP-5	kDa	LB
3	6	++	23	−
3	7	+	23	n.d.
3	8	+	23	n.d.
5	7	+	30	+
5	8	+	30	−
5	9	+	30	n.d.
5	10	+	30, 23	n.d.
5	11	+	30, 23	−
6	8	+++	16	+
6	9	+	16	+
7	2	+	20	−
7	3	+	23, 20	−
7	4	+	20	n.d.
7	8	++	16	+

LB, ligand-blot analysis; n.d., not determined.



Analysis of reduced and amidoalkylated C-terminal IGFBP-5 peptides

Peptide	Calculated mass	Electrospray mass	Mass difference
IGFBP-5 144-187	5091	6036±0.6	947
IGFBP-5 188-252	7787	7780±0.6	–

Fig. 4. Sequence and mass spectrometric analysis of the IGFBP-5 peptides from hemofiltrate – plasma proteolysis of IGFBP-5. The cysteine-rich conserved regions of IGFBP-5 are indicated as shaded boxes. Sequenced amino acids are in bold letters. Plasma proteolysis of IGFBP-5 was detected in the central non-conserved region and between cysteine 1 and cysteine 2 generating three different (glycosylated) peptides comprising the C-terminal domain with molecular masses of 13.2–13.5 kDa. In addition, one smaller 3-kDa peptide of the central region was found. Note the intramolecular cleavage site between Arg-188 and Met-189 and *O*-glycosylation of Thr-152. The analysis of the reduced and amidoalkylated C-terminal IGFBP-5 peptides was in accordance with the data obtained from the native peptides.

For initial purification, we selected fraction 25 of pH pool 6 (corresponding to lane 8 in Fig. 1) which contained an immunoreactive IGFBP-5 peptide in the range of 16 kDa and additionally showed a significant binding affinity for IGF-I (Table 1; Fig. 3A).

For isolation, the selected fraction (marked in Fig. 2B) was loaded onto a preparative RP-C18 column. The run was performed using a linear acetonitrile gradient and the generated fractions were analyzed for immunoreactivity and IGF-I binding (Fig. 2C). Those fractions showing both immunoreactivity and IGF-binding capacity were applied to a cation exchange column and separated by a linear sodium chloride gradient in phosphate buffer (Fig. 2D).

The generated fractions were analyzed as mentioned above. To achieve further purification the selected fractions (marked in Fig. 2D) were chromatographed using an analytical RP-C4 column (Fig. 2E). The strong immunoreactive fractions shown in Fig. 2E were sequenced using conventionel Edman chemistry. The N-terminal (double) sequences were (K)FVGGAENXAHPRII and MVPRAVYLPNXDRKG which correspond exactly to the sequence of human IGFBP-5, amino acid residues 144–159, 145–159, and 189–204, indicating plasma proteolysis of IGFBP-5 C-terminal to amino acids Gln-143, Lys-144, and Arg-188 (Fig. 4). MALDI and ESI mass spectra of the fraction marked in Fig. 2E revealed the existence of three different IGFBP-5 peptides (Fig. 3B). The ESI masses of the IGFBP-5 peptides were determined to be 13 542 ± 2.8 Da, 13 462 ± 2.1 Da, and 13 253 ± 2.1 Da (Fig. 3B). Sequencing of N-terminal Met-189 revealed that one peptide contains an intramolecular cleavage. Since only molecular masses higher than 13 kDa were determined, the generated fragments must be connected by a disulfide bond.

3.3. Biochemical characterization of the peptides

Since the three purified peptides were very similar in sequence (Fig. 4), mass (Fig. 3B), and structure we were not able to separate the single, native substances from each other by chromatography. Therefore, we reduced and alkylated the peptides at cysteine residues. After separation of the amidoalkylated peptides by RP-HPLC, mass spectrometric and sequence analysis revealed the occurrence of IGFBP-$5^{144-187}$ (M_r 6036) and IGFBP-$5^{188-252}$ (M_r 7780) (Fig. 4). The peptide IGFBP-$5^{144-187}$ has a calculated mass of 5091 Da, including one carboxyamidoalkylated cysteine, and therefore showed a mass increase of 947 Da caused by glycosylation.

The theoretical mass of the C-terminal IGFBP-5 domain (from Lys-144 to Glu-252), calculated from the cDNA of IGFBP-5 [21], amounts to 12 503 Da and is lower than all three masses of the native fragments determined by ESI-MS. Therefore, glycosylation of the three peptides seems to be probable. Although *N*-linked glycosylation is described for IGFBP-3 [22] and -4 [23] and extensive *O*-linked glycosylation is described for IGFBP-6 [24,25], little is known about *O*-glycosylation of IGFBP-5 [26]. When sequencing the native and the alkylated peptides, no amino acid was detectable at position 152 (Thr). This indicates a blocked Thr-152 by an *O*-glycosylation. The molecular mass difference between the calculated and the electrospray masses amounts to 947 Da (Fig. 4) which might be caused by a glycosylation containing one hexose, one *N*-acetylhexosamine and two *N*-acetylneuraminic acids. A corresponding *O*-glycosylation was described for IGFBP-6 [25].

3.4. Plasma proteolysis of IGFBP-5

The isolation of a circulating C-terminal IGFBP-5 domain is in agreement with earlier observations of IGFBP proteolysis

in vitro, where the generation of two main proteolytic products of IGFBPs was observed – one representing the N-terminal domain, the other representing the C-terminal domain [27,28]. The C-terminal IGFBP-5 fragments obviously retained a significant IGF-I binding affinity (Fig. 3A), which might be lower compared to the mature IGFBP-5. In general, proteolysis of the IGFBPs generates fragments with lower or no IGF affinity and therefore enhances the IGF availability for the IGF-I receptor [1].

In addition, we purified a fragment with M_r 2722 of the central IGFBP-5 region from pH pool 6 of our hemofiltrate peptide bank by routinely performed sequence analysis of circulating human peptides. The fragment showed the sequence HTRISELKAEAVKKDRRKKLTQS, therefore spanning from Thr-121 to Ser-143 of IGFBP-5 (Fig. 4). In conclusion, our data indicate that plasma proteolysis of human IGFBP-5 occurs C-terminally to amino acids Lys-120, Ser-143, Lys-144, and Arg-188. Although the proteases responsible for its degradation remain as yet undefined, enzymes with specificity such as trypsin might be responsible for plasma proteolysis of IGFBP-5, since basic amino acids seem to be favored cleavage sites. In addition, we were able to detect further IGFBP-5 immunoreactive peptides in the hemofiltrate peptide bank ranging from 12 to 25 kDa. Based on the knowledge of the circulating IGFBP-$5^{121-143}$, the existence of an N-terminal IGFBP-5 fragment spanning residues 1–120 would be expected.

3.5. Conclusions

Human hemofiltrate was recently described to be a valuable source for the isolation of regulatory peptides with $M_r < 20\,000$ [14,15]. Our approach to isolate a binding protein by its immunoreactivity, the IGF-I binding capacity, and MALDI-MS analysis of selected fractions should be of special advantage for isolation of IGFBP fragments. Nevertheless, smaller IGFBP fragments with reduced or lost affinity for the IGFs, or with only weak immunoreactivity could not be detected by the methods used. MALDI-MS was recently shown to be a suitable method for the purification of bioactive peptides from complex biological mixtures if their molecular masses are known [16].

In bone metabolism, IGFBP-5 was found to contribute to osteoblast proliferation and bone formation in an IGF-independent manner [5]. IGFBP-5 and IGFBP-5 fragments are described to act stimulatively in synergism with the IGFs – possibly acting via a membrane receptor of its own [5,6,29]. Interestingly, a C-terminal fragment of IGFBP-5 was shown to inhibit the interaction of IGFBP-5 to its putative 420-kDa signaling receptor [6]. C-terminal residues of IGFBP-5 were also found to be involved in the intracellular translocation and the putative nuclear interaction of IGFBP-5 [7]. Therefore, it seems to be probable that the circulating C-terminal IGFBP-5 fragments may have an intrinsic biological effect on cell growth and differentiation.

Meanwhile, we are currently in the process of isolating further fragments of IGFBPs from human hemofiltrate. Further characterization of the peptides will include structural aspects and will examine the biological relevance of the peptides with respect to the important (patho)physiological role of the IGF system, focusing on intrinsic, IGF-independent effects of IGFBP fragments.

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