Purification of the human fibroblast growth factor 2 using novel animal-component free materials

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ABSTRACT

This paper analyzes the use of animal-component free chromatographic materials for the efficient purification of the human fibroblast growth factor 2 (hFGF-2). hFGF-2 is produced in *Escherichia coli* and purified via three different chromatography steps, which include a strong cation exchange chromatography as a capture step, followed by heparin affinity chromatography and an anion exchange chromatography as a polishing step. The affinity chromatography step is based on the animal-derived material heparin. Chemically produced ligands provide a viable alternative to animal-derived components in production processes, since they are characterized by a defined structure which leads to reproducible results and a broad range of applications. The alternative ligands can be assigned to adsorber of the mixed-mode chromatography (MMC) and pseudo-affinity chromatography.

Eight different animal-component free materials used as adsorbers in MMC or pseudo-affinity chromatography were tested as a substitute for heparin. The MMCs were cation exchangers characterized with further functional residues. The ligands of the pseudo-affinity chromatography were heparin-like ligands which are based on heparin's molecular structure. The alternative methods were tested as a capture step and in combination with another chromatographic step in the purification procedure of hFGF-2. In each downstream step purity, recovery and yield were analysed and compared to the conventional downstream process.

Two types of MMC – the column Foresight™ Nuvia™ cPrime™ from Bio-Rad Laboratories and the column HiTrap™ Capto™ MMC from GE Healthcare Life Sciences – can be regarded as effective animal-component free alternatives to the heparin – based adsorber.

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1. Introduction

The glycosaminoglycan heparin is a highly sulphated polysaccharide which is important for various biological interactions [1,2]. Heparin is known for its function as an inhibitor in the coagulation cascade to maintain blood flow in the vasculature system [3]. Based on this property, heparin has seen widespread clinical use [4,5]. The biological activities of heparin are associated with its interaction with various proteins, which have led to its use in protein purification in biotechnological applications [6,7]. Diverse biologically active heparin-binding proteins, such as the human fibroblast growth factor 1 (hFGF-1) [8], hFGF-2 [9,10], the human bone morphogenic protein 2 (hBMP-2) [11], the serine protease throm-

bin [12], and the glycoprotein anti-thrombin III (ATIII) [13], can be successfully purified by heparin affinity chromatography in a very effective and simple process [7,14–16]. However, heparin is an animal-derived material, which is most frequently obtained from bovine or porcine tissue. Animal-based components are ethically problematic and carry the risk of virus contaminations [14], requiring strict quality controls and the validation of effective good manufacturing practice (GMP) implementation. Adequate animal-free components are therefore of considerable interest.

There are two options to replace conventional heparin in affinity chromatography. One option is to use chemically synthesized heparin [17,18], chemoenzymatically synthesized heparin [17,19], or bioengineered recombinant heparin, e.g. from genetically modified Chinese Hamster Ovary (CHO) cells [20]. Such bioengineered heparins have been applied in pharmaceuticals and for structure analysis but are very expensive. To our knowledge, there have not been

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studies on the use of bioengineered heparin for purification methods to date.

The second option is the use of animal-component free chromatographic methods, applying MMC and pseudo-affinity chromatography as an alternative to heparin affinity chromatography. Ligands of MMCs are characterized by a multimodal functionality compared to traditional single-mode chromatographic ligands [21,22]. These materials are characterized by the availability of different ligands in one material. Various functional groups can build up different types of interactions (ionic interactions, hydrogen bonds, Van-der-Waals interactions) which can lead to a high affinity to different proteins, enabling a broad range of applications [9,23–25]. MMCs are salt-tolerant due to their hydrophobic functionalities. This behaviour shortens the purification process time through fewer desalting steps and thus reduces the purification costs. This is advantageous as each additional purification step is associated with protein loss and higher purification costs [22,25].

The pseudo-affinity chromatography is related to the affinity chromatography but is based on pseudo-specific ligands which are chemically synthesized. In contrast to the ligands in the traditional affinity chromatography, the ligands of the pseudo-affinity chromatography are easier and cheaper to produce and are more robust to chemical or biochemical degradation [26].

Several groups have already tested animal-component free materials to purify heparin-binding proteins [25,27–29]. They all have in common that the purified proteins are produced as inclusion bodies. These inclusion bodies have already a purity of 80 % which makes further purification steps easier.

In this study we produced the cytokine hFGF-2 in recombinant *Escherichia coli* (*E. coli*) in a soluble form. After cell disruption and centrifugation, the experiments were performed with the soluble cell fraction. Eight different animal-component free materials (different mixed-mode and pseudo-affinity chromatography materials) were tested in bead-based column chromatography or membrane adsorbers technology (Table S1).

2. Materials and methods

2.1. Plasmid, strain, cultivation method

The *E. coli* strain BL21(DE3) (Novagen, Germany) with the plasmid pET29(+)-hFGF-2 was constructed by Hoffmann et al. [30]. The cultivation method for the production of hFGF-2 has been previously described by Li et al. [31].

2.2. Cell disruption

Cell pellets were resuspended in 15 mL lysis buffer (25 mM phosphate buffer, 100 mM NaCl, 3 mM DTT, 1 mM EDTA, pH 7.5) per gram cell pellet and disrupted with a high pressure homogenizer (M-110L, Microfluidics, USA) at 9000 psi in 10 cycles. After centrifugation the supernatant was filtrated through a 0.2 µm sterile filter (Minisart® high flow; Sartorius, Germany).

2.3. Chromatographic methods

All chromatographic procedures were performed at 4°C with the Äkta-Pure liquid chromatography system (GE Healthcare, USA). For the purification of hFGF-2 different chromatographic materials, including membrane adsorbers, were tested. The cytokine hFGF-2 (theoretical isoelectric point = 9.58) is negatively charged at the pH of the used buffers (pH 7.5). The tested materials were various types of cation exchanger adsorbers decorated with further functional groups or heparin-like structures mimicking the heparin's molecular structure (Table S1). The sample was loaded onto

a membrane adsorber or bead-based columns which were pre-equilibrated (10 column volume (CV), 1 mL·min⁻¹) with binding buffer (25 mM phosphate buffer, 3 mM DTT, 1 mM EDTA, pH 7.5). Subsequently, the systems were washed with binding buffer (20 CV, 1 mL·min⁻¹). The elution step was performed with a linear gradient (10 CV) of 0 to 2.0 M NaCl in the binding buffer with 1 mL·min⁻¹. The flow-through and elution peaks were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Cleaning in place (CIP) was carried out according to the manual instructions for the different bead-based columns and membrane adsorbers.

2.4. Cation exchange chromatography as a capture step and the desalting step for experiments in 3.2

For cation exchange chromatography two connected membrane adsorber capsules (MA) Sartobind® S nano (Sartorius AG, Göttingen, Germany) each with a membrane volume of 3 mL were used. The MA was equilibrated with 10 column volumes (CV) binding buffer and a flow rate of 10 mL·min⁻¹. The MA was loaded with the protein solution with a flow rate of 5 mL·min⁻¹. Next, a washing step with 15 CV binding buffer and a flow rate of 10 mL·min⁻¹ was performed. The elution with the elution buffer was an isocratic elution with three steps (15 %, 30 % and 50 % elution buffer), which corresponds to a NaCl concentration of 300 mM, 600 mM and 1 M. Each stage consisted of 20 CV and the entire elution was performed at a flow rate of 10 mL·min⁻¹. hFGF-2 was eluted in the second step at a NaCl concentration of 600 mM. The MA was then cleaned with 1 M NaOH (50 CV, 10 mL·min⁻¹). hFGF-2 fractions were desalted by desalting column HiPrep™ 26/10 (GE Healthcare, Chicago, USA) with a column volume of 53 mL. The column was pre-equilibrated with 5 CV binding buffer (10 mL·min⁻¹). The sample was subsequently loaded directly onto the column and the fractions were collected. NaCl and hFGF-2 are separated by their size using this type of column.

2.5. Desalting/Buffer exchange before anion exchange chromatography

hFGF-2 was rebuffed for the last purification and the following lyophilisation step. This was performed by a HiPrep™ 26/10 desalting column (GE Healthcare, Chicago, USA). The column was pre-equilibrated with 5 CV Tris-HCl buffer (25 mM Tris-HCl, 100 mM NaCl, 3 mM DTT, 1 mM EDTA, pH 7.5) with a flow rate of 10 mL·min⁻¹. The sample was then loaded directly onto the column and the fractions were collected. Using this type of column, NaCl and hFGF-2 were separated by their size and the protein was transferred into the anion exchange/lyophilisation buffer (Tris-HCl buffer, pH 7.5).

2.6. Anion exchange chromatography as a polishing step

The final step is performed by anion exchange chromatography using the membrane module Sartobind® Q75 (Sartorius AG, Göttingen, Germany, membrane volume 2.1 mL) [9]. This step is performed as a polishing step to remove endotoxins and DNA. First, the chromatography system was washed using washing medium 1 (2 M KOH, 30 % ethanol) to make the system endotoxin free. The membrane adsorber was installed and washed with 5 CV and a flow rate of 10 mL·min⁻¹ with washing medium 2 (0.1 M KOH, 20 % ethanol, 1.5 M NaCl and pyrogen-free water) and later equilibrated with Tris-HCl buffer (5 CV, 10 mL·min⁻¹). The sample was loaded onto the membrane adsorber for a flow-through mode. The flow-through fractions contained purified hFGF-2. Furthermore, the bound endotoxins and DNA were eluted with washing medium 2.

2.7. Lyophilisation

Freeze-drying was carried out with the freeze-drying device (Alpha 1-4 LCS, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). First, the protein sample was concentrated to a concentration of $1 \text{ mg} \cdot \text{mL}^{-1}$ using a centrifugal concentrator (VivaSpin 20, MWCO 3000 Da, Sartorius AG, Göttingen, Germany). Secondly, the concentrated sample was mixed with 1/10 volume (v/v) of a 20% sucrose solution, sterile-filtered ($0.2 \mu\text{m}$ pore size) and frozen at -80°C for 48 h. Freeze-drying was performed at a sample temperature of less than -25°C , a condensation temperature of -60°C and a vacuum of 0.12 mbar for 24 h to 36 h. The freeze-dried samples were stored at -20°C until further use.

2.8. SDS-PAGE analysis

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini-PROTEAN 3 Cell (Bio-Rad, USA) using a 5 % loading gel and a 16 % separating gel. The sample was diluted at 1:2 in reducing sample buffer (50 mM Tris-HCl (pH 6.8), 5 % (w/v) SDS, 20 % (v/v) glycerol, 0.1 mg mL^{-1} bromophenol blue, 2 % (v/v) β -mercaptoethanol) and heated at 95°C for 10 min. $10 \mu\text{L}$ of sample volume was loaded onto the gel and the electrophoresis was performed at 100 V for 2 h. Gels were stained with colloidal Coomassie Brilliant Blue solution and the destained gels were scanned on an Epson perfection V750 pro scanner (Epson, Japan).

2.9. Protein quantification and purity

Protein quantification was performed by Bradford protein assay (Thermo Fisher Scientific, USA). Absorbance was detected at 595 nm using UV/Vis spectrophotometer. Bovine Serum Albumin (BSA) was used as standard. The relative content (purity) of hFGF-2 was measured by densitometry analysis of the Coomassie Brilliant Blue stained gels by using the software GelAnalyzer.

2.10. Protein yield and recovery

In current research, the yield generally refers to the relative yield, which is the quotation of the actual yield and the total hFGF-2. The actual yield refers to the hFGF-2 in the elution fraction after purification. It is determined by the relative content of hFGF-2 in this elution fraction multiplied by the protein concentration in this fraction and the fraction volume. The total hFGF refers to the total hFGF-2 before purification. This is determined by the hFGF-2 concentration before purification multiplied by the relative content of hFGF-2 in this sample and the sample volume.

The recovery of hFGF-2 is the quotation of the actual yield of hFGF-2 in all peaks during purification (flow through, elution peaks, CIP peaks) and the total yield of hFGF-2 before purification.

2.11. Endotoxin assay

The endotoxin content of purified hFGF-2 was determined using an Endosafe-PTSM system (Charles River Laboratories, USA) with a cartridge ranging from 0.1 to 10 EU/mL (EU = endotoxin unit; product code PTS201). The test was carried out according to the manual instructions using the Limulus amoebocyte lysate (LAL) assay.

2.12. Bioactivity test

The bioactivity test was performed with NIH-3T3 fibroblast cells (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cells were incubated in 96-well plates in DMEM medium supplemented with 10 % (v/v) foetal

calf serum (FCS) and 1 % penicillin-streptomycin (PAA Laboratories, Pasching, Austria) with a cell concentration of 4000 cells/well. The plates were incubated at 37°C in a humidified atmosphere of 5 % (v/v) CO_2 in air. After 24 h the medium was changed to minimal medium (DMEM with 1 % FCS and 1 % penicillin-streptomycin) and incubated for a further 24 h. Then, the medium was changed to minimal medium containing hFGF-2 from different purification procedures and in different concentrations and further incubation took place for 72 h. Cells cultivated in minimal medium without hFGF-2 served as a negative control. The evaluation of the bioactivity test was performed by the CellTiter-Blue cell viability assay (Promega, Southampton, UK). Fluorescence measurements were performed on the Fluoroskan Ascents microplate reader (Thermo Scientific, Dreieich, Germany) and the results are presented as percentage of the negative control.

3. Results and discussion

All chromatographic techniques were tested with the soluble cell fraction, which was obtained after cell disruption and centrifugation. The relative content of hFGF-2 in the overall protein content was around 12 %, the remaining 88 % were *E. coli* host cell proteins. The established method [9] consisted of three chromatographic steps (Fig. 1A), a cation exchange chromatography as a capture step, the heparin affinity chromatography as an intermediate purification step and an anion exchange chromatography step as a polishing step. All purification conditions used for the heparin affinity chromatography (same buffers, CV of sample application, CV of washing step, CV of elution) were used for the alternative animal-component free purification methods. These alternatives were tested as a capture step (Fig. 1B) since fewer purification steps are attended with a faster purification process and with less protein loss. Both result in a less expensive hFGF-2 production. In addition, we tested the alternatives as an intermediate step in combination with a cation exchange chromatography as a capture step (Fig. 1C). The different chromatographic methods were compared by evaluating purity, recovery and yield of hFGF-2 after purification.

3.1. Alternative purification methods used as a capture step

For this experiment 21 g cell pellet solved in 315 mL lysate buffer were used. After cell disruption, the cell lysate was centrifuged and the supernatant sterile filtrated ($0.2 \mu\text{m}$ cut-off). The sample volume used for each downstream experiment was adjusted to the column/membrane volume (CV; Table 1). The sample volume was calculated with 20 mL per mL CV. The total protein concentration was $7.6 \text{ mg} \cdot \text{mL}^{-1}$. The relative content of hFGF-2 were 12 % which led to a hFGF-2 concentration of $0.91 \text{ mg} \cdot \text{mL}^{-1}$.

The heparin affinity chromatography and the cation exchange chromatography were tested in comparison to the animal-component free alternatives (Table S1).

The experiments were performed in triplicate.

By using heparin affinity chromatography as a capture step, the highest protein purity and yield were achieved. The MMC Prototype B and the pseudo-affinity chromatography Capto™ DeVirS as well as the Sartobind® Sulphated Cellulose (SUCA) were identified as the best animal-component free alternatives regarding the purity of hFGF-2. Consequently, the following section focuses on these chromatograms and the corresponding SDS-PAGE analyses in detail (Figs. 2 and 3). The other animal-component free materials resulted in a low purity, recovery and hFGF-2 yield (chromatograms and SDS-PAGE analyses are not shown).

In Fig. 2, the chromatogram of the purification by the heparin affinity chromatography shows two overlapping elution peaks and one single elution peak. Based on the SDS-PAGE gel the eluted

Fig. 1. Process charts showing the chromatographic methods for the purification of soluble hFGF-2 from the cell supernatant. In (A) the established method consisting of a cation exchange chromatography as capture step, followed by the heparin affinity chromatography as intermediate purification step and an anion exchange chromatography step as polishing step. (B) and (C) describes the hFGF-2 purification without heparin affinity chromatography. In (B) the animal-component free purification methods are used directly as capture step and in (C) the alternative purification methods are used in a multi-step purification as intermediate purification step.

Table 1

Sample volumes for the experiments 3.1 and 3.2.

	CV	Sample volume for 3.1 (20 mL per 1 mL CV)	Sample volume for 3.2 (2.5 mL per 1 mL CV)
Membrane adsorber technology			
Sartobind S75	2.1	42	5.25
Prototype B	1	20	2.5
Prototype C	1	20	2.5
Prototype D	1	20	2.5
Prototype E	1	20	2.5
SUCA	3	60	7.5
Bead-based Column Chromatography			
HiTrap™ Capto™ MMC	1	20	2.5
Foresight™ Nuvia™ cPrime™	1	20	2.5
Capto™ DeVirS	4.7	94	11.75
HiTrap™ Heparin HP	1	20	2.5

proteins in the overlapping elution peaks (Fig. 2A; SDS-PAGE gel: fractions 3 – 5) can be assigned to impurities and the single peak (Fig. 2A; SDS-PAGE gel: fraction 6) to hFGF-2. The hFGF-2 protein fractions had a purity of 86 % with a yield of 52 % and a recovery of 82 %.

Purification by the MMC Prototype B resulted in one elution peak. The SDS-PAGE gel detected hFGF-2 in the whole elution peak (Fig. 2B; SDS-PAGE gel: fractions 3 – 6). The densitometric analysis resulted in a hFGF-2 purity of 57 %, a yield of 35 % and a recovery of 78 %.

Purification by the pseudo-affinity chromatography SUCA resulted in one elution peak. The SDS-PAGE gel indicated that hFGF-2 was present in all fractions of the elution peak (Fig. 2C; SDS-PAGE gel: fractions 3 – 10) and had an average purity of 60 % with a yield of 41 % and a recovery of 78 %.

Purification of hFGF-2 by the pseudo-affinity chromatography Capto™ DeVirS resulted in overlapping elution peaks. Based on the SDS-PAGE gel, hFGF-2 was detected in the latest elution peak (Fig. 3; SDS-PAGE gel: fractions 13 – 19) with a purity of 60 %, a yield of 73 % and a recovery of 82 %.

All results for the hFGF-2 purification using animal-component free purification materials in the capture step are summarized in Fig. 4 (and Table S2). In summary, in comparison to the results achieved in the conventional process, single-step purification of hFGF-2 using alternative purification materials was not possible. The high amount of host cell proteins of *E. coli* affected the interactions between hFGF-2 and the ligands. Heparin affinity chromatography resulted in the highest protein purity and yield under these conditions. Prototype B, SUCA and Capto™ DeVirS were identified as the best animal-component free alternatives regarding the pu-

Fig. 2. Chromatogram of the FPLC runs (left) and the SDS-PAGE analyses (right) of the capture steps of the hFGF-2 purification. The bands of hFGF-2 are highlighted at 18 kDa. (A) Heparin affinity chromatography: M: protein ladder; 1: sample; 2: flow through; 3 – 5: fractions of the overlapping elution peaks, 6: fractions of the hFGF-2 elution peak. (B) Prototyp B: M: protein ladder; 1: sample; 2: flow through; 3 – 6: fractions of the elution peak. (C) SUCA: M: protein ladder; 1: sample; 2: flow through; 3 – 10: fractions of the elution peak.

Fig. 3. Chromatogram of the FPLC run (top) and the SDS-PAGE analysis (bottom) of the Capto™ DeVirS as capture step in the hFGF-2 purification. The bands hFGF-2 are highlighted at 18 kDa. M: protein ladder; 1: sample; 2: flow through; 3 – 12: fractions of the overlapping elution peaks; 13 – 19: fractions of the hFGF-2 elution peak.

Fig. 4. Results of the hFGF-2 purification. Yield (dark grey), recovery (medium grey) and purity (light grey) of the hFGF-2 after one-step purification are plotted in a bar chart with standard deviation. The standard deviation is obtained by performing the experiments in triplicate. The vertical dotted lines highlight the individual systems (heparin affinity chromatography, CEX, MMC, pseudo-affinity chromatography).

Fig. 5. Purification of hFGF-2 from the cell supernatant by two connected cation exchangers (Sartobind® nano, 3 mL, Sartorius AG, Göttingen, Germany). Left: chromatogram of the FPLC run, right: gel of the SDS-PAGE analysis. M: protein ladder; 1: sample; 2: flow through; 3: elution peak at 300 mM NaCl; 4: hFGF-2 elution peak at 600 mM NaCl; 5: elution peak at 1 M NaCl. The bands hFGF-2 are highlighted at 18 kDa.

rity of hFGF-2 at 60 %. The Capto™ DeVirS was the best alternative in terms of yield and recovery.

These results show that two-step purification is necessary to reduce the amount of host cell proteins prior to a selective purification step for hFGF-2. Thus, the different methods were compared after a cation exchanger step like the established method [9] to see if one of the combinations could achieve similar efficiencies to the heparin affinity chromatography.

3.2. Alternative purification methods as an intermediate purification step

Purification of the hFGF-2 from the cell supernatant by alternative bead-based column materials and membrane chromatographic systems resulted in a maximum purity of 60 %. In order to achieve higher purities, the alternative methods were tested as intermediate purification steps after a previous strong cation exchanger capture step.

After centrifugation and sterile filtration (0.2 µm pore size) of the supernatant, the whole sample was purified by a cation exchange chromatographic step. The elution was optimized by an isocratic elution with three steps (300 mM, 600 mM and 1 M NaCl) and hFGF-2 was eluted in the second step (Fig. 5).

The hFGF-2 containing fractions were combined, resulting in a purity of 65 %. The concentration of hFGF-2 was 3.3 mg·mL⁻¹. To ensure that the hFGF-2 sample was purified under the same conditions as in the previous experiments (3.1), the fractions were first desalted. The sample has a hFGF-2 concentration of 0.6 mg·mL⁻¹ after desalting. The sample volume used for each downstream experiment was adjusted to the column/membrane volume (CV; Table 1). The sample volume was calculated with 2.5 mL per mL CV. The experiments were performed in triplicate.

In total, higher purities were obtained using this two-step purification method. The use of the heparin affinity chromatography for an intermediate purification step achieved the highest protein purity and yield. In this case, the MMCs HiTrap™ Capto™ MMC and Foresight™ Nuvia™ cPrime™ were identified as the best animal-component free purification materials for the purification of hFGF-2. Therefore, we have focussed on these chromatograms

and the corresponding SDS-PAGE analyses in the following section (Fig. 6).

Applying the chromatogram for the heparin affinity chromatography as an intermediate step resulted in two elution peaks. Based on the SDS-PAGE gel hFGF-2 was only present in the second elution peak (Fig. 6A; SDS-PAGE gel: fractions 8 + 9) with a purity of 94 %, a yield and recovery of 90 %. The purification of hFGF-2 using the MMCs HiTrap™ Capto™ MMC and the Foresight™ Nuvia™ cPrime™ both resulted in one elution peak. The SDS-PAGE gel indicated that hFGF-2 was present in all fractions of the elution peak (Fig. 6B; SDS-PAGE gel: fractions 4 + 5; Fig. 6C; SDS-PAGE gel: fractions 4 – 6) with a purity of 90 %. The yield and recovery were 85 %. The whole results in purity, yield and recovery are summarized in a bar chart in Fig. 7 (and Table S3).

Based on these experiments, it can be concluded that the use of heparin affinity chromatography showed the best downstream results for the hFGF-2 under these conditions. The structure of heparin leads to a special combination of interactions to hFGF-2, which has a positively effect in the purification process. In contrast, both materials used for pseudo-affinity chromatography resulted in a worse purification of hFGF-2 (Capto™ DeVirS and SUCA in Fig. 7) despite the structure of their ligands is mimicking heparin's structure and characterized by sulphated polysaccharides. Compared to heparin, only electrostatic interactions can be formed which is in this case less sufficient. The different mixed-mode materials resulted in varied purification efficiency. The mixed-mode membrane adsorbers (Prototype B to E in Fig. 7) purified hFGF-2 to a purity of less than 60 %. The structure of the ligands of the mixed-mode membrane adsorbers was not available but this composition had probably weaker interactions to the protein, which led to a worse purification. However, the bead-based columns HiTrap™ Capto™ MMC from GE Healthcare Life Sciences and Foresight™ Nuvia™ cPrime™ from Bio-Rad Laboratories are promising animal-component free alternatives. Under the tested conditions, they achieved a product purity of 90 % with a yield of 85 %. The ligands of the bead-based columns are similar characterized by a positively charged carboxyl group, a phenyl group and a primary amine. This composition of ligands led to a combination of different interactions (ionic interactions, hydrophobic interactions and hydrogen bonds) between the protein and the ligands,

Fig. 6. Chromatogram of the FPLC runs (left) and the SDS-PAGE analyses (right) of the intermediate purification steps of the hFGF-2 purification after cation exchange chromatography. The bands of hFGF-2 are highlighted at 18 kDa. (A) Heparin affinity chromatography: M: protein ladder; 1: sample; 2 + 3: flow through; 4 – 7: fractions of the overlapped peaks, 8 + 9: fractions of the hFGF-2 elution peak. (B) Foresight™ Nuvia™ cPrime™: M: protein ladder; 1: sample; 2: flow through; 3: washing step; 4 + 5: fractions of the hFGF-2 elution peak. (C) HiTrap™ Capto™ MMC: M: protein ladder; 1: sample; 2: flow through; 3: washing step; 4 – 6: fractions of the hFGF-2 elution peak.

Fig. 7. Results of the hFGF-2 purification. Yield (dark grey), recovery rate (medium grey) and purity (light grey) of the hFGF-2 after two-step purification are plotted in a bar chart with standard deviation. The standard deviation is obtained by performing the experiments three times. The horizontal dotted lines highlight the individual systems (heparin affinity chromatography, MMC, pseudo-affinity chromatography).

Fig. 8. NIH-3T3 cell proliferation depending on hFGF-2 concentration. (A): purification by MMC (HiTrap™ Capto™ MMC); (B): purification by MMC (Foresight™ Nuvia™ cPrime™).

which resulted in an efficient purification of hFGF-2. Since these two methods appear suitable as heparin alternatives, the buffer exchange and final polishing step by anion exchange chromatography was undertaken to check the endotoxin level and biological activity.

3.3. Endotoxin content

The last purification step of hFGF-2 was corresponded to the previously established polishing step by the anion exchange

membrane adsorber Sartobind™ Q75 (Sartorius AG, Göttingen, Germany) [9]. This step uses animal-component free materials and removes endotoxins and DNA. Endotoxins and DNA are negatively charged and bind to the anion exchanger. The protein hFGF-2 is positively charged and directly elutes. For medical use or application in cell culture systems the endotoxin level of hFGF-2 must be within a safety limit (less than 0.5 EU · mL⁻¹). The endotoxin level was determined by limulus amoebocyte lysate assay (LAL assay). The endotoxin level in all hFGF-2 samples was below the critical safety limit.

3.4. Biological activity test

The biological activity was analysed by the proliferation of the NIH-3T3 cells which provided information about the mitogenic activity of hFGF-2. These analyses revealed that hFGF-2 was bioactive when purified by alternative animal-free methods. hFGF-2 shows a dose-dependent biological activity (Fig. 8). The EC₅₀ was determined as 38 ng·mL⁻¹ for hFGF-2 purified by HiTrapTM CaptoTM MMC and as 19 ng·mL⁻¹ for hFGF-2 purified by ForesightTM NuviaTM cPrimeTM.

4. Conclusion

In conclusion, we demonstrated two animal-component free chromatographic purification materials for the purification of the growth factor hFGF-2. The exchange of the heparin in the affinity chromatography through the MMCs HiTrapTM CaptoTM MMC and ForesightTM NuviaTM cPrimeTM purified the hFGF-2 up to 90 % with a yield and recovery of 85 %. Additionally, the purified protein was also endotoxin free and bioactive.

This bead-based column chromatography can be adapted to other heparin-binding proteins. In the medium term, the production costs of heparin-binding proteins can be reduced since proteins purified without animal-based materials are subject to less stringent quality controls and synthetic materials are less expensive.

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Ethical approval

No studies with human participants or animals were performed by any of the authors for this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2020.461367](https://doi.org/10.1016/j.chroma.2020.461367).

CRediT authorship contribution statement

Svenja Nicolin Bolten: Conceptualization, Writing - original draft, Methodology, Investigation, Writing - review & editing. **Anne-Sophie Knoll:** Validation, Investigation. **Zhaopeng Li:** Validation, Writing - review & editing. **Pia Gellermann:** Validation. **Iliyana Pepelanova:** Validation. **Ursula Rinas:** Supervision. **Thomas Scheper:** Supervision, Resources, Writing - review & editing.

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