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Anion-exchange purification of recombinant factor IX from cell culture supernatant using different chromatography supports

Daniel A. Ribeiro, Douglas F. Passos, Helen C. Ferraz, Leda R. Castilho*

Federal University of Rio de Janeiro (UFRJ), COPPE, Chemical Engineering Program, Caixa Postal 68502, 21941-972 Rio de Janeiro, RJ, Brazil

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ABSTRACT

Both recombinant and plasma-derived factor IX concentrates are used in replacement therapies for the treatment of haemophilia B. In the present work, the capture step for a recombinant FIX (rFIX) purification process was investigated. Different strong anion-exchange chromatography media (the resins Q Sepharose® FF and Fractogel® TMAE, the monolith CIM® QA and the membrane adsorber Sartobind® Q) were tested for their rFIX binding capacity under dynamic conditions. In these experiments, crude supernatant from CHO cells was used, thus in the presence of supernatant contaminants and mimicking process conditions. The highest dynamic binding capacity was obtained for the monolith, which was then further investigated. To study pseudoaffinity elution of functional rFIX with Ca^{2+} ions, a design of experiments to evaluate the effects of pH, NaCl and $CaCl_2$ on yield and purification factor was carried out. The effect of pH was not statistically significant, and a combination of no NaCl and 45 mM $CaCl_2$ yielded a good purification factor combined with a high yield of active rFIX. Under these conditions, activity yield of rFIX was higher than the mass yield, confirming selective elution of functional, γ-carboxylated rFIX. Scaling-up of this process 8 fold resulted in very similar process performance. Monitoring of the undesired activated FIX (FIXa) revealed that the FIXa/FIX ratio (1.94%) was higher in the eluate than in the loaded sample, but was still within an acceptable range. HCP and DNA clearances were high (1256 and 7182 fold, respectively), indicating that the proposed process is adequate for the intended rFIX capture step.

1. Introduction

Factor IX (FIX) is a vitamin K-dependent protein and plays an important role in the coagulation system. When activated by activated factor VII (FVIIa) and activated factor XI (FXIa), FIX interacts with activated factor VIII (FVIIIa), forming a complex that is responsible for activating factor X, in this way propagating blood coagulation [1]. Deficiency of FIX causes the disease known as haemophilia B, which is treated by replacement therapy with either plasma-derived or recombinant FIX. The coagulation factor should be in its non-activated form, in order to avoid risk of thrombosis [2].

Post-translational modifications in FIX are required for its functionality, especially the γ-carboxylation of 12 glutamic acid residues in the N-terminal region [3,4]. However, recombinant factor IX (rFIX) might not be totally γ-carboxylated when expressed in mammalian cells, since these cells are usually not able to process all translated rFIX due to the limitation of some enzymes involved in the γ-carboxylation process [5–9]. Selectivity for the functional, γ-carboxylated form of rFIX is thus a challenge in downstream processing development. A possible purification strategy explores the affinity of γ-carboxylated residues for Ca^{2+} ions. In the purification process of Benefix®, a commercially available rFIX product, the first purification step is a Q Sepharose® chromatography with pseudoaffinity elution by the addition of calcium chloride [10]. This concept can be applied to all vitamin K-dependent proteins [11].

The works that described the use of calcium elution in anion-exchange chromatography employed resins like Q Sepharose® or Fractogel® TMAE. However, these materials present inherent diffusional limitations, limiting operation to low flow rates, which results in low productivity. Other disadvantages of resin-based processes include the risk of having preferential channels inside the packed bed and the poor mechanical resistance that can lead to particle deformation and bed compaction problems. As a consequence, initial column packing is a critical and labour-intensive step in resin-based processes [12]. Due to these limitations, different materials have been investigated in the last decade as alternative supports for protein chromatography [13–15].

Membrane adsorbers were developed with the aim of increasing the productivity of protein chromatography processes due to the predominance of convective transport, which decreases diffusional limitations and allows the use of higher flow rates at low

* Corresponding author. Tel.: +55 21 25628336; fax: +55 21 25628300.
E-mail address: leda@peq.coppe.ufrj.br (L.R. Castilho).

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pressure drops. Additional advantages of using membranes as supports are the better mechanical resistance, the modular scale-up and the possibility of having disposable units. However, the lower binding capacity is usually a drawback of membrane adsorbers as compared to resins [13].

Monoliths are another type of supports that have been developed. They have been successfully used to purify many proteins and also large molecules, such as DNA and virus. In spite of the low diffusivity of large molecules, the large pores of monoliths enable convective mass transport, avoiding diffusional limitations, and allow high flow rates to be applied. Additionally, monoliths are easy to manufacture in different sizes and can be used in both preparative and analytical chromatography [14–17]. Although Factor IX is not a very large protein (55 kDa), some publications described the use of monoliths to purify FIX from plasma [18,19]. No reports on the use of monoliths for purification of recombinant FIX produced by mammalian cells have been found in the literature.

In this work we investigate the use of different supports (two resins and two convective media, a monolith and a membrane) bearing strong anion-exchange ligands for purification of rFIX from cell culture supernatant. Using the adsorbent with highest dynamic binding capacity, pseudoaffinity elution with calcium chloride was then investigated. The final process was evaluated at 8-fold larger scale and investigated for activated FIX (FIXa), HCP and DNA removal.

2. Experimental

2.1. Materials and equipment

Anion exchange monoliths CIM® QA (0.34-mL discs, and 1-mL and 8-mL columns) were supplied by Bia Separations (Slovenia). Sartobind® Q membranes were provided by Sartorius (Germany). Q Sepharose® FF (1-mL Hitrap® column and resin slurry) was from GE Healthcare (Sweden) and Fractogel® TMAE (resin slurry) from Merck (Germany).

All reagents and bovine serum albumin were purchased from Sigma–Aldrich (USA). FIX and FIXa activity kits were from Hyphen Biomed (France) and the standard plasma Unicalibrator was from Stago (France). The host cell protein ELISA kit (Kribiolisa) was from Krishgen Biosystems (India). The Qubit™ DNA quantification kit was from Invitrogen/Life Technologies (USA).

Chromatography runs were carried out in Äkta Purifier and Äkta Explorer systems (GE Healthcare, Sweden). Absorbance readings were carried out using a PowerWave XS microplate reader (BioTek, USA).

2.2. Production of recombinant factor IX

Chinese hamster ovary (CHO) cells expressing recombinant human factor IX were cultivated in a chemically defined, animal-component-free culture medium (TeutoCell AG, Germany). Cell culture supernatants used in this work originated from two different cultures: identical aliquots from a lot resulting from a pool of batch culture supernatants were used in the dynamic binding capacity study and in the experimental design runs. Aliquots from a fed-batch culture supernatant were used in the scale-up experiment. The batch cultures had approximately 6.0×10^6 cells/mL and 98% viability at moment of harvest, whereas the fed-batch culture had approximately 20.0×10^6 viable cells/mL and 70% of viability at harvest. The supernatant was clarified by centrifugation at $150 \times g$ and aliquots were stored at −70 °C until further use. Upon thawing, all supernatant aliquots were filtered through a 0.2 μm membrane prior to use.

Table 1
Design of experiments to evaluate elution conditions. Absolute values for pH, [Ca^{2+}] and [NaCl] are shown in parentheses, with concentrations given in mM.

Run	pH	[Ca2+]	[NaCl]	Y_{activ}	Y_{mass}	PF
1	−1 (6.5)	−1 (15)	−1 (0)	11.9	12.0	254
2	1 (8.5)	−1 (15)	−1 (0)	1.3	10.3	378
3	−1 (6.5)	1 (45)	−1 (0)	83.7	41.9	305
4	1 (8.5)	1 (45)	−1 (0)	76.8	31.4	334
5	−1 (6.5)	−1 (15)	1 (100)	83.2	69.4	138
6	1 (8.5)	−1 (15)	1 (100)	86.1	48.0	179
7	−1 (6.5)	1 (45)	1 (100)	97.3	93.6	86
8	1 (8.5)	1 (45)	1 (100)	104.5	97.5	132
9	0 (7.5)	0 (30)	0 (50)	87.9	39.6	270
10	0 (7.5)	0 (30)	0 (50)	91.6	37.8	253
11	0 (7.5)	0 (30)	0 (50)	82.9	33.1	179

Y_{activ}, yield in activity; Y_{mass}, yield in mass (ELISA); PF, purification factor.

2.3. Determination of the dynamic binding capacity for BSA and FIX

The dynamic binding capacity was determined for four materials: monolith CIM® QA (3×0.34-mL discs, totalizing 1.02 mL bed volume), Q Sepharose® FF (1-mL Hitrap column), Fractogel® TMAE (1-mL column) and Sartobind® Q membrane (75 cm^2 nominal area, equivalent to a packed volume of 2 mL). The dynamic binding capacity was defined as the amount of adsorbed protein at 10% breakthrough, as measured by absorbance at 280 nm (for BSA) and by an enzyme-linked immunosorbent assay (ELISA) (for rFIX).

Initial evaluation was done with bovine serum albumin (BSA) at 1 mg/mL as model protein, using the following buffers and flow rates: 20 mM Tris pH 7.5 for the monolith (3 mL/min) and the membrane (5 mL/min), and 50 mM Tris pH 8.3 (1 mL/min) for the resins. The conditions were selected according to the suppliers, when the information was available.

To determine the dynamic binding capacity for rFIX, 118-mL aliquots of the batch-culture supernatant lot (with no dilution nor buffer exchange) were applied to the chromatography media at 5 mL/min for the monolith and the membrane and 1 mL/min for the resins. Samples were collected and rFIX concentration was determined by ELISA.

2.4. Design of experiments (DOE) to evaluate elution conditions

A full factorial DOE with 3 variables and triplicate of the central point (Table 1) was carried out with the monolith CIM® QA (1-mL column) to investigate the following elution conditions: buffer pH, $CaCl_2$ concentration and NaCl concentration. The responses evaluated were purification factor, purity, activity yield and mass yield of Factor IX. Aliquots of the batch-culture supernatant lot were diluted three times with equilibration buffer (50 mM Tris–HCl, 0.15 M NaCl, pH 8), reaching a FIX protein concentration of 0.012 mg/mL (or 0.54 IU/mL). A volume of 225 mL of diluted supernatant was injected at 5 mL/min in every run, and 10-mL fractions were collected. After each run, the column was regenerated with 50 mM Tris–HCl, 2 M NaCl, pH 7.5 and sanitized for 1 h with 1 M NaOH. Data were qualitatively evaluated using cube plots and quantitatively analyzed (ANOVA and Pareto chart) using the software STATISTICA 7.

2.5. Process scalability and monitoring of critical contaminants

In order to evaluate scalability of the monolith-based process, 1- and 8-mL CIM® QA columns were compared. For the 1-mL column, a 3-fold diluted supernatant volume originating from the fed-batch lot, containing 350 UI of factor IX, was injected at a flow rate of 3.7 mL/min, and 2-mL fractions were collected. For the 8-mL column, pre-diluted supernatant containing 2500 UI of factor