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Process-scale purification and analytical characterization of highly gamma-carboxylated recombinant human prothrombin

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ABSTRACT

Prothrombin (coagulation Factor II) is a complex multidomain glycoprotein that plays a central role in blood coagulation. It is the zymogen precursor to the protease thrombin that catalyzes the formation of the fibrin clot and regulates a multitude of other cellular responses related to coagulation and hemostasis. For the biological activity of prothrombin, the vitamin K dependent posttranslational modification of glutamic acid residues to gamma-carboxylglutamic acid is of crucial importance. Prothrombin can be recombinantly expressed using mammalian cell culture. However, the product is a heterogeneous mixture of variants with different degrees of carboxylation, requiring separation of closely related charge isoforms. A second challenge for purification is the need to remove traces of the product-related impurity thrombin, a protease, to extremely low levels. In this work, we describe a purification strategy that provides solutions to both challenges and results in an efficient and robust process for active recombinant prothrombin. We also describe the analytical characterization of recombinant prothrombin by HPLC, LC–MS/MS, and complementary biochemical assays.

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1. Introduction

Human coagulation Factor II, or prothrombin, is the zymogen precursor of the protease thrombin that plays a central role in blood coagulation, thrombosis and hemostasis. Prothrombin is a single-chain, multidomain glycoprotein with a molecular weight of 72 kDa (Fig. 1). It is initially synthesized in the liver as a preproprotein that undergoes several posttranslational modifications: Upon import of the nascent polypeptide chain into the endoplasmic reticulum (ER), the signal peptide is removed by a microsomal signal peptidase, generating the pro-form of the molecule. The pro-peptide is recognized by the enzyme gamma-glutamyl carboxylase (GGCX) that catalyzes the carboxylation of ten glutamic acid residues in the amino-terminal portion (Gla-domain) of the molecule [1–3]. This process requires reduced vitamin K, oxygen and carbon dioxide [4]. In the course of this reaction, vitamin K is oxidized to the 2,3-epoxide form, which is subsequently converted back to the reduced form by the enzyme vitamin K-oxidoreductase (VKOR). This essential posttranslational modification confers metal-chelating properties on the protein, which allow the interaction with phospholipid membranes that concentrates prothrombin activation at the site of vascular injury [5]. Following gamma-carboxylation, the pro-peptide is cleaved off by a furin-like convertase. After attachment of three N-linked oligosaccharide chains, the mature zymogen is secreted from the hepatocyte into the blood.

Blood coagulation is a complex cascade of reactions that ultimately results in the formation of a fibrin clot and cessation of bleeding [6]. In both possible sequences, the intrinsic and extrinsic pathway, inactive enzyme precursors (zymogens) are proteolytically converted into the active proteases that then catalyze the subsequent step in the cascade. Both pathways merge into a final common pathway which involves the pivotal activation of prothrombin to thrombin. Thrombin then catalyzes the conversion of fibrinogen into the fibrin clot. The activation of prothrombin requires the assembly of the “prothrombinase” complex consisting of factor Xa, factor Va, calcium and anionic phospholipids [7]. An initial cleavage at residue R320 opens the active site and forms meizothrombin, and a second proteolysis at R271 generates thrombin and releases fragment 1.2 that contains the Gla-domain and two consecutive homologous domains characterized by three internal disulfide bonds, the so-called kringle domains. Thrombin consists of a light A chain disulfide-linked to the heavy B chain that contains the serine-dependent active site characterized by the chymotrypsin fold [8].

Besides the crucial role of prothrombin activation in blood coagulation and hemostasis, thrombin elicits various other cellular responses including the stimulation of platelet aggregation,

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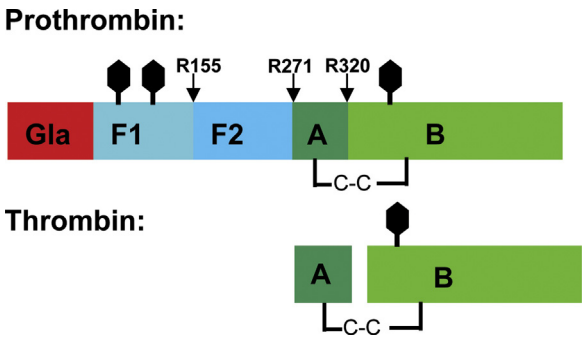

Fig. 1. Schematic representation of the primary structure of prothrombin and its proteolytic activation to thrombin. Prothrombin is a 72 kDa single-chain glycoprotein that contains the Gla domain, two kringle domains (part of fragments F1 and F2, respectively), and a serine protease domain consisting of the A and B chains. Cleavage at R320 by the prothrombinase complex generates the active site, and the subsequent cleavage at R271 creates the trypsin-like serine protease thrombin and releases fragment 1.2, which consists of the Gla domain and two tandem kringle domains. Solid hexagons indicate N-linked carbohydrate chains.

the proliferation of endothelial cells, mitogenesis of fibroblasts and various other processes mostly regulated through proteinase-activated receptors (PARs) [9]. Recent interest has focused on the role of prothrombin activation in angiogenesis and tumor growth [10,11].

In this work, we describe the process-scale purification and analytical characterization of recombinant prothrombin. Due to the extensive posttranslational modifications that are critical to the biochemical and pharmacokinetic properties of prothrombin, mammalian cells are the expression system of choice. To enhance the carboxylation of the protein, a suspension-adapted CHO cell line was developed that co-expresses the enzymes GGCX and VKOR. Together with the addition of vitamin K to the cell culture medium, this allows the efficient expression of prothrombin. Mass spectrometric analysis of the resulting recombinant protein revealed a distribution of gamma-carboxylation levels in the Gla domain. To investigate in detail the effect of carboxylation on biological activity, we separated charge variants and subjected them to structure–function studies combining mass-spectrometric peptide mapping, HPLC analysis and complementary biochemical assays. In contrast to other carboxylated coagulation factors like Factor VIIa or factor IX [12–15], a strong correlation was found between even minor changes in carboxylation of prothrombin and its bioactivity. The purification process was developed to enrich highly carboxylated prothrombin, while accommodating variability in Gla levels intrinsic to the recombinant expression system.

2. Materials and methods

2.1. Chemicals and recombinant protein

All chemicals used in the purification process were purchased from Sigma (St. Louis, MO, USA), VWR Scientific (West Chester, PA, USA) or Avantor Performance Materials (Center Valley, PA, USA). Human alpha-thrombin for spiking experiments was obtained from Enzyme Research Laboratories (South Bend, IN, USA). Recombinant human prothrombin (rh coagulation Factor II) was expressed in suspension-adapted CHO cells co-expressing two proteins for posttranslational modification (GGCX and VKOR).

2.2. Chromatography media and instrumentation

Q Sepharose Fast Flow (QFF) and Phenyl Sepharose HP were obtained form GE Healthcare (Piscataway, NJ, USA). Bakerbond XWP 500 Poly PEI-35 resin was obtained from Avantor (Center Valley, PA, USA). Macro-prep ceramic hydroxyapatite (CHT) type I (40 μm) was from Biorad (Hercules, CA, USA). Laboratory-scale chromatography experiments were performed using a GE Healthcare ÄKTA Explorer 100. Process-scale chromatography was performed using a K-Prime chromatography system obtained from Millipore (Billerica, MA, USA) or an AKTA Pilot from GE Healthcare. For process-scale, Quickscale columns from Millipore and BPG columns from GE Healthcare were employed, whereas for bench-scale experiments Millipore Vantage columns of 1.1 cm diameter or GEHC columns XK16 were used.

2.3. Analytical HPLC

Analytical high-performance ion exchange chromatography (HPIEC) was developed to monitor the charge heterogeneity in prothrombin. HPIEC was performed on an Agilent (Santa Clara, CA, USA) HPLC system using a Proteomix SAX NP10 column with 20 mM Tris, pH 8.5 as equilibration buffer and 20 mM Tris, 500 mM NaCl as elution buffer. The column was equilibrated at a flow rate of 1.0 ml/min and protein was eluted with a linear gradient from 150 to 375 mM NaCl in 10 mM Tris, pH 8.0. Elution profiles were monitored by UV absorbance at 280 nm.

Analytical size-exclusion chromatography (HPSEC) was performed on an Agilent HPLC system using a TSK-gel G3000SW$_{XL}$ column from Tosoh Bioscience (King of Prussia, PA, USA). The column was equilibrated at a flow rate of 0.5 ml/min with 16 mM sodium phosphate, 0.5 M sodium chloride, pH 7.4. 250 μg of protein was injected and eluted isocratically with the same buffer. Elution profiles were monitored by UV absorbance at 280 nm.

Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was employed to monitor fragment levels in prothrombin preparations. The method was performed using a YMC-Pack Protein-RP column (250 mm × 2.0 mm, S-5 μm) from YMC America (Allentown, PA, USA) with an Agilent HPLC1200 system. Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. The sample was eluted with a linear gradient of increasing mobile phase B at a flow rate of 0.4 ml/min. Elution profiles were monitored by UV absorbance at 280 nm.

2.4. Peptide mapping and LC–MS/MS analysis

Peptide mapping was performed to determine posttranslational modifications of prothrombin. Briefly, protein samples were first incubated with N-ethylmaleimide to cap all possible free thiols, and then mixed with guanidine for denaturation. The digestion procedure was performed by incubating samples with endoproteinase Lys-C overnight at neutral pH. Lys-C-generated fragments were separated by reversed-phase UPLC, and analyzed using a Thermo LTQ Orbitrap mass spectrometer (Thermo electron, San Jose, CA, USA) in positive ion mode. Each peptide was identified by the molecular weight as determined from its MS data and by the fingerprint fragmentation determined from its MS/MS data.

2.5. Analysis of N-linked carbohydrates

Protein glycosylation was analyzed by cleaving the N-linked oligosaccharides using PNGase F followed by fluorescent 2-AB labeling and separation using normal-phase HPLC with fluorescence detection and excitation and emission wavelengths of 330 and 420 nm, respectively [16]. In addition, an orthogonal sialic-acid determination method was employed based on acetic acid hydrolysis and DMB labeling followed by RP-HPLC separation [17].

2.6. Bioassays

2.6.1. Calibrated automated thrombogram (CAT) assay [18,19]

The CAT assay was used to characterize the thrombin generation potential of a prothrombin sample and to derive the potency relative to an internal reference standard and the NIBSC WHO International standard. Protein samples to be analyzed were prepared at four different concentrations, and were added to Factor II-deficient platelet-poor plasma (Beckman Coulter) and a 'trigger-solution' (containing Tissue Factor, Ca-ions, phospholipids). A slow fluorogenic thrombin substrate (Diagnostica Stago Inc., Parsippany, NJ, USA) was added and fluorescence generated by the action of thrombin was measured with the Fluoroskan Ascent Fluorometer, equipped with Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands), using an excitation wavelength of 390 nm and an emission wavelength of 460 nm. The Endogenous Thrombin Potential (ETP) was measured as the area under the thrombogram curve for each sample at four concentrations, and the relative potency was calculated by the slope-ratio method.

2.6.2. Clot assay

The clot assay measures the time taken to initiate clot formation in prothrombin-deficient platelet-poor plasma. Samples to be analyzed were added to prothrombin-deficient plasma in the presence of Tissue Factor, phospholipids and Ca-ions. The presence of prothrombin initiates clot formation, that in turn causes an increase in turbidity which was detected with the ACL TOP 500 CTS coagulation analyzer (Beckman Coulter, Brea, CA, USA). The time required to reach a set threshold of optical density (the prothrombin clot time) is directly proportional to the amount of prothrombin that can form active thrombin. Test sample biological activity was determined by comparing the test sample clot formation time to an in-house reference standard that has been calibrated to the WHO 3rd International standard for Factors II, VII, IX, and X (NIBSC 99/826).

2.6.3. Free thrombin assay

Residual thrombin was quantitated using the synthetic fluorogenic substrate SensoLyte-AFC (AnaSpec, Fremont, CA, USA). Substrate cleavage by the action of thrombin results in fluorescence of the released 7-amido-4-trifluoromethylcoumarin (AFC) group that was measured using an excitation wavelength of 380 nm and an emission wavelength of 500 nm. The value was regressed from a thrombin standard curve. The lower limit of quantitation in this assay was determined as 0.225 ng/ml (6.25 pM).

2.7. Detection of process-related impurities

Host cell protein (HCP) levels were quantitated using a sensitive fluorescent capture immunoassay on the Gyrolab platform (GE Healthcare, Piscataway, NJ, USA). Sheep anti-CHO HCP was used as a capture and detection antibody. The sheep anti-CHO HCP antibody has coverage of approximately 80% as determined by 2D Western Blot analysis. HCP content was calculated based on a standard curved using CHO HCP prepared from null mock-transfected cells. CHO DNA levels were determined by quantitative PCR using CHO-DNA specific primers. This method used forward and reverse primers targeting a unique and highly repetitive DNA sequence in the CHO genome. Prior to analysis, samples were combined with chaotropic salts and precipitated with isopropanol to isolate residual CHO DNA. CHO DNA content was calculated based on a standard curve using CHO genomic DNA.

2.8. Process chromatography and filtration

2.8.1. Q Sepharose FF capture chromatography

For the purification of product produced at 500 L bioreactor scale, a column with 30 cm diameter was employed and packed to a bed height of 11–15 cm. Optimum column load challenge is 4–11 g/L resin. The Q FF capture column was equilibrated with 10 column volumes (CVs) of 25 mM sodium citrate, 30 mM sodium chloride, pH 6.0, at a linear velocity of 200 cm/h. Conditioned media (CM) was adjusted to pH 6.0 with 250 mM sodium citrate, pH 4.75 and was loaded onto the column, followed by a 10 CV re-equilibration step. Bound protein was washed with 5 CVs of 25 mM sodium citrate, 173 mM sodium chloride, pH 6.0 and eluted with 25 mM sodium citrate, 308 mM sodium chloride pH 6.0. Elution of the product was monitored at 280 nm.

2.8.2. Q Sepharose FF flowthrough chromatography

For the second chromatography step, a separate Q FF column was packed and operated in flow-through mode, in contrast to the bind and elute operation of the previous step. For the purification at 500 L bioreactor scale, a 30 cm diameter column was employed with a resin bed height of 11–15 cm. Optimum column load challenge for this flowthrough step was 2–6 g/L. The column was equilibrated with 10 column volumes (CVs) of 25 mM sodium citrate, 400 mM sodium chloride, pH 6.0, at a linear velocity of 200 cm/h. Eluate from the Q FF capture column was adjusted to a conductivity of 41 mS/cm with 3 M NaCl and was loaded onto the column, followed by re-equilibration with 25 mM sodium citrate, 400 mM sodium chloride, pH 6.0. The product is in the non-bound fraction from this column. This elution pool is treated with a solvent/detergent mixture for the inactivation of enveloped viruses.

2.8.3. Poly PEI chromatography

At the 500 L bioreactor scale, a 25 cm × 13 cm column was employed. Optimum column load challenge was determined as 5–12 g/L. The Poly PEI column was pre-equilibrated with 125 mM HEPES, 0.4% citrate, 0.5 M NaCl, pH 6.5 for 5 CVs and subsequently equilibrated with 25 mM HEPES, 0.4% citrate, 0.5 M NaCl, pH 6.5 for 10 CVs. The eluate pool from the Q FF flow through column (with or without prior solvent–detergent treatment) was loaded onto the column at a linear flow rate of 140 cm/h. The column was re-equilibrated with 25 mM HEPES, 0.4% citrate, 0.5 M NaCl, pH 6.5 for 8 CVs, then washed with 25 mM HEPES, 0.4% citrate, 1.04 M NaCl, pH 6.5 for 5 CVs. Elution was achieved with a linear gradient from 1.04 to 2.1 M NaCl (in 25 mM HEPES, 0.4% citrate, pH 6.5) over 18 CVs.

For initial experiments aimed at evaluating charge variant separation at bench scale, the elution gradient was performed from 0.5 to 1.86 M NaCl over 25 CVs. For these experiments, a 1.1 cm × 13 cm column was used. Both at process-scale and at bench scale, fractions were collected and analyzed by HPIEC to determine pre-peak level and to characterize the extent of gamma-carboxylation.

2.8.4. Phenyl Sepharose HP chromatography

For the 500 L process-scale purification, a 25 cm × 13 cm column was used. For this step, optimum column load challenge was determined to be 3–10 g/L. The Phenyl HP column was equilibrated with 25 mM HEPES, 0.4% citrate, 1 M sodium sulfate, pH 6.5. The eluate pool from the PPEI column was adjusted with 25 mM HEPES, 0.4% citrate, 1 M sodium sulfate, 1.86 M NaCl pH 6.5, at a ratio of 1:0.66 (w/w) and loaded onto the column at a linear flow rate of 100 cm/h, followed by re-equilibration with 25 mM HEPES, 0.4% citrate, 1 M sodium sulfate, pH 6.5. Bound protein was washed with 25 mM HEPES, 0.4% citrate, 0.75 M sodium sulfate, pH 6.5, and elution is performed with a linear gradient from 0.75 to 0 M sodium sulfate in the same buffer.

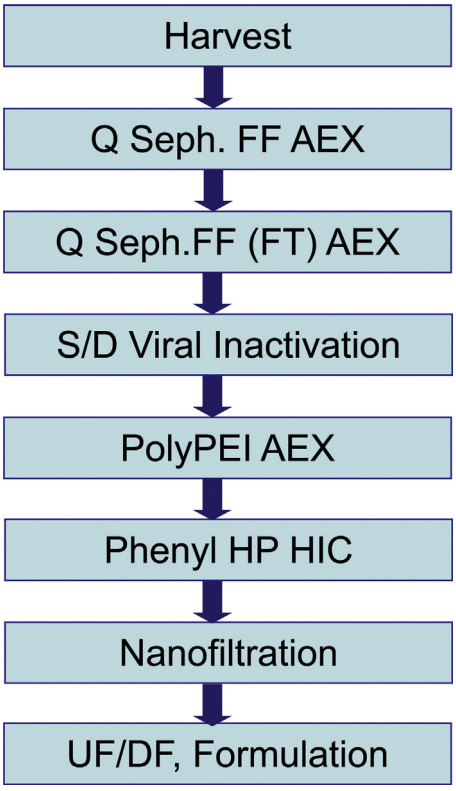

Fig. 2. Overview of the downstream process used to manufacture recombinant human prothrombin.

2.8.5. Ceramic hydroxyapatite chromatography

For the small scale preparative separation of prothrombin charge variants, a 1.1 cm × 13 cm CHT column was employed. The column was equilibrated with 20 mM sodium phosphate, 1 M NaCl, pH 6.5, for 10 CVs. Protein in the same buffer was loaded onto the column at 100 cm/h, followed by re-equilibration for 5 CVs. Elution was achieved with a linear gradient to 200 mM sodium phosphate, 2 M NaCl, pH 6.5 over 7 CVs.

2.8.6. Viral filtration and final UF/DF

Virus filtration was performed using Planova 20N membranes (Asahi Kasei, Tokyo, Japan) at a capacity of ≤30 L/m^2 and a differential pressure of 12 psig with 20 mM HEPES, 14 mM sodium citrate, pH 6.5, as the buffer system. For final product concentration and diafiltration, Pellicon 2 Biomax membranes (Millipore, Billerica, MA, USA) were employed with a capacity of ≤50 g/m^2. Crossflow was 6 L/min/m^2 at a transmembrane pressure of ≤20 psig during concentration and ≤30 psig during diafiltration.

3. Results and discussion

3.1. Process description and overview

Recombinant prothrombin was expressed in suspension-adapted CHO cells that co-express two enzymes for post-translational modification, GGCX and VKOR. An overview of the downstream manufacturing process is provided in Fig. 2. As prothrombin is an acidic glycoprotein, an anion exchange step was developed for product capture. As typical for coagulation factor processes, viral inactivation is performed by solvent–detergent treatment. The Poly PEI anion exchange chromatography step is operated in gradient mode to allow enrichment of highly carboxylated prothrombin while accommodating variability in Gla levels produced in the bioreactor. The final hydrophobic interaction chromatography (HIC) column on Phenyl HP is likewise operated in gradient mode to allow tight control over thrombin removal.

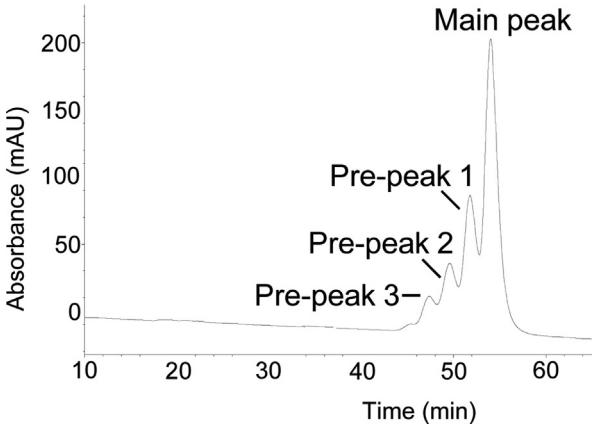

Fig. 3. Representative analytical HPIEC chromatogram for recombinant prothrombin after the first two downstream steps, illustrating the protein's charge heterogeneity.

3.2. Q FF capture and flowthrough chromatography

As human prothrombin is an acidic protein with an isoelectric point range of 5.1–5.5, an anion exchange capture step was developed using Q Sepharose FF. This step was optimized to efficiently reduce host cell proteins, DNA and other impurities such as bioreactor medium and feed components. Typical step yield for capture from a 500 L bioreactor is 65%. A subsequent second anion exchange step is operated in flow-through mode at higher conductivity, also using Q Sepharose FF and a buffer system of 25 mM sodium citrate, 400 mM sodium chloride. The product does not bind to the column under these conditions and is collected in the flowthrough fraction. At 500 L scale, a typical step yield of 90–95% is observed. After these two downstream steps, protein purity as determined by HPSEC is typically >98%. The eluate pool is then treated with a solvent/detergent mixture which serves as inactivation step for enveloped viruses.

3.3. Characterization of charge heterogeneity

Fig. 3 shows the HPIEC analysis after these first two downstream steps. The profile is characterized by one main peak and several pre-peaks that show run-to-run variability from 20 to 40% of the overall peak area. To characterize the variants giving rise to this profile, the HPIEC main peak and the pre-peaks were collected and subjected to comprehensive analysis, including mass spectrometry, oligosaccharide profiling, and bioassay testing. Peptide mapping was employed to characterize the extent of gamma-carboxylation of the ten glutamate residues forming the Gla domain of prothrombin.

As illustrated in Fig. 4, this technique in combination with enzymatic digest with the proteinase Lys-C allowed close monitoring of carboxylation in the Gla domain. LysC-generated fragment K1 (comprising residues 1–10 of mature prothrombin) encompasses the first two Gla residues, and peptide fragment K2 (residues 11–43) contains the remaining 8 Gla residues. Based on the intensity of peaks corresponding to the fully and partially carboxylated peptides, the relative extent of different species can be determined. Analytical results for the characterization of separated pre-peaks are summarized in Table 1. The charge variants giving rise to the indicated pre-peaks could be identified as protein molecules

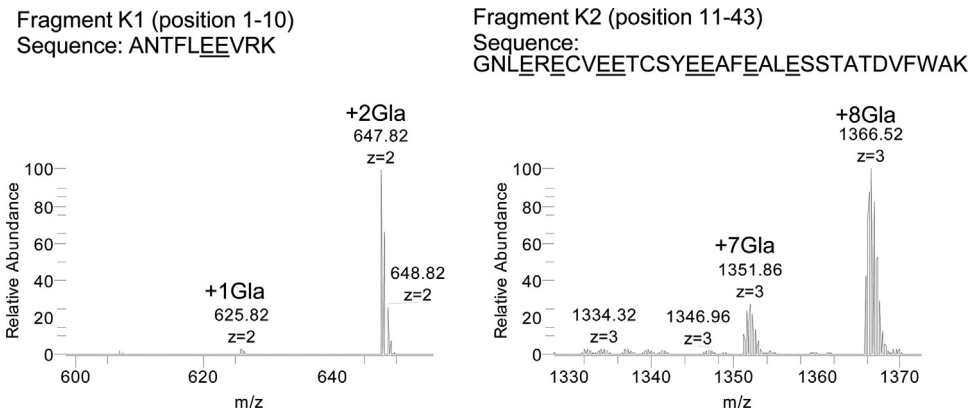

Fig. 4. Peptide mapping of recombinant human prothrombin to characterize the extent of carboxylation in the Gla domain. The left mass spectrum corresponds to fragment K1 after enzymatic digest with proteinase Lys C (containing residues 1–10 in the sequence of mature prothrombin and encompassing the underlined two carboxylation sites), and the right spectrum characterizes fragment K2 that contains the remaining 8 gamma-carboxylation sites. Based on these mass spectra, the relative extent of different species can be calculated.

Table 1
Characterization of main peaks and isolated pre-peaks observed in the HPIEC analysis of recombinant human prothrombin shown in Fig. 4.

Sample	Gla residues by MS peptide mapping[a]	Sialic acid content (mol/mol)	Clot bioassay, relative activity (%)	CAT bioassay, relative activity (%)
Main peak	9.5	1.3	92	108
Pre-peak 1	8.6	1.4	23	46
Pre-peak 2	7.7	1.4	9	33
Pre-peak 3	7.0	1.1	5	0

[a] Total Gla residues out of 10 possible gamma-carboxylation sites.

that are missing 1–3 Gla residues. These variants are lacking the corresponding amount of negative charges in their N-terminal region and therefore elute early from the anion exchange column. Differences in acidic oligosaccharide content do not contribute to the observed peak profile, as all isolated pre-peaks had similar sialic acid content and a similar overall oligosaccharide profile.

For recombinant prothrombin, these data suggest that missing one carboxyl group in the Gla domain significantly reduces the biological activity. Undercarboxylated variants therefore represent a product-related variant that is associated with reduced biological activity and needs to be well controlled in the manufacturing process.

Both peptide mapping and bioassays are laborious, low-throughput methods that are not suitable for routine process development. Therefore, for process development and manufacturing control, analytical HPIEC served as surrogate for characterizing levels of undercarboxylated variants corresponding to low biological activity.

3.4. Process-scale separation of charge variants

Several orthogonal separation modalities were evaluated for their potential to separate prothrombin charge variants. Such separations are often challenging at process-scale, due to mass transfer limitations and concurrent band broadening as a result of the larger bead size in preparative media. Fig. 5 shows the results for the bench-scale separation using ceramic hydroxyapatite (Panel A) and the weak anion exchange resin Bakerbond XWP 500 Poly PEI-35 (Panel B). Of all resins evaluated (including Source 15Q, 500-PolyQuat-35, DEAE, Q HP) these two allowed the best resolution. In both cases, the main elution peak is preceded by several pre-peaks. Fractions corresponding to individual peaks were pooled and subjected to bioactivity testing, pre-peak characterization by HPIEC

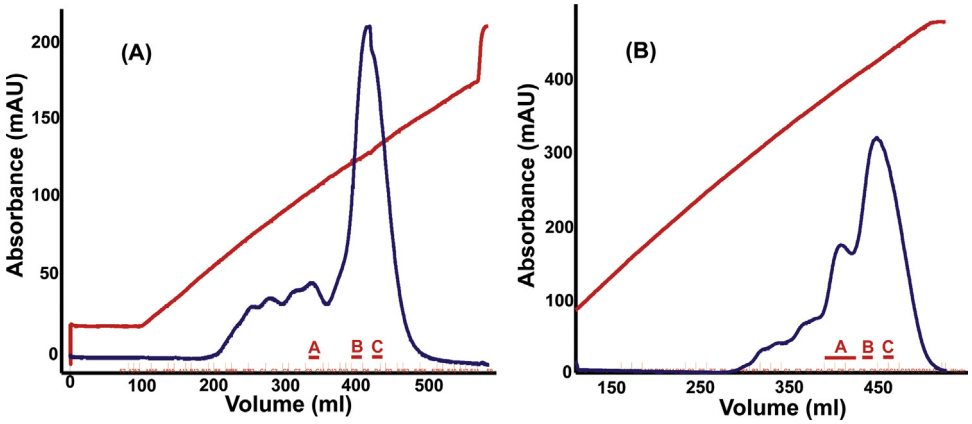

Fig. 5. Preparative separation of prothrombin charge variants on CHT (panel A) and Poly PEI (panel B) at bench scale. Partially purified prothrombin was bound to the resins and eluted with a shallow linear gradient. Exemplary fractions characterized in Table 2 are indicated as A through C.

Table 2
Characterization of prothrombin charge variants isolated by bench-scale CHT and Poly PEI columns.

Fraction	Peptide mapping: total missing Gla[a]	HPIEC pre-peak (%)	CAT bioassay, relative activity (%)	Clot bioassay, relative activity (%)
Separation on CHT				
A	1.35	90.7	24	23
B	0.5	22.3	57	78
C	0.29	10.6	78	106
Separation on Poly PEI				
A	1.31	24.5	25	21
B	0.51	29.8	63	81
C	0.18	1.8	81	106

[a] This value represents the average missing Gla residues in the respective sample (out of a total of 10).

and mass spectrometric peptide mapping. Results for exemplary fractions are summarized in Table 2, and overall results are illustrated by Fig. 6. As expected for elution behavior on an AEX resin, fractions eluting later in the gradient show more complete carboxylation, similar to results reported for the fractionation of Factor IX subpopulations with varying degree of carboxylation [15]. Corroborating the data from the analytical variant separation, our structure–function study for prothrombin revealed a clear correlation between missing Gla residues and reduced bioactivity, and highlights the need for the downstream process to control the gamma-carboxylation level in the final product. The chromatographic profile for the Poly PEI column mimics very closely the profile observed for the analytical HPIEC column, illustrating that undercarboxylated prothrombin charge variants can successfully be separated on preparative chromatography resins. This column allowed an easier process fit than CHT and was selected for the downstream process. Spiking studies with commercially available thrombin illustrate that this protein, which lacks the highly charged Gla and the F1 domains, binds poorly to the column under the conditions chosen and is found mainly in the flow through and the wash fraction.

Fig. 7 shows a representative chromatogram of the Poly PEI column operated at process-scale and illustrates the scalability and reproducibility of prothrombin charge variant separation on this anion exchange resin. The in-process control strategy uses analytical HPIEC for fraction analysis. The gradient operation on this column enables overall process robustness and manufacturing consistency. This pivotal process step can accommodate variability in product quality and column feed stream and ensures the consistent manufacture of a final product with acceptable biological activity, while achieving a yield of 50–75% (depending on the level of undercarboxylated material in the column load).

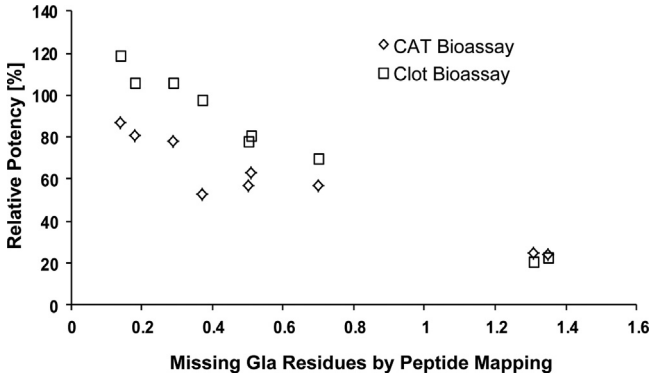

Fig. 6. Correlation of the degree of gamma-carboxylation as determined by mass spectrometric peptide mapping and observed bioactivity for prothrombin charge variants. Fractions separated on bench-scale CHT and Poly PEI columns were analyzed.

3.5. HIC polishing step

The second challenge for any prothrombin manufacturing process is to reduce the level of its proteolysis product, thrombin, to extremely low levels. Thrombin can degrade its own precursor prothrombin, resulting in product fragmentation and reduced stability. High levels of free thrombin also raise concerns for patient safety. The need to efficiently remove thrombin is exacerbated by the fact that traces of thrombin present in the product pool can not only fragment prothrombin, but were found to autocatalytically generate more thrombin, especially at ambient temperature (Fig. 8). A HIC step using Phenyl HP was developed to efficiently remove thrombin and also clear any fragments of prothrombin formed in the production process. Thrombin spiking studies were employed to delineate in detail the elution behavior of thrombin on this column. Whereas prothrombin fragment F1 elutes earlier from this column than prothrombin, the more hydrophobic protease thrombin binds considerably tighter to the Phenyl HP column and elutes at lower conductivity and in the water strip peak. To allow the tightest control of product-related impurity clearance, this column is also operated in gradient mode. Operated in this manner, thrombin can be efficiently removed below the lowest limit of quantitation (6.25 pM) in the fluorescence-based thrombin assay. An HPLC based in-process control strategy was not required for this unit operation, and product collection is performed from set UV absorption criteria. Typical step yield at process-scale is 90–99%. This step also further reduces levels of host cell proteins (HCPs) below 40 ppm. Fig. 9 shows a representative Phenyl HP chromatogram at process-scale.

3.6. Process performance and product quality summary

Table 3 illustrates process performance, product quality, and impurity clearance at the 500 L bioreactor scale. Analytical results demonstrate that this process results in recombinant prothrombin with very low levels of host cell DNA and HCPs, while also

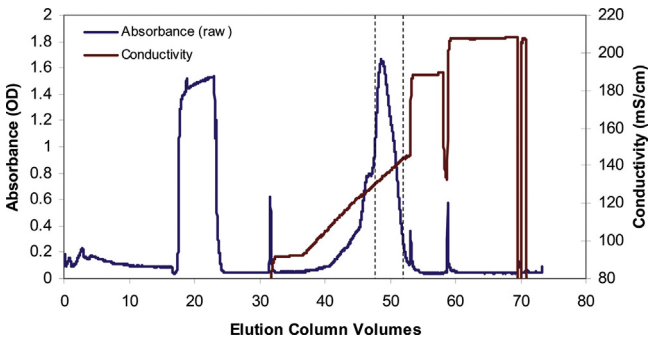

Fig. 7. Representative process-scale chromatogram obtained from Poly PEI chromatography with a 25 cm × 14 cm column. Dashed vertical lines correspond to pooled fractions. The large flowthrough peak during the load step, at approximately 20 CV, represents absorbance due to the solvent detergent mixture.

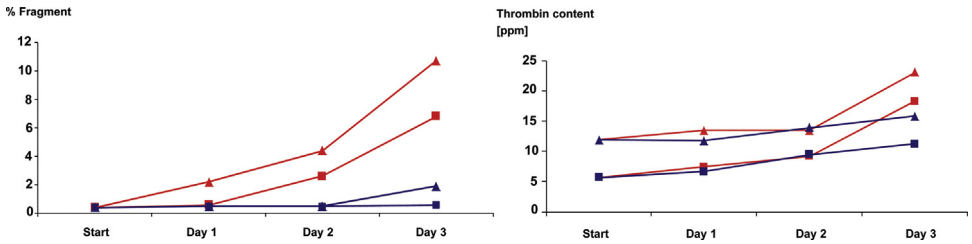

Fig. 8. Traces of thrombin (with a starting concentration of 5 ppm (squares) or 11 ppm (triangles)) in purification process intermediates lead to fragment formation (left panel) and also autocatalytically generate more thrombin (right panel). Commercial thrombin was spiked into protein samples from the Q FF flowthrough elution pool and material was incubated either at room temperature (red lines) or at 4 °C (blue lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
Process performance and in-process product quality at 500 L bioreactor scale.

Performance parameter or quality attribute	CM	Q FF capture	Q FF FT	Poly PEI AEX	Phenyl HP HIC
Step yield (%)[a]	–	67.2	113	76.5	99
CHO DNA (ng/mg)	–	0.038	0.027	0.03	0.0004
CHO HCP (ng/mg)	–	5609	5940	<66	<24.5
Pre-peak by HPIEC (%)[b]	–	21.5	20.3	13	11.8
Missing Gla by peptide mapping[c]	0.84	–	–	–	0.58
Purity by HPSEC (%)	–	98.3	98.4	99.1	99.8
p*I* by cIEF (%)	5.1–5.5	5.1–5.5	5.1–5.5	5.1–5.5	5.1–5.5
Fragment by RP HPLC (%)	–	3.1	1.5	1.2	1.0
Thrombin (ng/mg)	16.5	1.13	1.5	<0.4	<0.3
Clot bioassay, relative activity (%)	–	–	–	–	87

[a] Yields for the first two column steps are calculated based on RP HPLC concentration values, whereas all later step yields are based on A280 absorbance measurements.
[b] This pre-peak value represents the sum of all 3 pre-peaks corresponding to undercarboxylated prothrombin variants.
[c] Average missing Gla residues (out of 10).

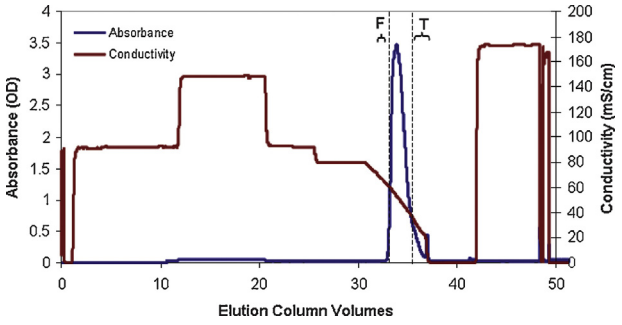

Fig. 9. Representative process-scale chromatogram of Phenyl HP chromatography with a 25 cm × 13 cm column. Dashed vertical lines correspond to the collected eluate pool. Areas labeled "F" and "T" indicate parts in the elution gradient where prothrombin fragment 1 ("F) and thrombin ("T") elute, respectively.

achieving extremely low levels of fragment and thrombin. As the Poly PEI column serves to enrich highly carboxylated prothrombin and the pooling decision for the gradient fractions is based on in-process pre-peak analysis, the yield for this chromatography step can vary depending on the degree of prothrombin carboxylation in the bioreactor. From harvest to formulation and filtration, this process typically has an overall downstream yield of 20–25%.

4. Conclusion

This work describes the development of a process-scale purification for human prothrombin that results in the robust manufacture of a highly carboxylated, biologically active recombinant protein. The initial high-resolution separation of prothrombin variants with different levels of carboxylation allowed detailed structure–function studies that revealed the crucial impact of gamma-carboxylation on bioactivity. A convenient HPIEC assay reliably served as a surrogate for the structural and functional characterization, proving crucial to characterize the extent of carboxylation for process development and manufacturing control.

To enable the challenging separation of closely related charge variants at process-scale, a robust and scalable anion exchange step in gradient mode was developed that accommodates variability in product quality and ensures the enrichment of highly carboxylated prothrombin. This unit operation ensures the consistent manufacture of a final product with high biological activity. Another critical aspect for process development was the control of the product-related impurity thrombin that represents the natural proteolysis product and is a product stability and safety concern. Process development focused on the efficient removal through a final polishing HIC step that controls levels of thrombin and also of prothrombin-fragment F1 to extremely low levels.

This downstream process provides another illustrative example of the importance of a systematic approach to product understanding and process development, especially for novel proteins that are characterized by a complex structure–function relationship and limited platform knowledge.

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