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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.

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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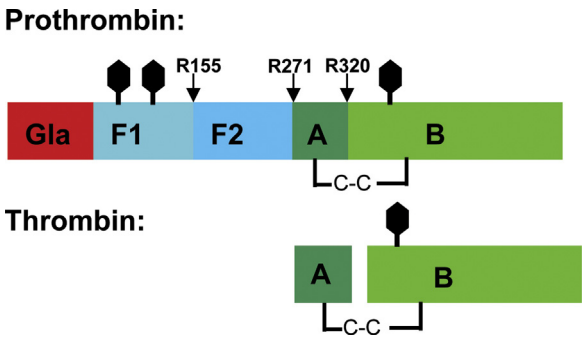

Fig. 1. Schematic representation of the primary structure of prothrombin and its proteolytic activation to thrombin. Prothrombin is a 72 kDa single-chain glycoprotein that contains the Gla domain, two kringle domains (part of fragments F1 and F2, respectively), and a serine protease domain consisting 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].