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Characterization of Recombinant Human Coagulation Factor XII

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Abstract: Coagulation factor XII (FXII) is an 80-kDa plasma glycoprotein, which initiates the intrinsic pathway of blood coagulation reactions. FXII is auto-cleaved after the Arg353 residue upon contact with a negatively charged surface, to yield active protease FXII (FXIIa). In this study, we prepared PA-tagged recombinant human FXII (PA-hFXII) in a mammalian expression system using Expi293F cells, analyzed the contact-activation response, protease activity, and glycosylation, and compared them to those of human plasma FXII (hFXII). PA-hFXII was purified using affinity chromatography on anti-PA antibody-conjugated Sepharose from the conditioned medium of the transfected Expi293F cells. PA-hFXII was autoactivated when it came in contact with the negatively charged lipid, sulfatide, and its protease activity was found to be similar to that of hFXII. Two potential N-glycosylation sites underwent glycosylation in both, PA-hFXII and hFXII. O-glycosylation in the proline-rich region appeared more homologous in PA-hFXII than in hFXII. These results showed that recombinant PA-hFXII was suitable for the investigation of the molecular mechanism underlying FXII activation.

1. Introduction

Blood coagulation occurs via two separate pathways, the extrinsic and the intrinsic, which converge at a common pathway. Both involve the sequential activation of plasma coagulation factors, which are the proenzymes of trypsin-like serine proteases. The intrinsic pathway is initiated by the activation of factor XII (FXII), when it comes in contact with a negatively charged surface (e.g., sulfatide (Fig. 1B), polyphosphates, and misfolded proteins) [1][2], however, the exact molecular mechanism underlying its activation remains unknown.

FXII is an 80-kDa glycoprotein biosynthesized in the liver and secreted into the blood stream in an inactive form. Starting at the N-terminus, the protein structure of FXII consists of the fibronectin type-II domain (FnII), epidermal growth factor like 1 domain (EGF1), fibronectin type-II domain (FnI), epidermal growth factor like 2 domain (EGF2), kringle domain, proline-rich region (PRR), and the protease domain (Fig. 1A). Upon autoactivation and the subsequent amidolytic cleavage of the Arg353-Val354 bond just before the protease domain, FXII is converted to active FXII (FXIIa) comprising of a 50-kDa heavy chain (FXIIa-HC) and a 30-kDa light chain (FXIIa-LC), where a catalytic triad, Ser544 linked to His393 and Asp442 in a hydrogen-bonded network is located. FXII has a potential N-glycosylation sequence (Asn-X-Ser/Thr) in the kringle and protease domains, putative

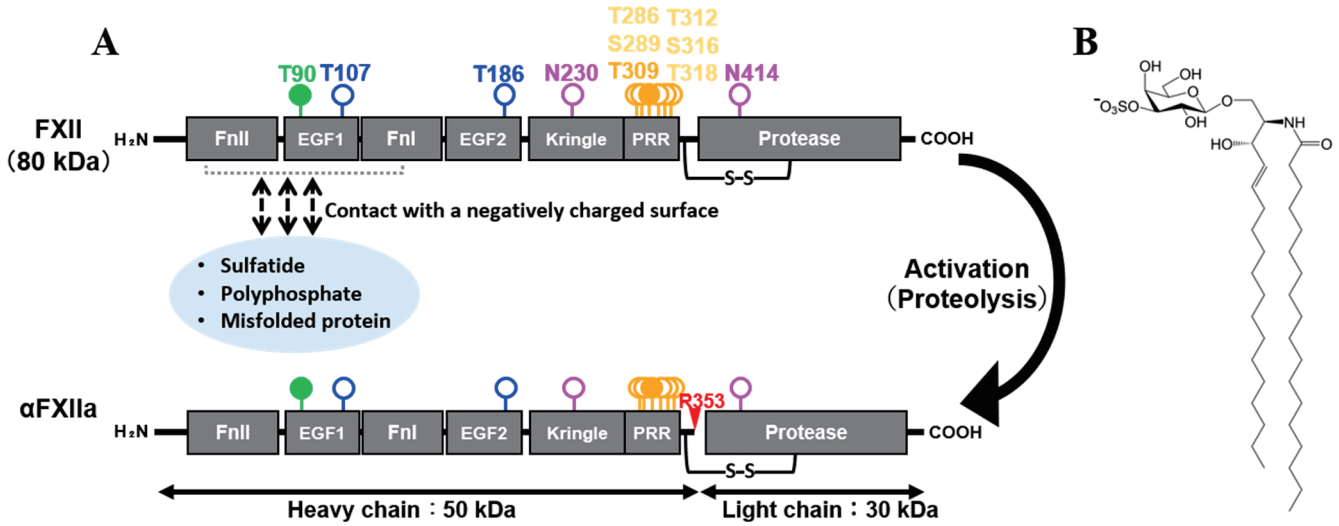

Figure 1. Schematic domain structure of the human FXII.
(A) hFXII comprises of the fibronectin type-II domain (FnII, 22–69), epidermal growth factor like 1 domain (EGF1, 77–112), fibronectin type-II domain (FnI, 114–154), epidermal growth factor like 2 domain (EGF2, 157–191), kringle domain (198–276), proline-rich region (PRR, 277–330), and the protease domain (354–596). FXII is cleaved after Arg353 and converted to FXIIa. Closed circles and open circles indicate identified glycosylation sites and potential glycosylation sites, respectively: O-fucosylation (green), O-GlcNAcylation (blue), N-glycosylation (pink), and mucin-type O-glycosylation (orange). (B) The structure of sulfatide.

mucin-type O-glycosylation sites in the PRR, and putative O-linked Fuc/GlcNAc modification sites in the EGF domains. Glycoforms of FXII have not been completely analyzed and their contribution towards the activation or the activity of FXII remains to be elucidated. Given that FXII defective in Thr309-linked mucin-type O-glycosylation (Thr-Hex-HexNAc-NeuAc) displays higher activity compared to the wild-type FXII and induces hereditary angioedema type III [3], we hypothesized that the glycosylation of FXII would contribute to its activity and structural stability.

In this study, we aimed to prepare a glycosylated recombinant human FXII that showed protease activity in response to contact with a negatively charged surface. We also examined the involvement of glycosylation in the molecular mechanism of FXII activation.

2. Materials and Methods

Materials

The Expi293F Expression Medium, ExpiFectamine 293 Transfection kit, and Expi293F cells were obtained from Thermo Fisher. Anti-PA tag Antibody Beads (NZ-1 Sepharose), PA tag peptide, and sulfatide sodium salt (from bovine spinal cord) were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). S-2302 (H-D-Pro-L-Phe-L-Arg-pNA dihydrochloride, a chromogenic substrate for FXIIa was obtained from Chromogenix (Milan, Italy). Human factor XII was procured from Haematologic Technologies, Inc. (Essex Junction, VT). Glycopeptidase F was obtained from Takara Bio Inc. (Shiga, Japan).

Expression of recombinant human FXII containing an N-terminal PA tag

Expi293F human cells (derived from Human Embryonic Kidney cells) were grown in the Expi293 medium on a shaker at 120 rpm at 37°C, in an 8% CO2

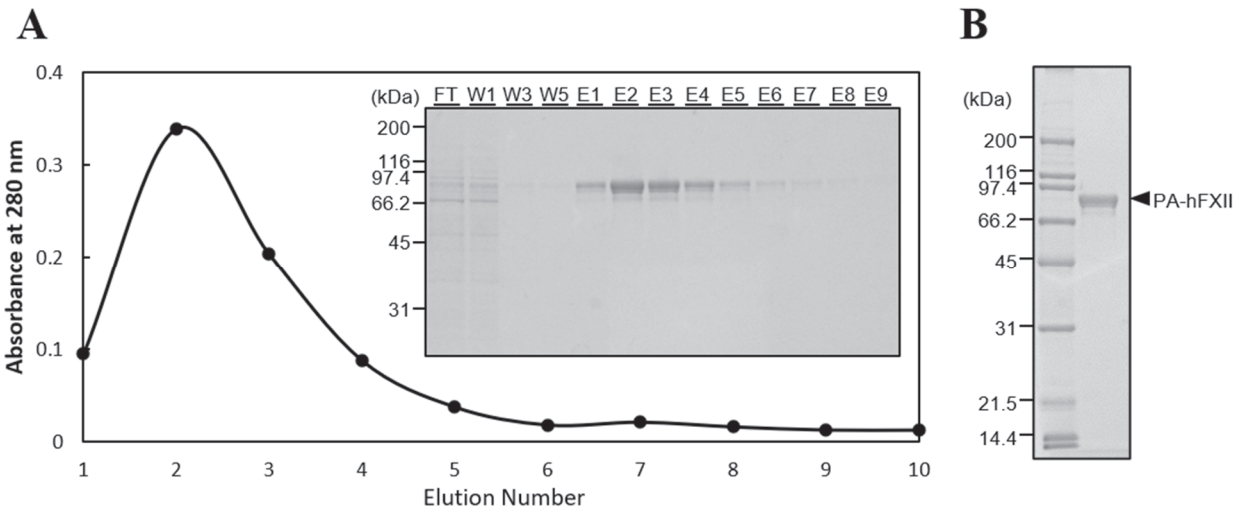

Figure 2. Purification of PA-hFXII.
(A) NZ-1 Sepharose binding PA-hFXII was washed with TBS (pH 7.5). Samples of flow-through, wash fractions, and eluted fractions were subjected to SDS-PAGE (12.5% gel) and stained with CBB. Absorbances of eluted fractions were measured at 280 nm. (B) Purified PA-hFXII was dialyzed and concentrated, and analyzed using SDS-PAGE (12.5% gel) and CBB staining.

atmosphere. Human FXII containing an N-terminal PA tag (PA-hFXII) was expressed using the Expi293 Expression System. Briefly, 7.5 x 107 Expi293F cells in 30 mL of the medium were transfected with pSec-PA-hFXII (30 µg), and 81 µL of the ExpiFectamine 293 Reagent was added. The next day, 150 µL of theExpiFectamine™ 293 Transfection Enhancer 1, and 1500 µL of Enhancer 2 were added. Cells were cultured for a further 72 h and the supernatants containing PA-hFXII were harvested.

Purification of recombinant PA-hFXII

Culture supernatants containing PA-hFXII were passed through a GD/X syringe filter (nylon; diameter, 25 mm; pore size, 0.2 µm; GE Healthcare), and the filtrate was mixed with 300 µL of 1 M Tris-HCl (pH 8.0) and then with a 400-µL bed volume of NZ-1 Sepharose gel. After incubating overnight at 4°C, the gel was poured into a column and the flow-through fraction (FT in Fig. 2A inlet) was stored. The column was washed with TBS (Tris-HCl, pH 7.5, 150 mM NaCl) and 800-µL fractions were collected (fractions W1–W5 in Fig. 2A inlet). PA-hFXII adsorbed on the gel was eluted with TBS containing 0.1 mg/mL PA tag peptide (EGGVAMPGAEDDVV) and 400-µL fractions were collected (fractions E1–E9 in Fig. 2A inlet). These multiple fractions were analyzed using SDS-PAGE and the fractions containing PA-hFXII were pooled and dialyzed against the HEPES buffer (20 mM HEPES, 100 mM NaCl, pH 7.4), which yielded purified PA-hFXII.

SDS-PAGE

Protein samples were dissolved in PAGE sample buffer [60 mM Tris-HCl (pH 6.8), 2% SDS, 0.001% bromophenol blue, 10% glycerol, 2% 2-mercaptoethanol], heated at 98°C for 5 min, and loaded onto 12.5% e-PAGEL (ATTO). After electrophoresis, gels were stained with Coomassie brilliant blue or the silver staining reagent (Silver Stain KANTOIII, Kanto Chemical Co., Inc., Japan).

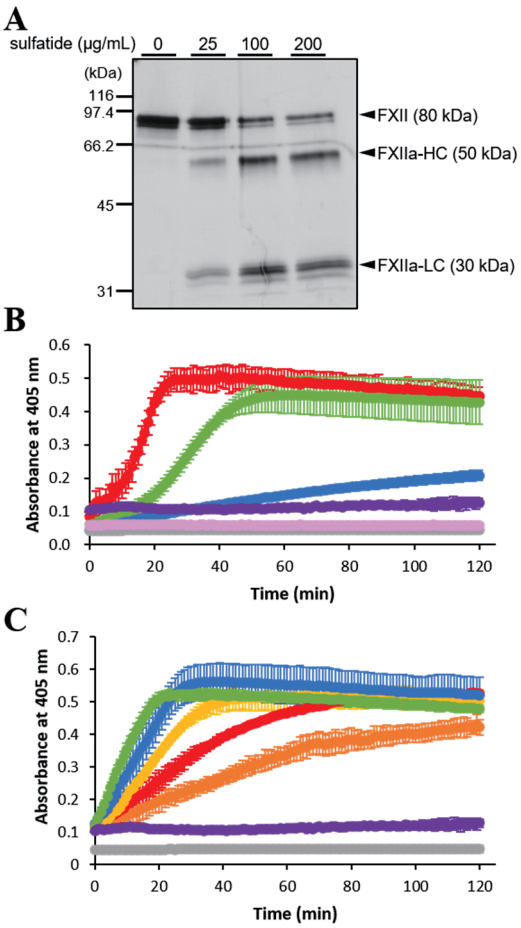

Figure 3. Autocatalytic activation of PA-hFXII. (A) PA-hFXII (16 μg/mL) was activated via exposure to sulfatide (0 μg/mL, 25 μg/mL, 100 μg/mL, 200 μg/mL). Samples were analyzed using SDS-PAGE (12.5% gel) and the proteins were visualized using silver staining. (B) PA-hFXII (16 μg/mL), S-2302 (200 μM), and sulfatide (0 μg/mL: blue, 25 μg/mL: green, 100 μg/mL: red) were incubated at 37°C. Change in absorbance at 405 nm was monitored for 120 min. Gray line indicates S-2302 in the absence of PA-hFXII and sulfatide. (C) S-2302 (200 μM), sulfatide (100 μg/mL), and PA-hFXII (1 μg/mL: orange, 2 μg/mL: red, 4 μg/mL: yellow, 8 μg/mL: blue, 16 μg/mL: green) were incubated at 37°C. Change in absorbance at 405 nm was monitored for 120 min. Gray line indicates S-2302 in the absence of PA-hFXII and sulfatide. Pink and purple lines indicate S-2302 in the presence of 25 μg/mL and 100 μg/mL sulfatide respectively.

Activation of PA-hFXII with sulfatide

PA-hFXII (16 μg/mL) and sulfatide at various concentrations (0 μg/mL, 25 μg/mL, 100 μg/mL, 200 μg/mL), mixed in Standard Buffer [20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% PEG-6000, 10 μM ZnCl2], were taken in PEG-6000 coated tubes and incubated at 37°C for 1 h. The reaction was arrested by adding sample buffer and analyzed using SDS-PAGE.

Chromogenic assays

Chromogenic assays were performed as per a previously described method [4]. The 96-well microtiter plate was coated with PEG-6000. Plasma FXII or PA-hFXII (4 μg/mL), and 200 μM S-2302 were mixed in Standard Buffer. The reaction was initiated by the addition of sulfatide (100 μg/mL) and incubated at 37°C. The total reaction volume was adjusted to 100 μL. Change in absorbance at 405 nm was monitored using a Cytation3 imaging reader (BioTek, Winooski, VT).

Glycopeptidase F -digestion

The digestion of glycopeptidase F (GPF) was performed according to the manufacturer's instructions. Intact PA-FXII and plasma FXII (8 μg/mL), and sulfatide-activated PA-FXII and plasma FXII (16 μg/mL) were digested with GPF (40 mU/mL) under denaturing conditions at 37°C overnight.

3. Results and Discussion

Expression and purification of recombinant PA-hFXII

Recombinant PA-hFXII protein was expressed in the Expi293F cells using plasmid pSec-PA-hFXII. PA-hFXII was secreted into the conditioned medium and subsequently purified using affinity chromatography with anti-PA tag antibody-conjugated Sepharose. PA-hFXII was detected as a single protein band in SDS-PAGE of the eluted fractions 1-6 (Fig. 2A, E1–E6). These fractions were pooled, dialyzed to remove the PA tag peptide, and concentrated to yield approximately 160 μg of the purified PA-hFXII (Fig. 2B).

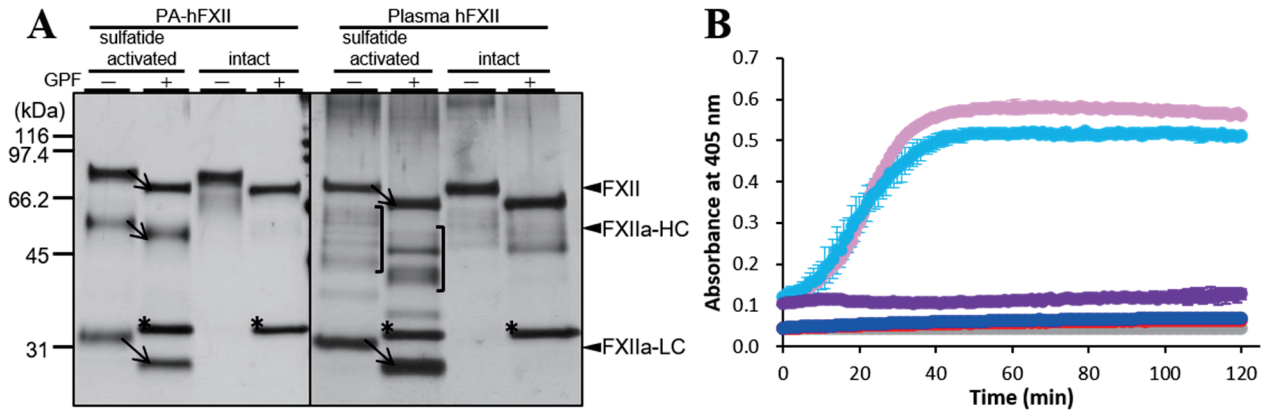

Figure 4. Comparison between PA-hFXII and plasma hFXII.
(A) Sulfatide-activated hFXII (15 μg/mL) or intact hFXII (8 μg/mL) were digested using GPF, subjected to SDS-PAGE (12.5% gel), and proteins were visualized using silver staining. Asterisk (*) indicates GPF. Arrows indicate the decrease in molecular weight after GPF-digestion. (B) PA-hFXII or plasma hFXII (4 μg/mL) and 200 μM S-2302 were incubated in the presence or absence of sulfatide (100 μg/mL) at 37°C and the change in absorbance at 405 nm was monitored every min for 120 min. PA-hFXII (red), PA-hFXII with sulfatide (pink), native hFXII (blue), and native hFXII with sulfatide (light blue) have been shown. Gray line indicates S-2302 in the absence of PA-hFXII and sulfatide. Purple lines indicate S-2302 in presence of 100 μg/mL sulfatide.

Autocatalytic activation of PA-hFXII

We examined the activation response of PA-hFXII through contact with a negatively charged surface. As shown in Fig. 3A, PA-hFXII was cleaved after the Arg353 residue in a sulfatide dose-dependent manner to generate PA-hFXIIa-LC and PA-hFXIIa-HC. S-2302 was cleaved in a dose-dependent manner depending on the concentrations of sulfatide and PA-hFXII (Fig. 3B, C). These results confirmed the autocatalytic activity of PA-hFXII, which produced PA-hFXIIa upon binding to sulfatide.

Comparison between PA-hFXII and plasma hFXII

It is unknown whether the two potential N-glycosylation sites (Asn230, Asn414) of hFXII are actually N-glycosylated. The molecular weights of both, the intact and activated hFXII, decreased after GPF digestion. Similar results were obtained for PA-hFXII (Fig. 4A), indicating that both, the recombinant and native hFXII were most likely N-glycosylated at the Asn230 and Asn414 residues. In addition, SDS-PAGE after GPF digestion showed a single band for the FXII heavy chain derived from PA-hFXII, but multiple bands were observed for that from plasma hFXII. Given that the proline-rich region (PRR) within the FXII heavy chain has multiple mucin-type O-glycosylation sites, these results increase the possibility that the glycoforms of mucin-type O-glycans of PA-hFXII are homogeneous, while those of plasma hFXII are heterogeneous and consist of more extended glycans. Chromogenic assay in the presence and absence of sulfatide showed no differences between PA-hFXIIa and plasma hFXIIa (Fig. 4B), suggesting that the possible differences in glycoforms of mucin-type O-glycosylation in PRR were not involved in the protease activity of FXIIa. For further understanding of O- and N-glycosylation in PA-hFXII, we would like to analyze it using mass spectrometry in the future. Based on these findings, we successfully prepared PA-hFXII that could be converted to active PA-hFXIIa by autocatalysis. PA-hFXII could be a valuable tool for the

further investigation of the molecular mechanism of FXII activation.

References

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