THE ROLE OF N-LINKED GLYCOSYLATION ON HUMAN FIBRINOGEN: A STRUCTURE-FUNCTION ANALYSIS UTILIZING AFFINITY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

by

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A Signature Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

by

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May, 2023

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I hereby declare I am the sole author of this thesis. The writing and data analysis is my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree. The affinity column was developed in collaboration with Nicholas Kirby, Kristine Peterson, Grega Popovic; the ELISA assay and scramble peptide column Western blot was performed by Nicholas Kirby; the altered glycan study was expanded upon the work of Grega Popovic; and the Mathematica file was developed by Heather Belcher.

Signed: Caroline Daub Date: 05/04/2023

The Role of N-Linked Glycosylation on Human Fibrinogen: A Structure-Function Analysis Utilizing Affinity and High-Performance Liquid Chromatography

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ABSTRACT - Fibrinogen (Fgn) is a soluble, 3-chain homodimeric glycoprotein found in blood plasma that is involved in forming blood clots. It is the precursor to fibrin, an insoluble protein that forms the structural scaffold of blood clots. Fibrinogen is converted to fibrin when fibrinopeptides A and B are cleaved off fibrinogen by the enzyme thrombin, exposing polymerization sites termed knob 'A' and knob 'B.' Blood clots are formed when fibrin molecules laterally aggregate through knob:hole interactions. There are four N-linked glycosylation sites on fibrinogen, which have been suggested to influence the conversion of fibrinogen to fibrin. Furthermore, a relationship is suspected between the pattern of N-linked glycosylation and fibrinopeptide release due to physiological changes during pregnancy. The concentration of fibrinogen in blood plasma rises and the sialic acid content on the N-linked glycans are reduced, which put pregnant women higher potential risk for impaired blood clotting patterns that can lead to stroke or heart attack. However, little is known about the link between glycan structure and the efficiency of thrombin processing the fibrinopeptides. In this preliminary study, a fibrinopeptide release assay (FPRA) using high-performance liquid chromatography (HPLC) was developed to quantify the differences in clotting patterns between native fibrinogen and altered N-linked glycan fibrinogen. Additionally, an affinity column purification system was developed to purify fibrinogen from human blood plasma. In

the future, the combination of the affinity column purification system and the FPRA can be used to assess the clotting patterns of pregnant patient fibrinogen samples.

Acknowledgments

I would sincerely like to thank the people and organizations that made this study possible: Dr. Adam R. Offenbacher and Dr. Nathan E. Hudson for their knowledge, guidance, and patience; my research peers Nicholas Kirby, Grega Popovic, and Heather Belcher for their assistance in this study and their friendship; my parents, Cyndi and Steven Daub and my brother Ian; and the Departments of Biology, Chemistry, and Physics at East Carolina University. Funding was received from the National Institutes of Health and an East Carolina University Undergraduate Research and Creativity Award.

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Introduction

Fibrinogen (Fgn) is a soluble 340 kDa glycoprotein that is involved in forming blood clots. It is exclusively synthesized in the liver and is present in blood plasma ranging from 1.5-4.2 g/L (Maghzal, 2005). It is a homodimeric protein composed of two sets of three polypeptide chains designated as Aα, Bβ, and γ, which are interconnected by a series of disulfide bonds (Simurda, 2021). The N-terminus of each chain is disulfide-linked to form the central "E region" while the C-terminal of the Bβ, γ, and part of the Aα chains form two lateral "D regions" (Simurda, 2021). The two "D regions" contain polymerization sites termed hole 'a' and hole 'b' (Laudano, 1978).

There are 4 N-linked glycosylation sites at Bβ Asn364 and γ Asn52 (Maghzal, 2005). A maximum of eight sialic acid residues can be on a single fibrinogen molecule. However, in healthy adults, there are six sialic acid residues often detected per mol of fibrinogen from the analysis of *ex vivo* samples (Maghzal, 2005). The N-termini for the α and β chains are comprised of 15 amino acid segments termed fibrinopeptide A (FpA) and fibrinopeptide B (FpB) (Medved, 2009). The cleavage of fibrinopeptides A and B by the enzyme thrombin (Thr) exposes two polymerization sites, knob 'A' and knob 'B' (Fig. 1).

FpA is cleaved at the AαArg16-Gly17 peptide bond and FpB is cleaved at the BβArg14-Gly15 bond (Weisel, 2017). No peptides are cleaved from the γ chains, so the conversion of fibrinogen to a fibrin monomer can be described as $(A\alpha\ B\beta\ \gamma)_2 \rightarrow (\alpha\ \beta\ \gamma)_2 +$ 2FpA + 2FpB (Weisel, 2017). Normally, FpA is cleaved more rapidly from fibrinogen than FpB. The cleavage of FpA converts fibrinogen to desA fibrin $(\alpha,B\beta,\gamma)_2$, followed by the cleavage of FpB, which converts desA fibrin to desAB fibrin $(\alpha,\beta,\gamma)_2$ (Medved, 2009).

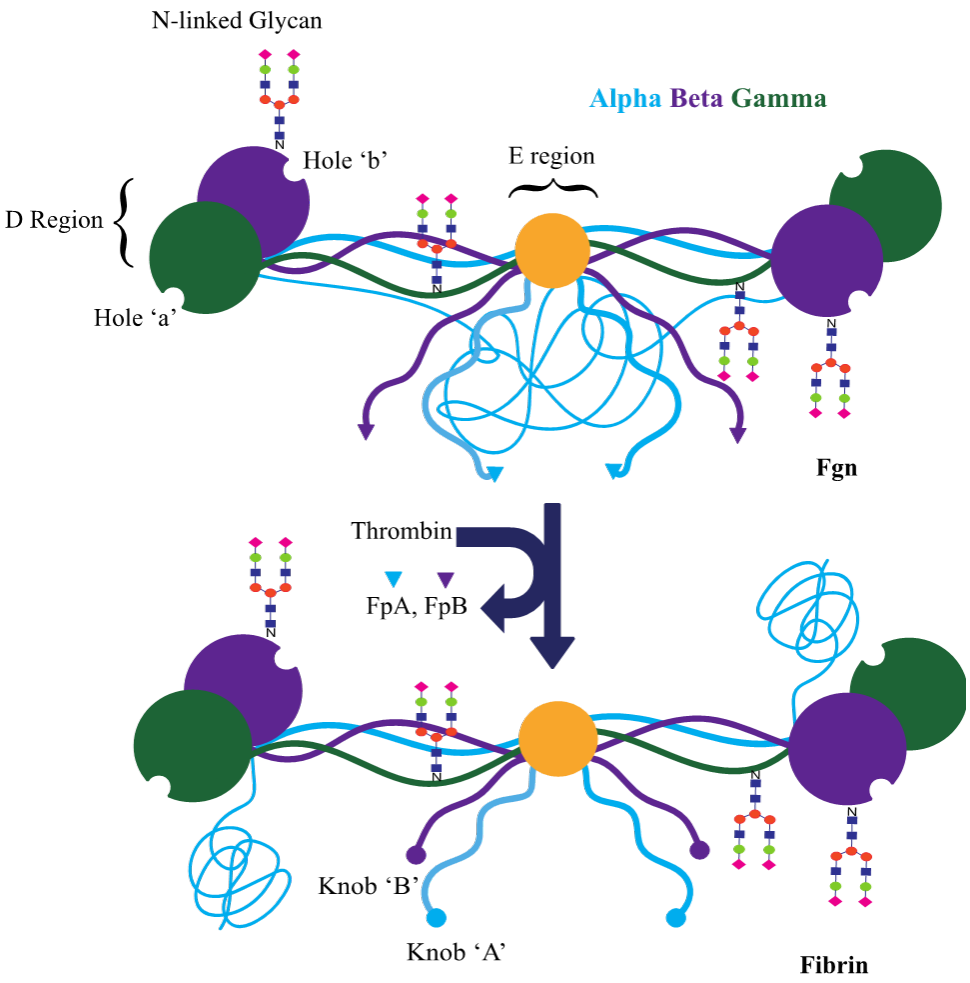


Figure 1: Cartoon representation of the conversion of fgn to monomeric fibrin.

The sialic acid content of fibrinogen's N-linked glycans is known to fluctuate due to various diseases or physiological states. An extreme case of one of these fluctuations is asialofibrinogen, a type of dysfibrinogenaemia where fibrinogen completely lacks sialic acid residues on the N-linked glycans (Maghzal, 2005). In healthy adults, asialofibrinogen is not present in circulation as it is rapidly removed and degraded by reticulo-endothelial tissues (Martinez, 1977). Asialofibrinogen has a shorter thrombin clotting time and yields thicker fibrin fibers, which suggests that sialic acid influences fibrin polymerization (Maghzal, 2005). A physiological state that affects the sialic acid content on fibrinogen's N-linked glycans is pregnancy, in both the pregnant woman and the foetus. In a preliminary study

performed by Maghzal et al, it was found that the average number of sialic acid residues per molecule of fibrinogen decreases from 5.3 to 5.0 during pregnancy. In contrast, human foetal fibrinogen has more sialic acid than adult fibrinogen and displays prolonged thrombin clotting times and delayed fibrin polymerization (Maghzal, 2005). Pregnant women are also in a hypercoagulable state because fibrinogen levels in plasma exceed the normal range of 1.5-4.2 g/L. The reduction of sialic acid residues puts pregnant women at higher potential risk for thrombotic diseases such as venous and arterial thrombosis due to the shorter thrombin clotting time and thicker fibrin fibers.

The purpose of this study was to develop an affinity column to purify fibrinogen from human blood plasma and develop an fibrinopeptide release assay (FPRA) that measures the time-dependent percentage of fibrinopeptides released from the conversion of fibrinogen to fibrin. For proof-of-concept, initial studies used the enzymes neuraminidase and PNGase F to alter or completely remove, respectively, N-linked glycan structures from native fibrinogen (Fig. 2).

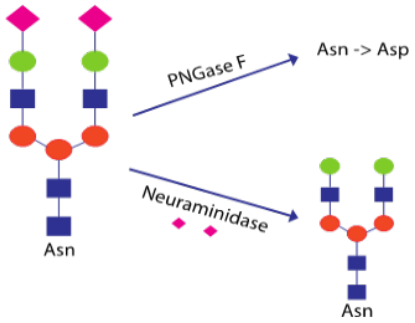


Figure 2: Cartoon representation of the effect of neuraminidase or PNGase F treatment on an N-linked glycan.

Neuraminidase removes sialic acid from the N-linked glycans on native fibrinogen (Fig. 2) converting it to asialofibrinogen, which polymerizes rapidly (Dang, 1989). PNGase F

is an endoglycosidase that completely removes the N-linked glycans from fibrinogen by converting the asparagine residue at the glycan removal site to aspartic acid (Fig. 2) (Freeze, 2010).

These altered glycan samples were intended to provide a loss-of-function approach to assess if N-linked glycans have a role in the rate of the release of FpA and FpB by thrombin. The conversion of fibrinogen to fibrin reaction scheme was used as a model to calculate molar concentration of fibrinopeptides released. For this study, the HPLC method was developed to quantify the amount of FpA and FpB in a reaction mixture at a given time. The Lambert function, applied in the context of Michaelis-Menten kinetics, was the method used to analyze the FPRA data for time-dependent kinetic parameters. The combination of the affinity column purification system and FPRA can be used in the future with pregnant patient plasma samples.

Materials & Methods

I. Affinity Column Development, original method

The synthetic peptide Fmoc-GPRPFPAWK, made by Genscript, is a knob 'a' mimic that was used to purify fibrinogen from human blood plasma (Fig. 2).

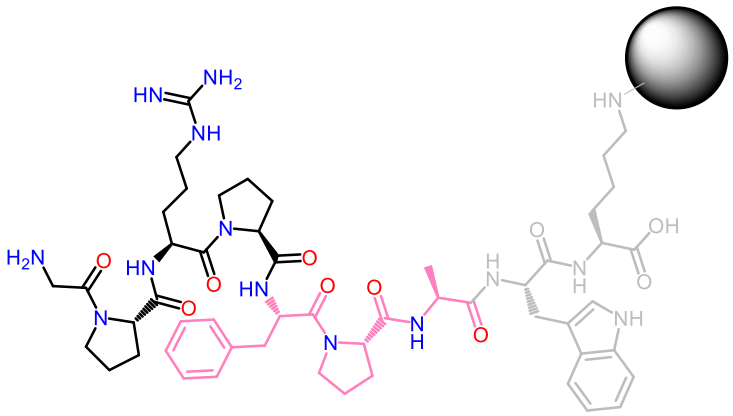


Figure 3: Chemical structure of the GPRPFPAWK synthetic peptide (Popovic, 2022)

The synthetic peptide was dissolved in DMSO and diluted with ddH_2O to create a 1 mM stock solution. The stock solution was combined with a 2x coupling buffer (0.4 M $NaHCO_3$, 1 M NaCl at pH 8.5) at a final peptide concentration of 0.4 mM (Popovic, 2022). NHS-activated Sepharose 4 Fast Flow beads were washed with cold 1 mM HCl to ensure that the NHS group remained unhydrolyzed (Popovic, 2022). The synthetic peptide was coupled to the Sepharose beads by combining both solutions at a 0.5:1 coupling solution: resin ratio for 3 hours at room temperature. The remaining NHS binding sites of the resin were then blocked with blocking buffer (0.1 M Tris-HCl, pH 8.5) for 3 hours at 4 °C. The Fmoc group protecting the N-terminal glycine of the peptide was de-protected with 4-Methylpiperidine for 3 hours at room temperature (Popovic, 2022). A glass column (Bio-Rad, Hercules, CA, USA) was then packed with resin by gravity. The column was washed with three column

volumes of low pH buffer (0.023 M sodium acetate, 0.077 M glacial acetic acid, 0.5 M NaCl, pH 4) followed by three column volumes of blocking buffer (Popovic, 2022).

II. Affinity Column Development, optimized method

The original affinity column coupling method was utilized for the data obtained in this study. However, an optimized coupling method was developed after the data was obtained. The synthetic peptide (Fmoc-GPRPFPAWK) was dissolved in DMSO and diluted with ddH_2O to create a 1 mL stock solution at concentration of 10 mM. A sample of the stock solution was combined with ddH_2O and coupling buffer (0.5 M $NaHCO_3$, 1M NaCl, pH 8.3) to create a 1 mL coupling solution containing 6 mg of peptide. The coupling solution was sonicated for 10 minutes before combining with NHS-activated Sepharose 4 Fast Flow beads at a 0.5:1 coupling solution: Sepharose bead solution (1 mL coupling solution: 2 mL Sepharose). The beads were washed with 10-15 column volumes (CVs) of cold 1 mM HCL (0-4 °C) to ensure that the NHS group remained unhydrolyzed.

The coupling solution was combined with the washed beads and the pH was adjusted to 7-9 with 0.8 M sodium hydroxide as necessary. To block the remaining Sepharose resin binding sites, three CVs (6 mL) of blocking buffer (0.1 M Tris-HCl, pH 8.5) was added to the beads and was rocked on a rotating platform for three hours at 4 °C. The N-terminal glycine of the peptide was de-protected by removing the Fmoc group with 4-methylpiperidine. Three CVs of 20% 4-methylpiperidine in dimethylformamide (1.2 mL 4-methylpiperidine and 4.8 mL dimethylformamide) were added to the beads and rocked on a rotating platform for 5 minutes under a fume hood (repeated for two total washes). Three CVs of 100% dimethylformamide were added to re-suspend the beads and slowly poured into a glass column (Bio-Rad, Hercules, CA, USA). A 10 mL ddH_2O wash was flowed over

the column to remove the dimethylformamide. To further pack the beads into the column, three CVs of blocking buffer were flowed over the column at a flow rate between 1.5-1.9 mL/min. The resin was washed with three CVs of low pH buffer (0.1 M acetate buffer, 0.5 M NaCl, pH 4) followed by three CVs of high pH buffer (blocking buffer) at a flow rate between 1.0-1.5 mL/min. This wash cycle was repeated six times. The column was stored in 20% EtOH wrapped in foil in a cold room.

III. Affinity Column Functionality Tests

The 2 mL GPRPFPAWK column was initially assessed using commercial fibrinogen (Peak 1 fibrinogen, Enzyme Research Labs, South Bend, IN, USA). The column was equilibrated with 3 column volumes (CVs) of loading buffer (20 mM HEPES, 20 mM $CaCl_2$, 150 mM NaCl, pH 7.4) at 0.5 mL/min (Popovic, 2022). The fibrinogen sample was diluted to a concentration of 0.17-0.3 mg/mL in loading buffer and flowed over the column at 0.3 mL/min. Three post-flow fractions (2 mL/fraction) were collected. The column was then washed with 5 CVs of loading buffer at 0.5 mL/min and 5 wash fractions were collected (2 mL/fraction). To elute fibrinogen off the column, 2.5 CVs of elution buffer (1 M NaBr, 50 mM sodium acetate at pH 5.3) was run over the column at 0.3 mL/min (Popovic, 2022). Five to six elution fractions were collected (1 mL/fraction) and were tested with pH strips and neutralized with 0.8 M sodium hydroxide as needed. To clean the column, 2 CVs of ddH_2O and 20% ethanol were run over the column.

The protein amounts (mg) in all the fractions were calculated by testing the fractions with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). The total amount of protein in all the fractions was calculated to determine the percentage of protein in each fraction. The

total percentage of the protein isolated in the fractions was compared to the total amount of protein in the pre-flow to determine the actual yield of the column.

IV. Plasma Purification

Human fresh frozen plasma (FFP) (Cone Bioproducts, Sequin, TX, USA) was run over the 2 mL GPRPFPAWK column. The 2 mL FFP aliquot was thawed at 30 °C for no longer than 1 hour and filtered with a 0.2 μm filter. Benzamidine HCL (1mM) was added to the FFP aliquot to prevent fibrin polymerization during the purification process. The treated FFP aliquot was added to the pre-equilibrated affinity column and flowed through by gravity. Post-flow, wash, and elution fractions were collected. All elution fractions were adjusted to a pH of 7 and exchanged into HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) using a PD10 column (Popovic, 2022).

V. Coomassie Gels

The collected column fractions were checked for fibrinogen presence routinely by SDS-PAGE analysis, employing standard techniques (Popovic, 2022). Fraction samples were mixed with a reducing loading dye and heated at 98 °C for 5 minutes before loading the 10% polyacrylamide gel. Electrophoresis was performed at 80 V for the initial stacking phase, followed by a separation phase at 120 V for 1 hour. The gel was rinsed with water and stained with Gelcode Blue-Safe Protein Stain (AQ-2), then de-stained before imaging on an Azure Biosystems c300 (Popovic, 2022).

VI. Western Blot

The Western blot followed the same procedure as the SDS-PAGE analysis, however after electrophoresis, the gel was transferred onto a PVDF membrane using a BIO-RAD Trans-Blot Turbo Transfer System (Popovic, 2022). The transfer was run for 30 minutes at

25 V. The membrane was removed and placed in a 40 mL 5% milk solution in TBS-T buffer and shook for 1 hour at room temperature or overnight at 4 °C. A 40 mL solution of 1:2500 diluted primary rabbit antifibrinogen antibody (Dako) in TBS-T buffer was added to the membrane and shook for 1 hour at room temperature. Next, a 30 mL solution of 1:10,000 diluted secondary mouse anti-rabbit antibody (Invitrogen) was added to the membrane and shook for 1 hour at room temperature. The membrane was washed with water three times after antibody treatment. Western blot detection reagents (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific, Waltham, MA, USA) were mixed at a 1:1 ratio and added on top of the membrane. After incubating for 1 minute, the reagents were poured off and the membrane was imaged using chemiluminescence on an Azure Biosystems c300 (Popovic, 2022).

VII. Clottability and Turbidity Assays

The functionality of the fibrinogen isolated from human FFP was assessed using a combination of clottability and turbidity assays. Peak 1 fibrinogen (Enzyme Research Labs, South Bend, IN, USA) was used as a comparison to fibrinogen purified from FFP (Popovic, 2022). The clottability assay assessed the percentage of fibrinogen that could successfully be converted to fibrin. Before preparing the samples, the concentrations of the fibrinogen used for testing were measured using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). A 1:1 volume ratio of 50 µL of fibrinogen at 1 mg/mL (in 20 mM HEPES, 150 mM sodium chloride, pH 7.4) and 50 µL of 0.1 U/mL thrombin (in 20 mM HEPES, 150 mM sodium chloride, 10 mM calcium chloride, pH 7.4) was combined and had a final concentration of 0.5 mg/mL fibrinogen and 0.05 U/mL thrombin (Popovic, 2022). After the samples were mixed, the reactions were incubated at 37 °C for 2 hours. The reactions were

then centrifuged for 1 hour at 13,000 rpm to remove the polymerized material. The supernatant that resulted was measured with a Nanodrop 2000c spectrophotometer. The percentage difference between the initial fibrinogen concentration and the final amount of fibrinogen present in the supernatant was calculated as a percentage of clottable material (Popovic, 2022).

The turbidity assay was performed in triplicate at 37 °C using a 96-well plate (Corning Costar®® black with clear flat bottom polystyrene assay plate). The fibrinogen samples were prepared at a concentration of 0.8 mg/mL at 75 µL per well and the thrombin samples were prepared at a concentration of 0.1 U/mL at 75 µL per well (Popovic, 2022). The final concentration of the fibrinogen/thrombin samples in each well was 0.4 mg/mL and 0.05 U/mL, respectively. The fibrinogen and thrombin samples were prepared in the same buffers used in the clottability assay. The fibrinogen solution was added to the 96-well plate first, followed by the thrombin solution, which was added simultaneously using a multichannel pipette. Readings were taken at 350 nm every 60 seconds for 1 hour using a Synergy™ multi-mode microplate reader (Bio Tek Instruments).

VIII. ELISA

Chicken IgY Antifibrinogen antibody (Thermo PA1-9526) was first diluted to 0.3 µg/mL in coating buffer (29 mM sodium carbonate, 71.4 mM sodium bicarbonate, pH 9.6) and added to each of the wells being used in a 96-well plate (Popovic, 2022). The 96-well plate was centrifuged at 500 rpm for 1 minute and incubated at 37 °C for 2 hours (alternatively, overnight at 4 °C). The wells were washed four times with 200 µL of PBS-T buffer (11.9 mM phosphates, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.05% Tween-20 *v/v*). The remaining protein binding sites were blocked by adding 200 µL of 2%

milk in PBS-T to each well and incubating for 60 minutes at 37 °C followed by four PBS-T washes (Popovic, 2022). The samples were fractions from the FFP purification, which included a sample of the FFP pre-flow. The samples were added to selected wells and incubated for 30 minutes at 37 °C. The concentration of the Peak 1 fgn standards ranged from 7 µg/L to 500 µg/L in HBS buffer (20 mM HEPES, 150 mM sodium chloride, pH 7.4) and were also added to selected wells. The controls were wells with only HBS buffer. All samples, standards, and controls were performed in triplicate.

After incubation, all wells were washed four times with PBS-T. Next, 50 µL of antifibrinogen-rabbit antibody (Dako, A0080) was added to each well at a concentration of 5 µg/mL in 2% milk in PBS-T, then incubated for 30 minutes at 37 °C (Popovic, 2022). Wells were then washed with PBS-T buffer followed by addition of 50 µL/well of goat anti-rabbit HRP antibody (31462, Invitrogen, Waltham, MA, USA), diluted to 10 µg/mL in 2% milk in PBS-T, then incubated for 30 minutes at 37 °C. Wells were then washed six times with 200 µL of PBS-T buffer. Lastly, 200 µL of ABTS substrate was added to each well and incubated at room temperature while absorbance readings at 405 nm were taken every 60 seconds with a Synergy™ multi-mode microplate reader (Bio Tek Instruments) (Popovic, 2022).

IX. Altered Glycan Fibrinogen Preparation

Peak 1 fibrinogen was treated with neuraminidase by combining a 5 mg/mL fibrinogen solution in 0.01 M of imidazole-HCl, pH 6.3, with a neuraminidase solution in 0.15 M NaCl (Okude, 1992). This resulted in a concentration of 0.005 U of neuraminidase per mg of fibrinogen. The combined solutions were incubated at 37 °C for 5 hours, then dialyzed 3 times against 0.15 M NaCl (Okude, 1992). Separately, fibrinogen was treated with PNGase F by combining 20 µg of fibrinogen in 1X PBS, pH 7.4 to a final volume of 18 µL,

followed by addition of 2 µL of PNGase F. The combined solutions were incubated at 37 °C beginning at 0.5 hours and up to 24 hours.

X. Ethanol Precipitation of Fibrinogen from Fresh Frozen Plasma

As a comparison to the affinity-based purification, an ethanol-precipitation purification method adapted from Doolittle, et al. (1967) was performed on 100 mL of human fresh frozen plasma (FFP) (Cone Bioproducts, Sequin, TX, USA). The FFP was thawed at 37 °C for no more than 1 hour and then immediately placed on ice. The FFP was decalcified by adding 3 mM ethylenediaminetetraacetic acid (EDTA; final concentration) while on ice for 5 minutes to inhibit fibrin polymerization. The FFP was then centrifuged at 18,000 x g for 20 minutes and the supernatant was collected. 0.22 original volumes (OVs) of a 10 mM HEPES, 50% ethanol solution (pH 7.1, 3 °C) was added to the supernatant, followed by centrifugation at 18,000 x g for 20 minutes. This centrifugation resulted in a supernatant and pellet. The supernatant was poured off and the pellet was washed with 0.5 OVs of a 10 mM HEPES, 7% ethanol solution (pH 6.5, 3 °C) and centrifuged at 18,000 x g for 20 minutes. The pellet was resuspended in 0.25 OVs of a 55 mM citrate buffer (pH 6.5, 30 °C) and cooled until it reached ~ 0 °C. The "cold insoluble material" was removed by addition of a 10 mM HEPES, 20% ethanol solution (pH 7.1, 3 °C) to a final concentration of 2%, followed by centrifugation at 18,000 x g for 30 minutes. The supernatant was retained, and the mucous-like pellet was discarded. A 10 mM HEPES, 20% ethanol solution (pH 7.1, 3 °C) was added to bring the ethanol concentration of the supernatant to 8%. The solution was centrifuged at 18,000 x g for 20 minutes, which resulted in a pellet of purified fibrinogen. The pellets were air dried and suspended in HBS buffer (20 mM HEPES, 150 mM sodium chloride, pH 7.4).

The fibrinogen isolated from FFP were further purified by using a fast-protein liquid chromatography (FPLC) system. A Cytiva HiLoad™ 16/600 Superdex™ 200 pg column was used for the purification. The fractions that resulted were the final purified fibrinogen aliquots from this ethanol precipitation method.

XI. Fibrinopeptide Release Assay

A fibrinopeptide release assay (FPRA) was developed using a Hitachi high-performance liquid chromatography (HPLC) system and PC/Chrom+ software, which helped to measure the time dependent amount of fibrinopeptides are released during fibrinogen/thrombin reactions. Integration of the HPLC peaks, identified as fibrinopeptide A (FpA) and fibrinopeptide B (FpB), provides the relative amount of FpA and FpB released at a particular time point (Haverkate, 1986).

A time series procedure was established to compare the integrations of the FpA and FpB peaks of samples with differing digestion times. The chosen digestion time points were 1, 5, 10, 20, 40, and 80 minutes. An additional 60-minute "infinite" timepoint with a higher concentration of thrombin was used to normalize the HPLC peak intensities and calculate the percentage of fibrinopeptide release from the chosen timepoints. The 1–80-minute samples were prepared by combining a 50 µL fibrinogen sample at 8 mg/mL in HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and a 50 µL thrombin sample at 0.066 U/mL in HBS with a buffered calcium solution (20 mM HEPES, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4).

Reactions were performed at 37 °C at one of the chosen time points. The 60-minute timepoint followed the same procedure and had the same concentration of fibrinogen, but the thrombin concentration was 0.2 U/mL. The final concentration was 4 mg/mL fibrinogen and 0.033 U/mL thrombin for the 1-80-minute samples, and 4 mg/mL fibrinogen and 0.1 U/mL

thrombin for the 60-minute samples. Pure FpA and FpB standards were also prepared at a concentration of 0.0723 μg/μL and 0.0730 μg/μL, respectively, in 100 μL of HBS buffer.

Polymerization was stopped by heat quenching the samples at 100 °C for 2 minutes followed by submersion in an ice bath. The samples were centrifuged at 10,000 x g for 8 minutes to pellet the fibrin clot that was generated from the reaction. The supernatant above the fibrin clot, containing the fibrinopeptides released from the fibrinogen/thrombin reaction, was filtered using dolphin tubes with 0.22 μm filters and then injected in the HPLC system.

Two mobile phases, A (25 mM sodium phosphate, 5% acetonitrile, pH 6.0) and B (500 mL of A, 500 mL acetonitrile), filtered by a 0.22 μm filter, were used in the HPLC system. A Waters Symmetry300 C18 3.5 μm 4.6x150 mm silica column was used for the analysis. The HPLC method (Table 1) ran at 1.5 mL/min at an absorbance of 205 nm and had step gradient between mobile phase A and mobile phase B at the 7-minute timepoint. The purpose of the step gradient was to increase peak resolution and provide faster elution times due to the steady increase of organic solvent. The method ran for a total of 17.5 minutes per sample.

Table 1: Reverse phase HPLC method for the FPRA with a step gradient from mobile phase A to mobile phase B.

Time (minutes)	Flow (mL/min)	%A	%B
Initial	1.5	100	0
7.0	1.5	60	40
9.0	1.5	0	100
14.0	1.5	0	100
14.5	1.5	100	0

17.5	1.5	100	0

Results

I. Affinity Column Functionality

The GPRPFPAWK column's selectivity for fibrinogen was initially tested using Peak 1 fibrinogen (Enzyme Research Labs, South Bend, IN, USA). The fractions resulting from a purification of 1 mg of Peak 1 fibrinogen were tested with a Western blot for fibrinogen content (Fig. 4A).

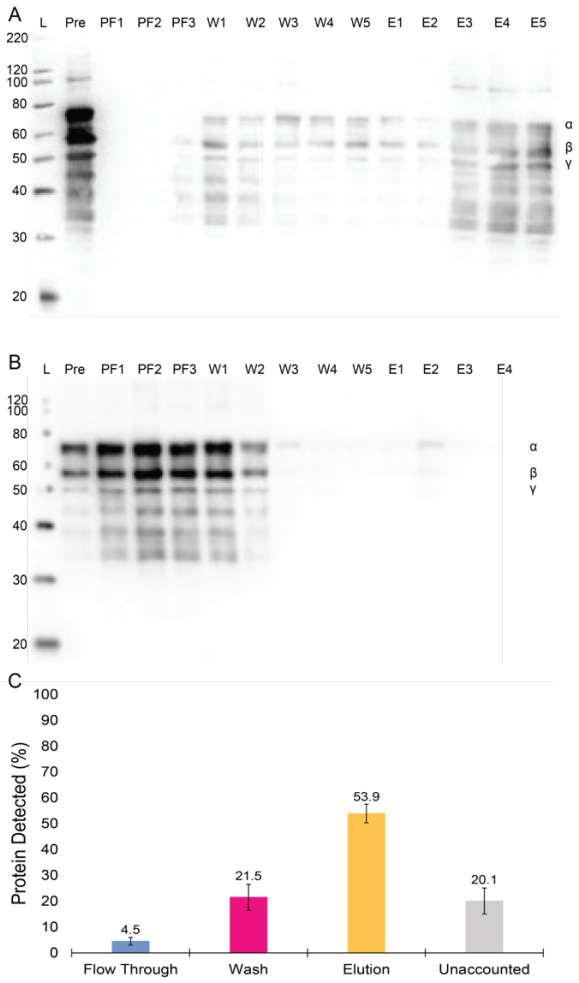


Figure 4: Column assessment for selectivity of fgn with Peak 1 fgn. **(A)** Western blot of the GPRPFPAWK affinity column fraction steps. Lane labels are the following: Ladder (L; kDa), pre-column loading (Pre), post-flow (PF1-PF3), washes (W1-W5), and elutions (E1-E5). **(B)** Western blot of the RPGPFAWPK scramble column. Lane labels are the same as

(A). **(C)** Bar chart of the average fgn yields of the column over 9 separate runs with standard error bars shown.

A negligible amount of fibrinogen was present in the post-flow and wash fractions (PF1-PF3 and W1-W5 in Fig. 4A), but most fibrinogen was detected in the elution fractions (E1-E5 in Fig. 4A). This indicated that fibrinogen binds to the column during the loading and the wash steps and successfully elutes of the column during the elution steps (Popovic, 2022).

To validate the selectivity of the 2 mL GPRPFPAWK column, a 2 mL scramble peptide column (RPGPFAWPK) was developed using the same column coupling procedure (Popovic, 2022). The fractions that resulted from a purification of 1.8 mg of Peak 1 fibrinogen with the scramble peptide column were tested with a Western blot (Fig. 4B). The Western blot shows that most of the fibrinogen was present in the post-flow and wash fractions (PF1-PF3 and W1-W5 in Fig. 4B) while a negligible amount was present in the elution fractions (E1-E4 in Fig. 4B). The results of the scramble peptide column are opposite to those of the GPRPFPAWK column, which validates the specificity of the GPRPFPAWK column for fibrinogen capture (Popovic, 2022).

The repeatability of the column was tested by a series of nine purification runs performed over nine separate days. All purifications were performed with the same amount of Peak 1 fibrinogen (1 mg at 0.17 mg/mL) and the same series of fractions were collected. The amount of fibrinogen in the fractions, determined spectrophotometrically, was nearly identical over the nine purifications (Fig. 4C). This data demonstrates the reproducibility of the capture and elution of fibrinogen, with an average elution yield of 54 ± 4% (Popovic,

2022). A minor amount of fibrinogen was lost during the washing steps based on UV-Vis analysis.

II. Plasma Purification

After validating that the GPRPFPAWK column successfully captures and elutes fibrinogen, the ability of the column to purify fibrinogen from a complex medium was examined (Popovic, 2022). Fibrinogen from 2 mL of human fresh frozen plasma (FFP) was purified using the same 2 mL column and series of fraction collection steps. The fractions were assessed with Coomassie stained SDS-PAGE and a Western blot (Fig. 5). The Coomassie gel shows high selectivity for fibrinogen in the middle elution fractions (E3-E5 in Fig. 5A). The Western blot confirmed that most of the fibrinogen appeared in the elution fractions (E1-E6 in Fig. 5B).

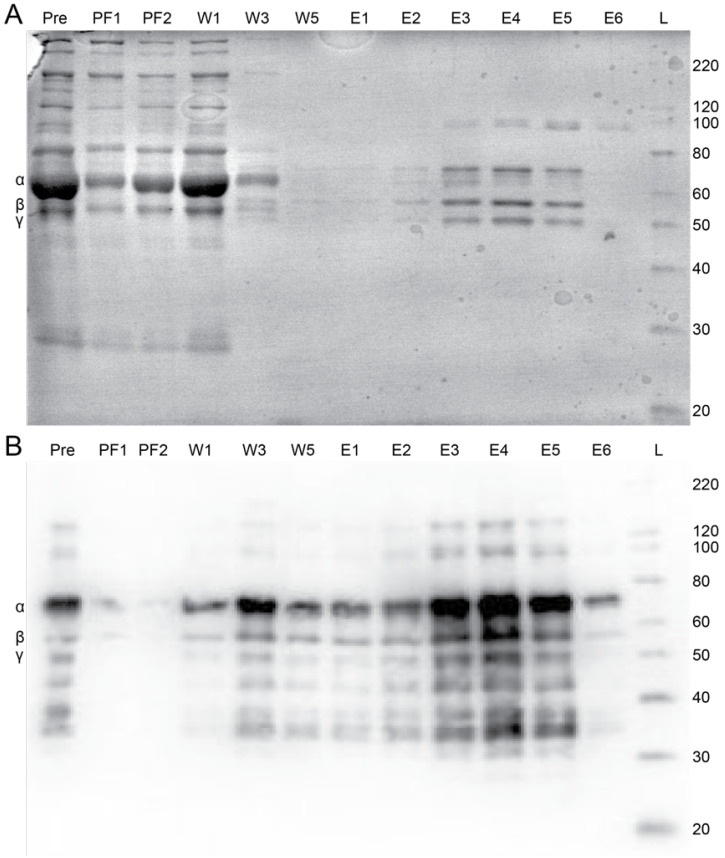


Figure 5: Purification of fgn from human fresh frozen plasma (FFP) with the 2 mL GPRPFPAWK affinity column. **(A)** Coomassie and **(B)** Western blot showing plasma proteins before and after running over the column, with similar fraction steps to Figure 4A and 4B.

From 2 mL of FFP, a combined 2.3 ± 0.4 mg of fibrinogen was collected in the elution fractions, based off an ELISA of a standard curve and the batch of FFP used for the purification (Figure 6A & 6B).

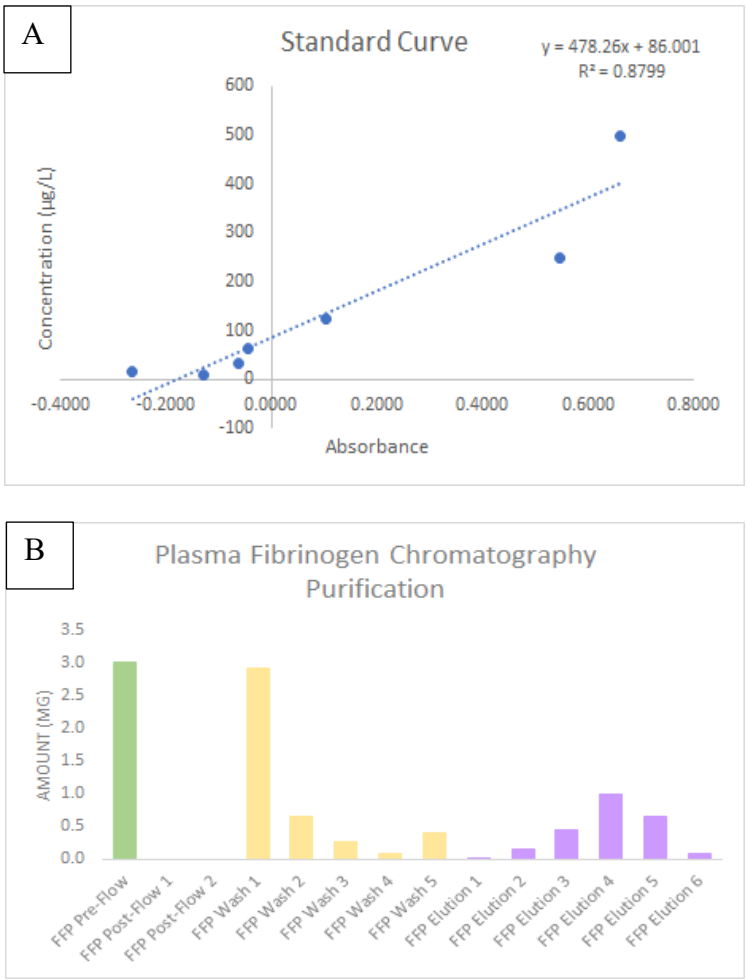


Figure 6: ELISA analysis of fgn content in original FFP sample compared to column elution fractions. **(A)** Standard curve of absorbance vs. concentration for Peak 1 fgn and **(B)**

Calculated amount (mg) of fgn in FFP pre-flow compared to column purification fractions using the standard curve equation. [1]Protein amounts estimated from elution fractions only.

The ELISA estimated 3 mg of fibrinogen total in the FFP pre-flow, which is within the range of 1.5-4.2 g/L of fibrinogen present in plasma (Fig. 6B) (Popovic, 2022). From the amount of protein calculated from the elution fractions, it was estimated that 75% of fibrinogen was recovered from the purification of FFP with the GPRPFPAWK column (Popovic, 2022).

III. Clottability and Turbidity Assays

To assess the purity and functionality of the fibrinogen isolated from FFP by the GPRPFPAWK column and ethanol precipitation, clottability and turbidity assays were performed. The clottability assay involved combining 0.5 mg/mL fibrinogen with 0.05 U/mL thrombin (final concentrations). Peak 1 fibrinogen, the ethanol purified fibrinogen, and the affinity purified fibrinogen were prepared with this procedure, and their clottability percentages were compared. Clottability is determined by the conversion of soluble fibrinogen to insoluble fibrin by thrombin (Popovic, 2022). The purified fibrinogen from FFP using the GPRPFPAWK column was found to be 86 ± 5% clottable, while the ethanol purified fibrinogen was found to be 80 ± 6% clottable (Table 2). The affinity purified fibrinogen had a much more comparable clottability value to the commercial, highly purified Peak 1 fibrinogen (Table 2).

Table 2: Clottability values of fgn purified by the GPRPFPAWK affinity column and ethanol precipitation compared to commercial Peak 1 fgn.

Fibrinogen Source	Clottability (%)
Peak 1 fgn (commercial)[1]	84 ± 3

Ethanol Purified	80 ± 6
Plasma, purified by GPRPFPAWK column	86 ± 5

[1] Note that Enzyme Research Labs estimates ≥ 95% clottable for Peak 1 fgn. The difference between the calculated value and the estimated value may be due to uncertainty in determining the amount of protein at the endpoint (Popovic, 2022).

The functionality of the purified fibrinogen from both methods was further evaluated by performing a turbidity assay to measure turbidity changes in the sample upon thrombin induced polymerization (Popovic, 2022). Turbidity monitors fibrin polymerization in real time through the change in light scattering at 350 nm (Popovic, 2022). The turbidity trace for ethanol purified fibrinogen had a shorter lag phase compared to the purified fibrinogen from FFP and Peak 1 fibrinogen. The turbidity trace for the purified fibrinogen from FFP had a nearly identical lag phase and polymerization rate to the commercial Peak 1 fibrinogen (Figure 7).

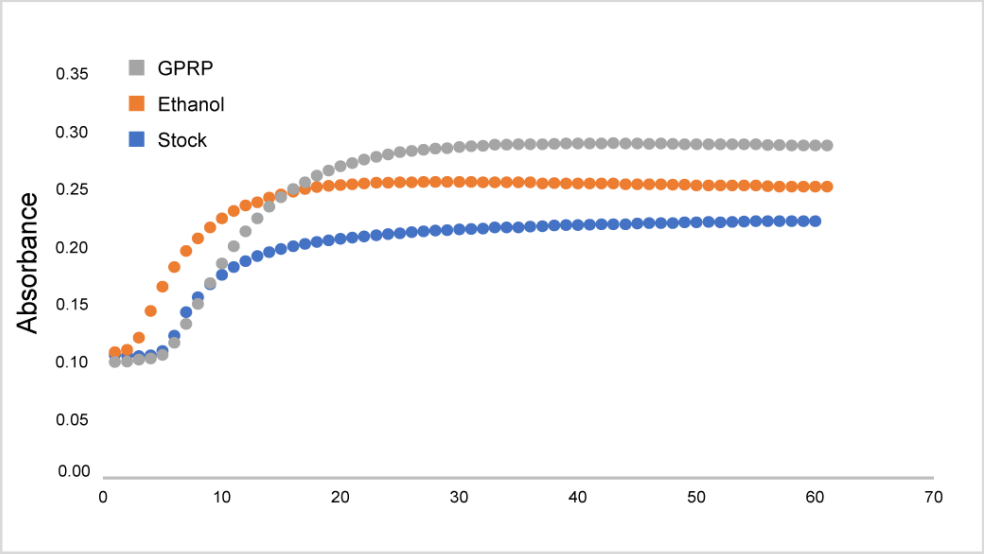
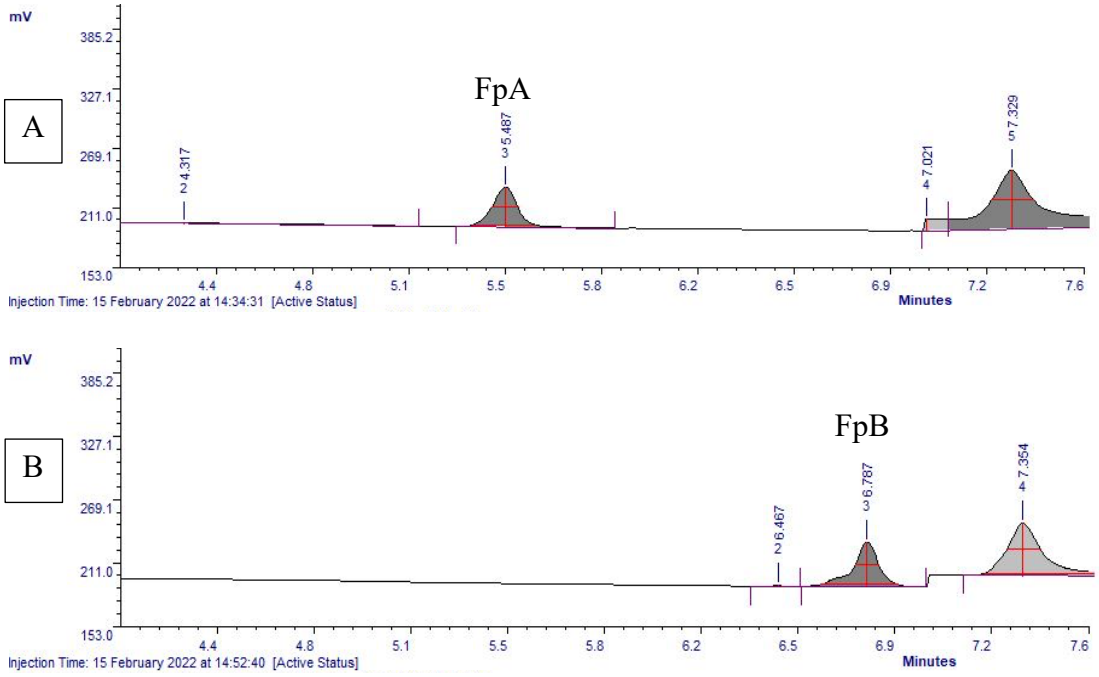


Figure 7: Turbidity traces at 350 nm for fgn isolated from FFP using GPRPFPAWK column (grey), ethanol precipitation (orange) and commercial fgn stock (blue).

The combination of the clottability and turbidity assays, along with the SDS-PAGE and Western blot analysis (Fig. 5), revealed that, in contrast to the ethanol-purified fibrinogen, the fibrinogen isolated from FFP by the GPRPFPAWK column is highly pure, functional, and comparable to commercial standards (Popovic, 2022).

IV. Fibrinopeptide Release Assay (FPRA)

The FPRA utilized pure fibrinopeptide standards to determine when FpA and FpB peaks eluted in the chromatograms of the three fibrinogen variants. As stated before, it was expected that the FpA peak would elute before the FpB peak due to more rapid cleavage of FpA from fibrinogen. This was confirmed by the chromatograms of pure FpA and FpB, where the FpA peak eluted at 5.487 minutes and FpB eluted at 6.787 minutes (Fig. 8A & 8B). These elution times were used as a comparison to select FpA and FpB peaks from the samples, which appeared at approximately the same times (Fig. 8C).



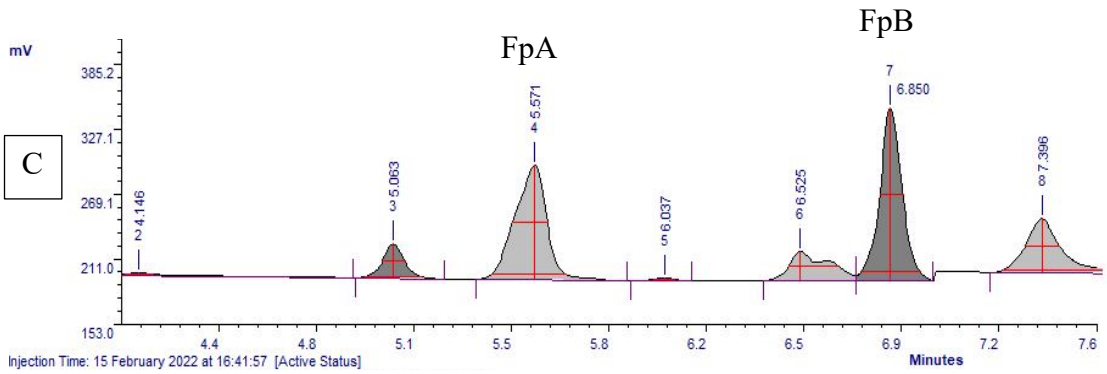


Figure 8: Chromatograms of fibrinopeptide standards compared to a chromatogram of a native fgn sample. **(A)** Chromatogram of pure FpA, elution time at 5.487 min, **(B)** Chromatogram of pure FpB, elution time at 6.787 min and **(C)** Chromatogram of native fgn 80-minute timepoint, FpA elution time at 5.571 min and FpB elution time at 6.850 min.

The raw peak integration data was plotted versus the selected digestion timepoints, where maximum peak area plateaued around the 20-minute timepoint (Fig. 9).

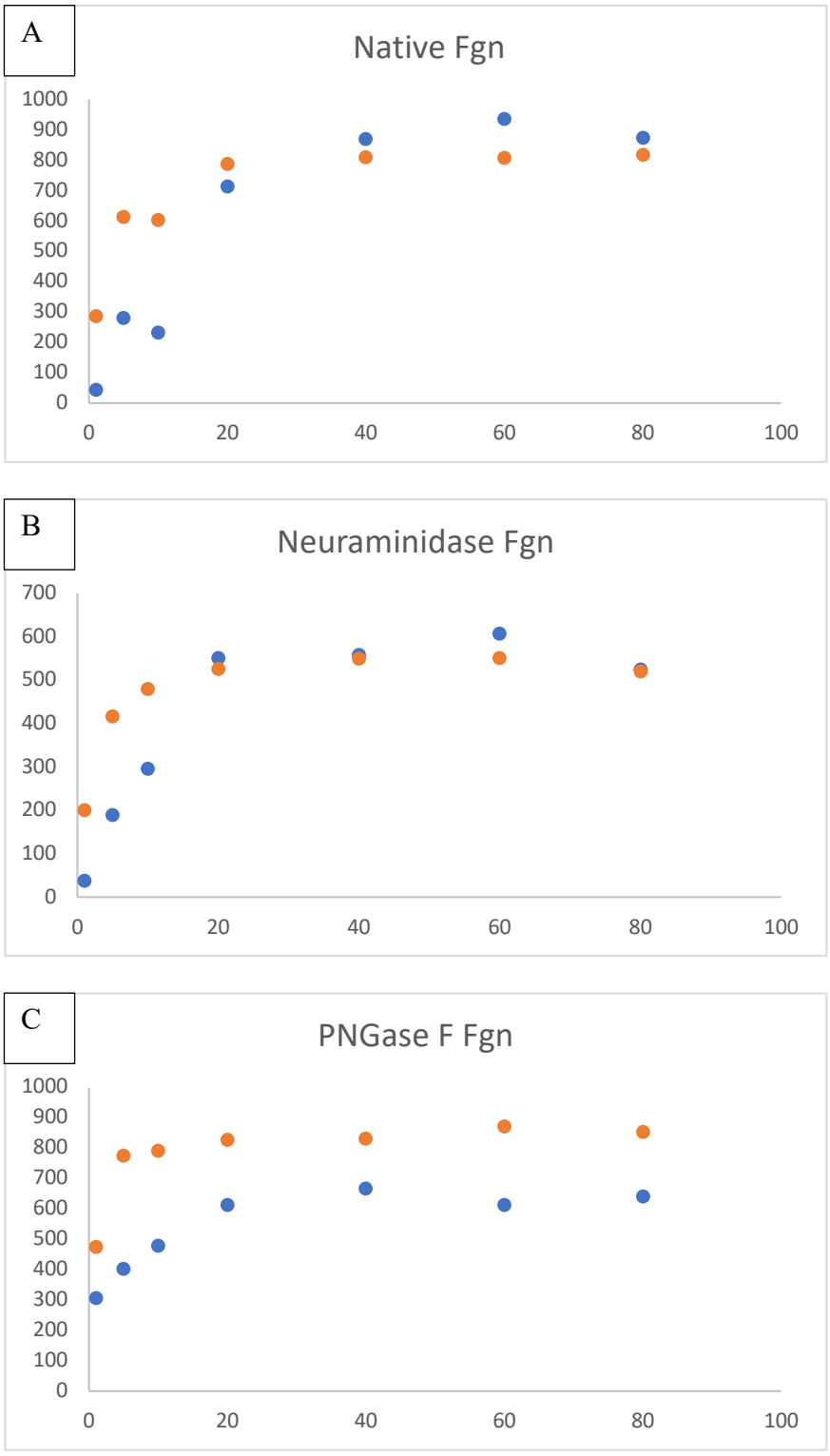
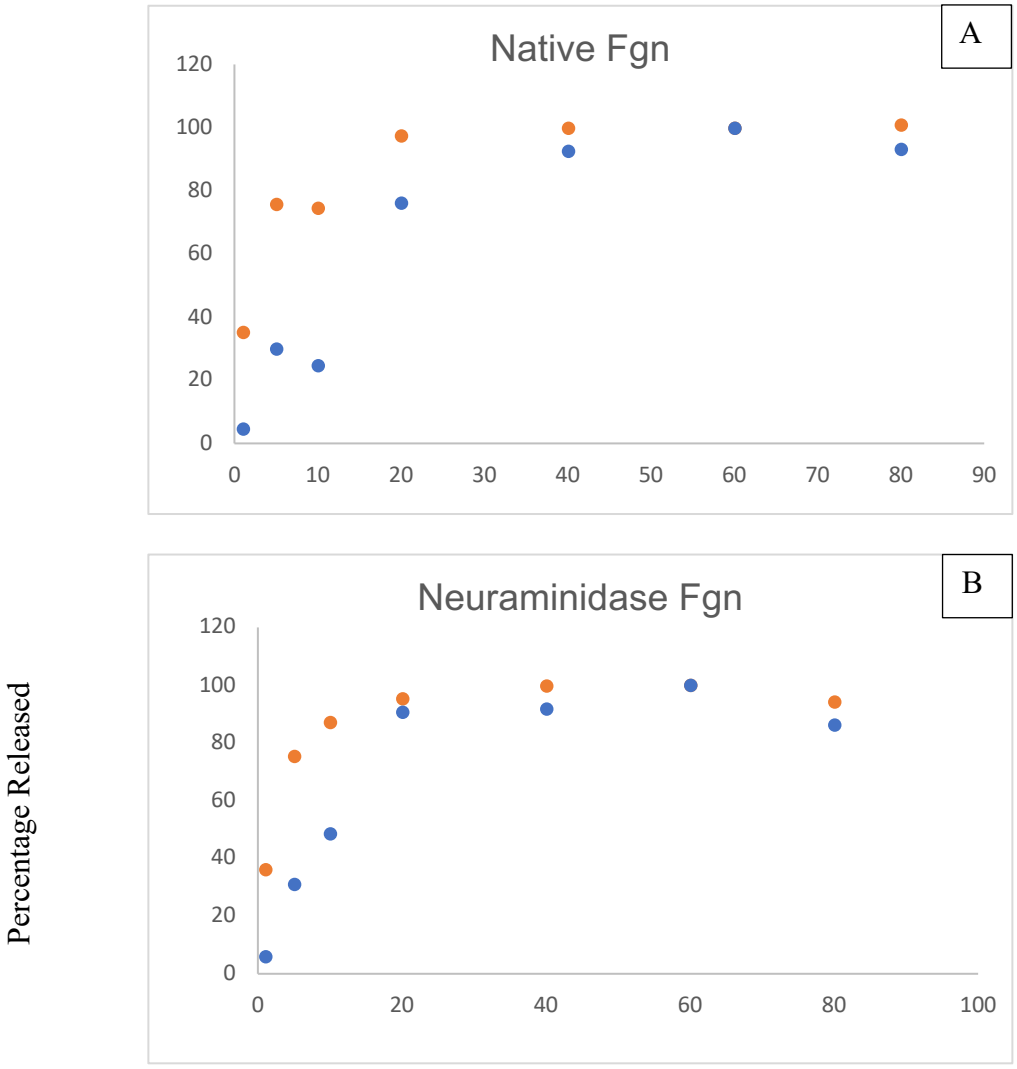

A
Native Fgn
1000
900
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
B
Neuraminidase Fgn
700
600
500
400
300
200
100
0
0
20
40
60
80
100
C
PNGase F Fgn
1000
900
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
Peak Area (mV)
Digestion Time (min)

Figure 9: Peak areas of FpA (orange) and FpB (blue) plotted versus digestion time. **(A)** Native fgn, **(B)** Neuraminidase fgn, and **(C)** PNGase F fgn.

The peak areas of the 60-minute "infinite" timepoint was used to normalize peak intensities and calculate the percentage of fibrinopeptide release from the chosen timepoints (Fig. 10). A sample calculation for the peak area of FpA for native fibrinogen at the 1-miunte timepoint is shown below:

$$\frac{FpA\ Peak\ Area\ (1\ min)}{FpA\ Peak\ Area\ (60\ min)} \times 100\% \rightarrow \frac{286.067\ mV}{808.438\ mV} \times 100\% = 35.385\%$$



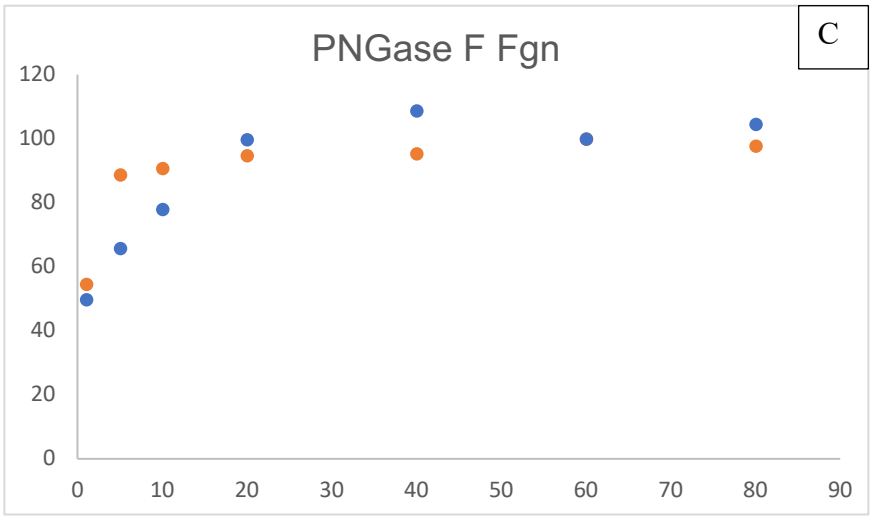


Figure 10: Percentage of FpA (orange) and FpB (blue) released plotted versus digestion time. **(A)** Native fgn, **(B)** Neuraminidase fgn, and **(C)** PNGase F fgn.

Qualitatively, PNGase F treated fibrinogen has a much more rapid release of fibrinopeptides than native and neuraminidase treated fibrinogen. This is especially seen in the comparison of the percentage of FpB released for all three variants at the 1-minute timepoint. The percentage of FpB released for native and neuraminidase fibrinogen was around 5%, while it was around 50% for PNGase F treated fibrinogen (Fig. 10A, B, C).

Discussion

A novel affinity column purification method was developed to purify fibrinogen from a complex media by utilizing the synthetic peptide Fmoc-GPRPFPAWK. This knob 'A' mimic peptide was chosen because knob 'A' interactions are stronger than knob 'B' interactions. In the presence of calcium, the binding affinity of a knob 'B' mimic, such as GHRP, is comparable to the binding affinity of GPRP. However, GHRP is readily displaced by GPRP, indicating that knob 'A' interactions are stronger than knob 'B' interactions (Stabenfeldt, 2010). This idea is supported due to the robustness of the affinity column, indicated by the specificity to fibrinogen in the Coomassie gel and Western blot (Fig. 4) and the high amount of protein recovery in purifications of both Peak 1 fibrinogen and human FFP (53.9% and 75% respectively, Fig. 4, 5, and 6). The fibrinogen isolated from FFP can also be classified as highly pure and functional, due to the similarity to Peak 1 fibrinogen in both the percent clottability values and the lag phases and polymerization rates shown in the turbidity traces (Table 2 & Fig. 7).

The FPRA successfully quantified the percentage and concentration of fibrinopeptide release from all three fibrinogen variants. To evaluate the FPRA, the Lambert function $W[a\exp(a-bt)]$ was applied to derive a Michaelis-Menten-like rate law (Williams, 2010). Classical M-M kinetics could not be fit to the FPRA data due to the concentration of fibrinogen staying constant over all experiments. When $p(t=0)=0$, the transformation of the M-M equation by the Lambert function yielded Equation 1,

$$p(t) = p_\infty - K_m^{app} W\left[\frac{p_\infty}{K_m^{app}} \exp\left(\frac{p_\infty - V^{app} t}{K_m^{app}}\right)\right] \quad \text{(Eq. 1)}$$

where p is the product concentration, p_∞ is a constant representing p at equilibrium, and V^{app} and K_m^{app} are the approximate velocity and K_m values, respectively (Williams, 2010).

It was hypothesized that this method would be appropriate for fitting to the FPRA data because it yields p_∞, V, and K_m as fitted parameters, allowing estimates of their values at a single substrate (fibrinogen) concentration. Comparison of these parameters between the three fibrinogen variants were intended to reveal how or if altered N-linked glycans affect blood clotting patterns.

The nonlinear fitting function procedure in Mathematica was used to fit the above equation to the concentration of fibrinopeptides released vs. digestion time. This fit yielded the parameter values p_∞, V^{app}, and K_m^{app}. The concentration of fibrinopeptides released was calculated based off the concentration of fibrinogen used for the FPRA samples and the reaction scheme $(A\alpha\ B\beta\ \gamma)_2 \rightarrow (\alpha\ \beta\ \gamma)_2 + 2FpA + 2FpB$, where two moles of both FpA and FpB are released when fibrinogen is converted to fibrin (Weisel, 2017). Equation 2 was used to calculate the concentration of both fibrinopeptides at 100% cleavage:

$$\frac{4\ mg\ Fgn}{340{,}000\frac{g}{mol}} \times \frac{2\ mol\ FpA/FpB}{1\ mol\ Fgn} = 2.353 \times 10^{-5} M \text{ (Eq. 2)}$$

The percentage of fibrinopeptide release data was multiplied by this concentration at 100% cleavage value to obtain the concentration of fibrinopeptides released for each timepoint. The concentration of fibrinopeptides released was plotted versus digestion time in Mathematica and the nonlinear fitting function was used to fit Equation 1 to the data (Fig. 11).

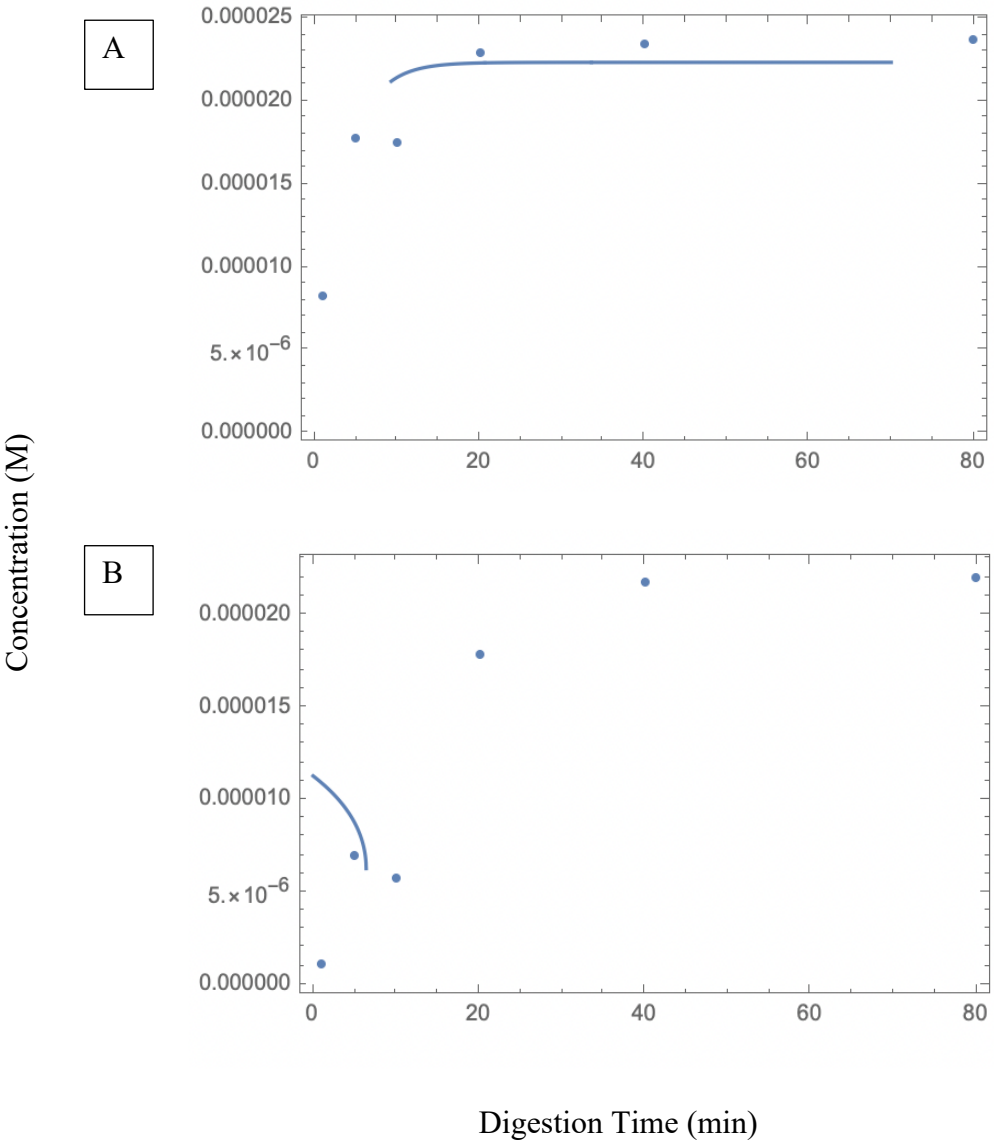


Figure 11: Concentration of fibrinopeptides released plotted versus digestion time for native fgn with nonlinear fitting function in Mathematica. **(A)** FpA for native fgn, and **(B)** FpB for native fgn.

Equation 1 did not appear to fit to the FPRA concentration versus digestion time data for both FpA and FpB (Fig. 11A & 11B). This may have been due to a lack of earlier timepoints for the FPRA. Since Equation 1 was unable to fit appropriately with the nonlinear fitting function in Mathematica, the parameters p_∞, V^{app}, and K_m^{app} could not be obtained for

the FPRA data. This could be remedied in the future by including earlier timepoints in the FPRA or lowering the thrombin concentration to allow for initial reaction rates to be obtained.

While the attempted data analysis with Mathematica was unsuccessful, previous work has provided additional evidence of PNGase F affecting the speed of fibrinopeptide cleavage by thrombin. In a turbidity assay of all three fibrinogen variants, the PNGase F treated fibrinogen exhibited higher rates of fibrin polymerization and a shorter lag phase when compared to the native and neuraminidase treated fibrinogen (Fig. 12A & 12B).

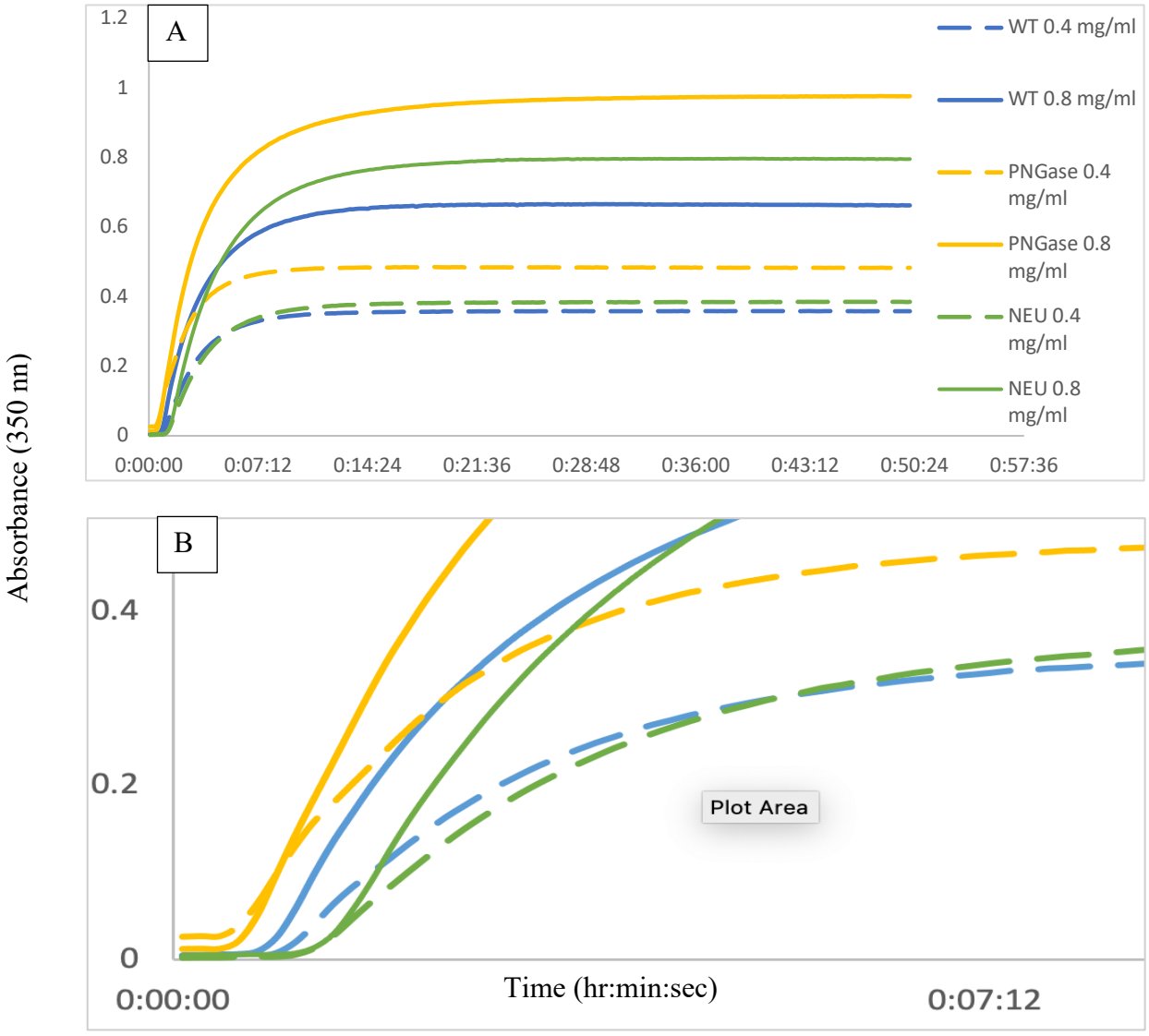


Figure 12: Turbidity assay of wild type (native) fgn (blue), neuraminidase treated fgn (green), and PNGase F treated fgn (yellow) at concentrations 0.4 mg/mL and 0.8 mg/mL. **(A)** Full traces and **(B)** zoomed-in traces of the lag phase and initial polymerization rates.

This same pattern was seen in the FPRA data, where at the 1-minute timepoint the percentage of FpB released was about 5% for the native and neuraminidase variants, but for the PNGase F variant, it was about 50% (Fig. 10A, B, C). This may be due to no steric hindrance from the N-linked glycans since PNGase F removes the glycans at Bβ Asn364 and γ Asn52 (Langer, 1988). This allows more availability of high affinity binding sites for thrombin to cleave the fibrinopeptides. The combination of the turbidity assay and the FPRA has suggested that the removal of N-linked glycans by PNGase F increases the efficiency of thrombin, which in turn increases the rate of fibrin polymerization.

Conclusions

The original GPRPFPAWK column successfully purified functional fibrinogen from human blood plasma. There were issues with reproducing the original column coupling method due to fibrinogen not successfully binding to the column, so an optimized coupling method was introduced. This optimized coupling method included a higher concentration of the synthetic peptide stock solution and a shorter shaking time with 4-methylpiperidine, which prevented degradation of the synthetic peptide. The current FPRA successfully quantifies the percentage of fibrinopeptides released from the conversion of fibrinogen to fibrin for all three fibrinogen variants. While the Lambert function can provide estimates of K_m and V at a single substrate concentration, in the future the FPRA can be modified to fit classical Michaelis-Menten kinetics with earlier timepoints and changing substrate concentrations. In all, the GPRPFPAWK column is a robust purification system that is suitable for human blood plasma and the FPRA successfully quantified both the percentage and concentration of fibrinopeptides released. The FPRA, in combination of the turbidity assay of all three fibrinogen variants, suggests that thrombin efficiency is increased when N-linked glycans are removed from fibrinogen. In the future, the optimized GPRPFPAWK column and FPRA can be used to purify and analyze fibrinogen from pregnant patient plasma samples.

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