CASE STUDY

Characterization of a Low Molecular Weight Product-related Impurity in a Manufactured Fc-Fusion Protein

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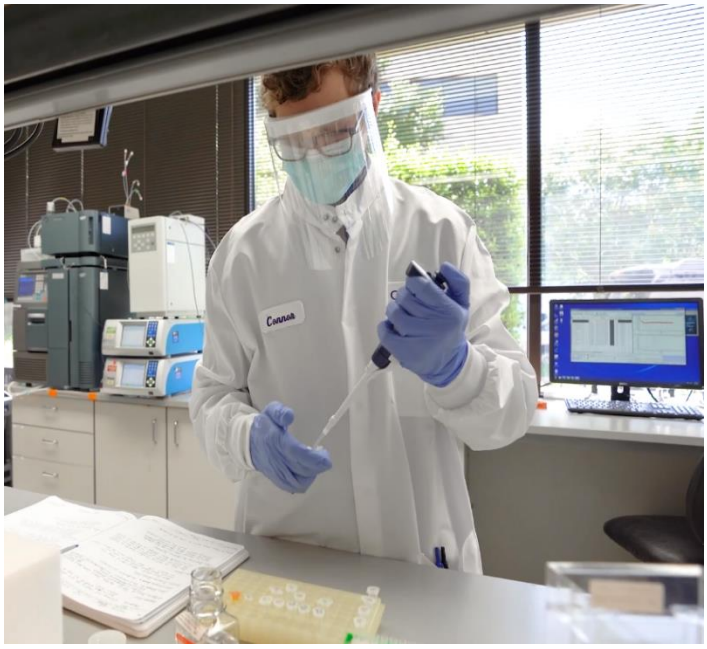
INTRODUCTION

Fragment crystallizable (Fc)-fusion proteins are a growing class of biopharmaceuticals that have gained popularity due to the manufacturability benefits and extended drug half-life afforded by the Fc fragment. One such fusion protein, consisting of a protein receptor fused to a human IgG Fc fragment (hereafter referred to as Protein X), was manufactured by Cytovance Biologics. Size Exclusion High-Performance Liquid Chromatography (SE-HPLC) is an analytical method that is routinely performed to determine size-based purity of manufactured lots of Protein X to support product release. Numerous manufacturing runs were performed for Protein X and results from the SE-HPLC analyses indicated that the purity of a recent lot was lower than expected and outside of the established specification. Upon further investigation, it was determined that the low Protein X purity was due to an increase in a poorly resolved, unidentified shoulder species eluting just after the main Protein X peak. Characterization studies were performed to both identify and measure the functionality of this low molecular weight (LMW) impurity and more fully understand if the species was a critical attribute. The results of our analyses suggest that the shoulder



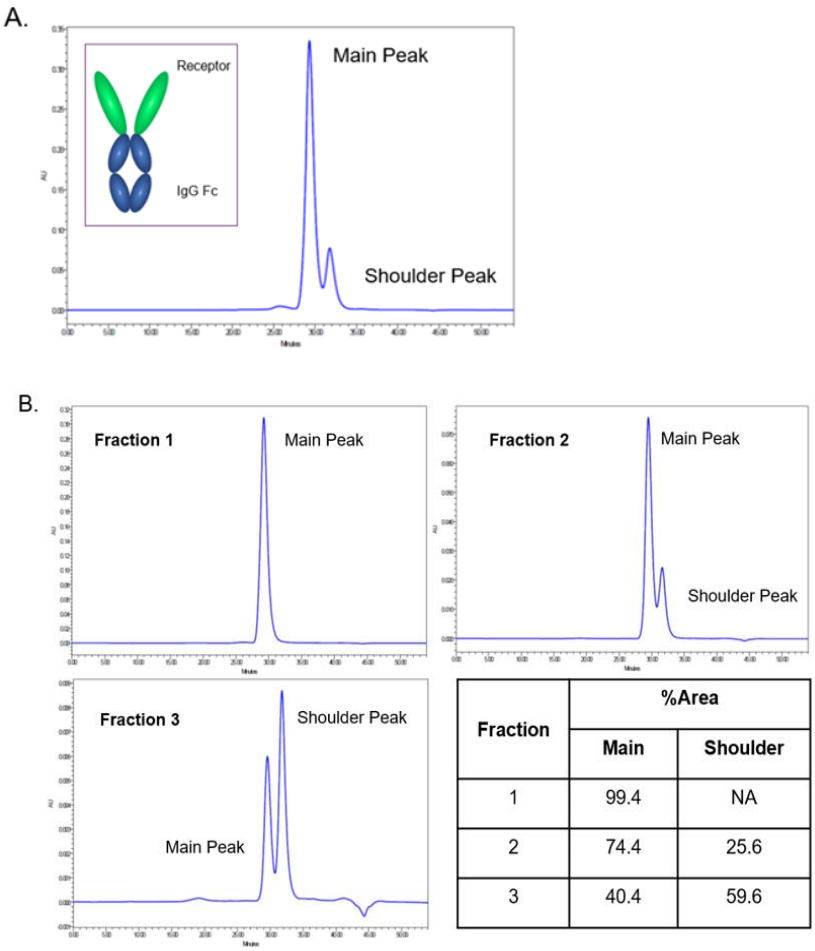
species represents a Protein X heterodimer comprised of one full-length monomer and one monomer lacking the receptor sequence. These findings defined a critical quality attribute and led to the closure of a CGMP investigation.

RESULTS AND DISCUSSION

Protein X is comprised of a human protein receptor fused to human IgG Fc (Figure 1A, inset). Monomers each contain three N-linked glycosylation sites and interact through the Fc portion to generate the Protein X dimer. Separation by size-exclusion chromatography (SE-HPLC) typically results in a main Protein X peak and four less prevalent peaks. In the Protein X Reference Standard (RS), the major species consists of >85% of the total area count and elutes at approximately 28 minutes. Two less predominate peaks that elute before the main species from approximately 20 – 25 minutes are assumed to be oligomers of Protein X and are labeled as high molecular weight (HMW) species. The two less prevalent peaks that elute later than the main species represent lower molecular weight (LMW) peaks. The first LMW peak (labeled Shoulder Peak; Figure 1A) represents approximately 8% of the total area count and exhibits incomplete resolution from the main species. The second LMW peak elutes at approximately 34 minutes.

A CGMP lot of Protein X was manufactured and analyzed via the SE-HPLC test method, resulting in 77% main peak percent area and 2% combined HMW peaks. These results were outside of the specified acceptance range for purity in this assay, which are >80% main peak, <5% combined HMW peaks. A significant increase in the LMW shoulder was also apparent (21% versus 8% in RS). Following an out-of-specification investigation, it was determined the results were valid and the material did not conform with preset purity requirements. Cytovance was asked by the client to characterize these size-based species.

In order to more fully understand and characterize the shoulder peak, fractions were collected during SE-HPLC to isolate and enrich the main and shoulder protein species (Figure 1B). Fractionation was performed on a total of 75 injections of 100 µg of Protein X GMP Drug Substance (DS), collecting 1-minute fractions



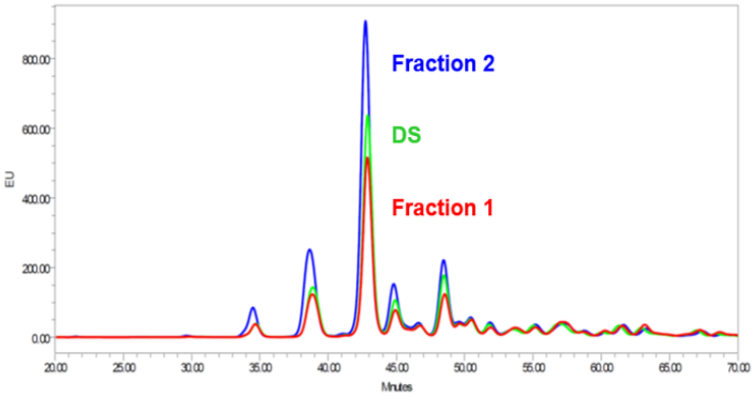
Fraction	%Area	
	Main	Shoulder
1	99.4	NA
2	74.4	25.6
3	40.4	59.6

Figure 1. Enrichment of Protein X SE-HPLC Shoulder Species. A) (inset) Schematic representation of Protein X which is comprised of a human protein receptor fused to human IgG Fc. Monomers interact through the Fc portion to form Protein X dimer. Separation by SE-HPLC results in a main Protein X peak and an unresolved shoulder species peak. B) Fractions were collected to enrich the main and shoulder species for further analysis with the percent of the main and

Table 1. Approximate molecular weight of fractions determined by SEC-MALS

SEC-MALS		
Sample	**Main Peak MW (kDa)**	**Shoulder Peak MW (kDa)**
DS	135	95
Fraction 1	138	*NA*
Fraction 2	140	117
Fraction 3	126	95
Protein X Glycosylated Dimer Expected MW: 130 kDa		

from 20-38 minutes. Each fraction was concentrated using 10 kDa molecular weight cut-off filters and buffer exchanged into PBS. The content of each fraction was assessed by SE-HPLC and fractions containing similar peaks were combined, concentrated, and buffer exchanged. A second round of fractionation, pooling and concentrations was performed on fractions containing multiple species. Fraction 1 demonstrated a successful purification of the main peak with minimal contamination of other protein species (Figure 1B, top left chromatogram). Both Fraction 2 and 3 contain mixtures of main and shoulder peak at different ratios (~75% main, ~25% shoulder in Fraction 2 and ~40% main, ~60% shoulder Fraction 3; Figure 1B, top right and lower left chromatograms and Table). While complete isolation of the shoulder peak was not achieved, the purified main peak in Fraction 1 allowed sufficient comparison to Fractions 2 and 3 to ascertain critical characteristics of the shoulder variant.



N-linked Glycan Profile					
Sample	G0F	G0FB	G1F(3)	G1F(6)	G2F
DS	39.8%	7.7%	1.8%	3.3%	4.2%
Fraction 1	36.8%	6.7%	2.3%	3.6%	5.6%
Fraction 2	42.3%	8.2%	1.6%	2.7%	3.4%

Figure 2. Glycan profile is similar in fractions containing shoulder species. N-linked glycans were removed from Protein X samples, labeled, purified and then separated by HPLC. The glycan profiles of Protein X DS and the enriched fractions were compared in the overlaid chromatograms. The percentages of specific glycans that were identified in Protein X DS and the enriched fractions is presented in the N-Linked Glycan Profile Table.

Using the enriched fractions, the molecular weight of the protein species was assessed using SE-HPLC separation combined with multi-angle light scattering (MALS). The calculated molecular weight of the main peak was consistent across all samples with an average of 134 kDa (Table 1). This molecular weight correlates with the estimated weight of the glycosylated Protein X dimer (~130 kDa). The shoulder peak was detected in Protein X DS (unfractionated), Fraction 2, and Fraction 3, and was calculated to be ~102 kDa. This weight is higher than the Protein X monomer but lower than the dimer, suggesting the shoulder may be comprised of a variant dimer form.

In order to determine if the size differences observed between the main and shoulder peaks was due to a possible loss of glycosylation in the shoulder variant, glycan mapping by HPLC was performed on the Protein X fractions. The Protein X DS and Fractions 1 and 2 were digested with PNGase F, releasing attached glycans. Released glycans were isolated, fluorescently labeled, and separated by Normal-Phase HPLC. Overall, a similar glycan profile was observed for all samples (Figure 2), indicating the size difference of the LMW shoulder is not due to loss of glycosylation.

To assess the purity and relative size of proteins within the isolated fractions, reduced and non-reduced SDS-PAGE was performed. A dark, main Protein X band was present ~140 kDa in all non-reduced samples (Figure 3; left). A doublet between 80-110 kDa was observed in all samples except the purified main peak (Fraction 1; Figure 3), which is consistent with the loss of the LMW shoulder in this sample as demonstrated by SE-HPLC (Figure 1B; top left chromatogram). This doublet was also more prominent in the Protein X DS sample compared to the Protein X RS, which is consistent with the higher percentage of the shoulder species in the Protein X DS (21%) compared to the Protein X RS (8%). A main band of ~60 kDa was present in all samples

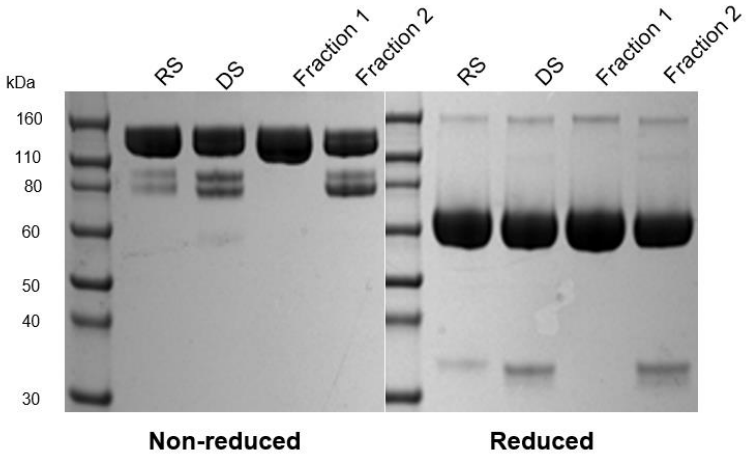


Figure 3. SDS-PAGE analysis demonstrates different banding pattern for should species in Reduced gels. Non-reduced and reduced SDS-PAGE analysis was performed to assess the purity and size of proteins within Protein X DS and the enriched fractions. The Reduced gel analysis indicated the presence of a small protein that is shared in samples containing a higher percentage of the shoulder species (DS and Fraction 2).

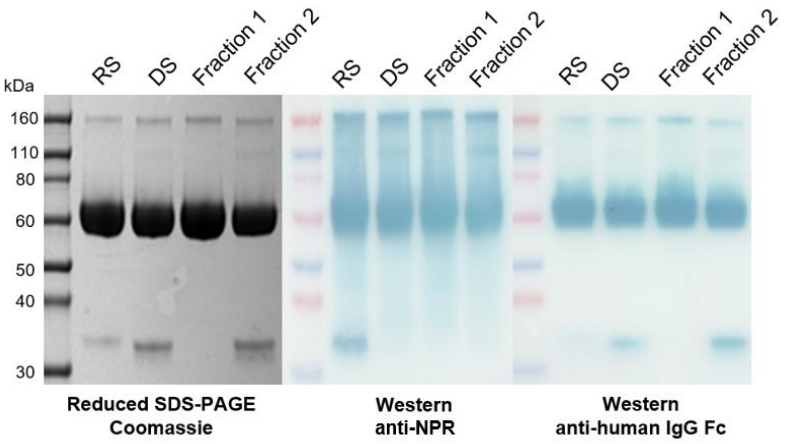


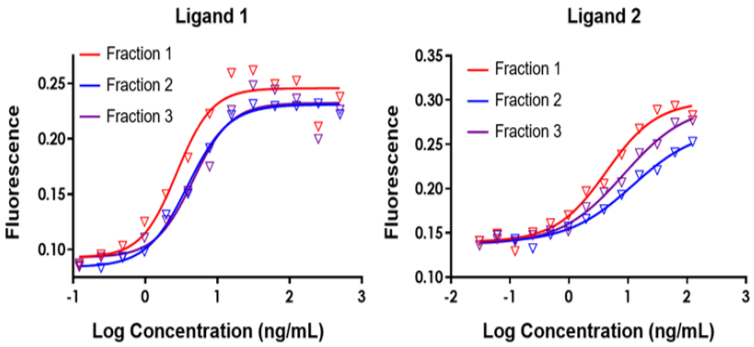
Figure 4. Immunoblot analysis of Protein X DS and fractions suggests the shoulder species is a truncated Protein X variant. Reduced SDS-PAGE gels were used for immunoblot analysis using the indicated antibodies. The combined results suggest the LMW shoulder species is a truncated variant of the protein due to the size of the protein band and the antibody recognition.

separated by reduced SDS-PAGE (Figure 3, right). A ~32 kDa band was observed in the Protein X RS, Protein X DS and Fraction 2 (Figure 3) but was absent from the purified main peak fraction (Fraction 1; Figure 3). The absence of the 32 kDa band in the main peak fraction combined with the known purity of this sample by SEC indicates that this band is composed of the LMW shoulder species. Interestingly, the 32 kDa band in the Protein X RS appears to run slightly slower in the gel, perhaps suggesting that the protein in this band is slightly different than that found in the CGMP Protein X DS and fractions containing the shoulder.

The SDS-PAGE data presented in Figure 3 indicated that the ~32 kDa LMW shoulder band observed in reduced samples may differ slightly in size between the Protein X RS and Protein X DS. Based on this data it was hypothesized that this band represents a cleavage product of full-length Protein X monomer. Moreover, the apparent molecular weight of the band is consistent with the expected molecular weight of either the novel protein receptor (NPR) fragment (~31 kDa) or the Fc fragment (~25 kDa) of the Protein X monomer. To determine the presence of the NPR and Fc sequence in these samples, Western blot analysis was performed using either polyclonal anti-NPR or polyclonal anti-IgG Fc. Under reducing conditions, Protein X DS and Fraction 2 LMW bands (~35 kDa) reacted with anti-IgG Fc but not anti-Protein X antisera (Figure 4). Taken together, these results suggest that i) the LMW shoulder species in reduced Protein X RS and Protein X DS is smaller than the full length Protein X monomer, likely indicative of a truncated variant of the protein in each lot; ii) the protein species within the LMW shoulder of the Protein X RS differs from that in the Protein X DS lot based on antibody recognition; iii) the LMW shoulder of Protein X RS contains adequate NPR sequence to be recognized by a polyclonal antibody while Protein X DS appears to lack NPR sequence required for anti-NPR recognition.

To assess the functional impact on the binding ability of Protein X to its ligands (Ligand 1 and Ligand 2), the fractions were assessed by homogeneous time-resolved fluorescence resonance energy transfer (HTRF) method. The purified main peak (Fraction 1) exhibited approximately 1.5-fold higher binding activity to Ligand 1 compared to fractions containing the shoulder species, indicating that the main peak possesses the majority of binding ability in the

sample (Figure 5). Fractions 2 and 3 exhibited binding activity 66% and 60% of the purified main peak, respectively. Fraction 2 is comprised of 75% main peak and 25% shoulder, while Fraction 3 is 40% main peak and 60% shoulder (Figure 1B). Thus, the ligand binding data correlates reasonably well with the ratio of full-length Protein X and shoulder fragment in the two samples and suggest that the shoulder fragment does not exhibit equivalent binding activity as the full-length protein. The purified main peak (Fraction 1) also exhibited approximately 1.5-fold higher binding to Ligand 2 compared to fractions containing the shoulder species (Figure 5). The shoulder and LMW fractions exhibited 44% and 52% of main peak binding, respectively. These data correlate with the Ligand 1 binding and suggest that the shoulder fragment does not convey full-length Protein X binding.



Fraction	%Area		%Binding	
	Main	Shoulder	Ligand 1	Ligand 2
1	99.4	NA	100.0	100.0
2	74.4	25.6	65.8	43.5
3	40.4	59.6	59.7	52.1

Figure 5. Functional analysis of fractions by HTRF indicates the shoulder species has reduced binding compared to full-length Protein X. The enriched fractions were assessed for binding activity and showed that the higher percentage of shoulder species corresponded with a lower capacity to bind to Protein X.

Several pieces of data presented above suggest that the Protein X LMW shoulder is a fragment of the full-length molecule. To determine the portion of the fusion protein that is present in the shoulder, peptide mapping via liquid chromatography coupled with mass spectrometry (LC-MS) was performed at the Oklahoma Medical Research Foundation. Protein X DS material was separated by reduced SDS-PAGE and protein bands were stained with Coomassie. The main band of approximately 60 kDa and LMW shoulder band around 32 kDa were separately in-gel digested with trypsin, and the resulting peptides were extracted. Isolated peptides were separated using capillary reversed-phase chromatography (RP-HPLC), high resolution MS analysis was performed, and detected peptides were mapped to the sequence of Protein X. Mapping of peptides isolated from the 60 kDa main band revealed good coverage across the entire Protein X peptide sequence (Figure 6; red). In contrast, only peptides mapping to the IgG Fc fragment and the C-terminal 10 amino acids of the receptor were identified in the 32 kDa shoulder band (Figure 6; green). To identify the N-terminus of each species, the 60 kDa main band and 32 kDa LMW shoulder band were excised from Ponceau stained PVDF membrane and submitted for Edman degradation analysis.

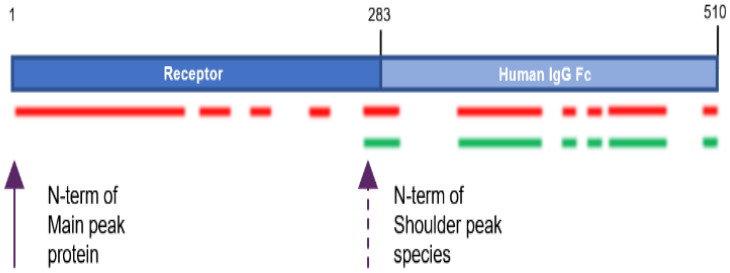


Figure 6. Schematic representation of LC-MS and Edman Degradation results confirming the shoulder species is a fragment of Protein X. Peptide mapping of the main band peptides showed coverage across the entire Protein X peptide sequence (red), however the shoulder species mapped to the IgG Fc fragment and the C-terminal 10 amino acids of the receptor.

N-terminal sequencing confirmed the peptide mapping data, with the expected N-terminus of full-length Protein X identified as the N-terminus of the 60 kDa main band protein and the last 10 amino acids of the receptor identified as the N-terminus of the 32 kDa shoulder band (Figure 6 arrows and data

not shown). Taken together, these data demonstrate that the Protein X shoulder species is a fragment that contains the last 10 amino acids of receptor and all of the IgG Fc sequence.

CONCLUSIONS

Based upon the data collected during this investigation and presented in this case study, the observed SE-HPLC shoulder is a product-related impurity consisting of a heterodimer of full length and truncated Protein X monomers (Figure 7). To determine if the species was a critical attribute, it was necessary to both identify and measure the functionality of the impurity. To identify this impurity, the LMW species was collected, enriched (Figure 1) and characterized via glycan mapping (Figure 2), SEC-MALS (Table 1), SDS-PAGE, Immunoblot (Figures 3 and 4), N-Terminal analysis by Edman Degradation and LC-MS peptide mapping (Figure 6) to gain insight into the molecular size and sequence. The functional consequence of the Protein X cleavage product was assessed through homogenous time resolved fluorescence (HTRF) (Figure 5).

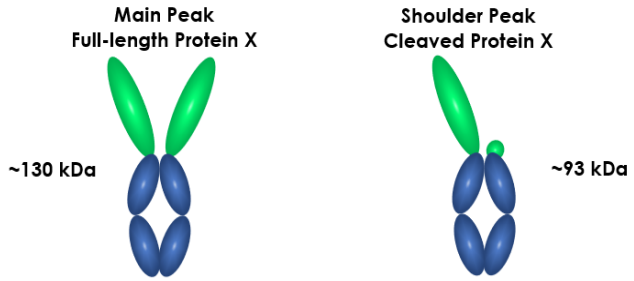


Figure 7. Schematic representation of shoulder species compared to full-length Protein X. The results of these studies indicate the Protein X shoulder is a cleavage product lacking a large portion of the NPR.

Characterization of the identity and functionality of this product-related impurity defined a critical quality attribute and lead to the closure of GMP investigations. In addition, characterization of this impurity provided sufficient evidence to support the recommendation and design of additional process steps to control the impurity levels and allowed informed adjustments to the manufacturing process to reduce generation of this fragment.

MATERIALS AND METHODS

Size Exclusion -High Performance Liquid Chromatography (SE-HPLC) and MALS

Fractions were collected during SE-HPLC to isolate and enrich the main and shoulder protein species. SE-HPLC was performed using a TSK-GEL SuperSW3000 (Tosoh) column on a Waters Alliance with UV detection at 280 nm. Fractions were collected using a Fraction Collector II (Waters). Light scattering data were collected using a miniDAWN Treos MALS detector and T-rEX RI detector (Wyatt Technologies) in sequence with the SE-HPLC TSK-GEL SuperSW3000 column.

SDS-PAGE and Immunoblot

Protein X DS SE-HPLC fractions were separated on NuPage 4-12% Bis-Tris Polyacrylamide gels (Invitrogen) in the presence or absence of reducing agent. Gels were stained with Coomassie Brilliant Blue (Sigma).

For immunoblots, separated protein was transferred to PVDF (BioRad), blocked with milk buffer, probed with anti-Protein X / rabbit anti-goat HRP (R&D Systems) or anti-human IgG Fc HRP (Bethyl Labs) and detected using TMB chromogenic substrate (Invitrogen).

Glycan Mapping

N-linked glycans were removed from Protein X samples by PNGase F (Sigma) digestion under denaturing conditions. Released glycans were labeled with 2-AB (Sigma) and purified with acetonitrile (Fisher) via vacuum filtration. Labeled glycans were separated using a GlycoSep N column (Prozyme) on a Waters Alliance with fluorescent detection at 425 nm.

Peptide Mapping

Protein X DS was separated by reduced SDS-PAGE and stained with Coomassie. Full-length and shoulder bands were excised and digested with

Trypsin. Peptides were separated with Phenomenex Aeris Peptide XB-C18 beads (Phenomenex) on an Ultimate 3000 HPLC (Thermo Scientific) and LC-MS performed on a Q Exactive Plus MS system (Thermo Scientific) with Skyline software (MacCoss Lab Software) at Oklahoma Medical Research Foundation, Oklahoma City, OK.

Edman Degradation

Protein X DS was separated by reduced SDS-PAGE and stained with Coomassie. Full-length and shoulder bands were excised. Edman Degradation was performed using an ABI Procise 494 sequencer at Alphalyse (Palo Alto, CA).

Homogenous Time Resolved Fluorescence (HTRF)
Homogenous time resolved fluorescence (HTRF) measured energy transfer between d2-labeled ligand and Tb-labeled anti-human IgG and was performed according to established SOP (Cisbio). EC_{50} values were calculated for each sample and compared to the enriched main peak.

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