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BIOMÉRIEUX

Recombinant Factor C Characterization Studies Confirm the Published Structure and Function of Manufactured rFC

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INTRODUCTION

With the advent of first the EU Pharmacopeial Chapter 2.6.32 (2020) and now the publication of USP Chapter <86> (2024) on the use of recombinant Factor C assays, the manufactured products supplied must meet customer confidence expectations in terms of r-Protein characterization. Both current and historical QC characterization tests are described here for ENDOZYME II. The known and therefore expected structure of the complex molecule is shown below from: Shibata et al. 2018 "Intermolecular autocatalytic activation of serine protease zymogen factor C through an active transition state responding to lipopolysaccharide" (1).

CONCLUSION: RECOMBINANT FACTOR C (ENDOZYME® II) PRODUCED BY BIOMERIEUX HAS BEEN SHOWN TO BE HIGHLY CHARACTERIZED AND HIGHLY REPRODUCIBLE, LOT TO LOT, ON A HISTORICAL BASIS.

BACKGROUND

The basic domain structure of Factor C is shown below followed by a more detailed structural map.

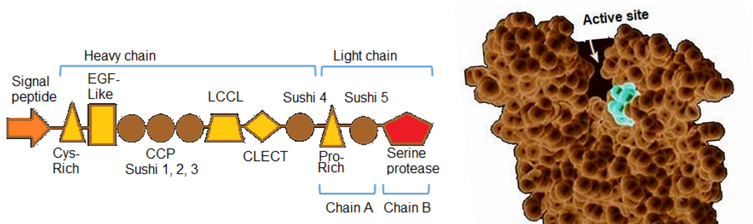

Figure 1. A simplistic domain map of Factor C (left) and a generalized example of a globular type protein (right).

Factor C is a multi domain protein with a globular domain at its C-terminal end that acquires a structure as shown above at right. The globular structure surrounds the serine protease active site that is activated by endotoxin. The proper formation of the structure is critical for the appropriate response as a zymogen to endotoxin.

The structure shows multiple domains that include, importantly, (a). the Cys-rich endotoxin binding region, (c) the 5-complement control protein (CCP) or Sushi domains, and (f) the activated Serine protease domain. These are significant domains to verify for QC characterization study purposes.

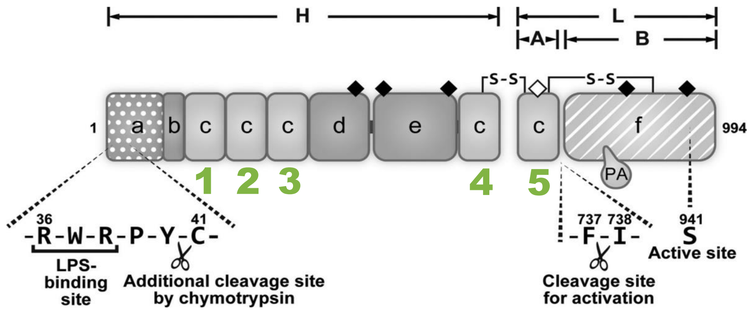

Figure 2. A schematic domain structure of factor C. A. Cys-rich domain, B. epidermal growth factor domain, C. 5 complement control protein domains (Sushi domains), D. Limulus factor C-Coh 5b2-Lg11 domain, E. C-type lectin domain, F. Serine protease domain. The two interchain disulfide bonds are indicated by bars. PA indicates the position of the PA tag inserted between Trp^{758} and Leu^{759}. Closed diamonds show N-linked glycosylation sites and the fourth N-glycosylation site indicated by the open diamond is partially modified [2].

References:

[1] Shibata et al., Intermolecular autocatalytic activation of serine protease zymogen factor C through an active transition state responding to lipopolysaccharide, J. Biol Chem. 302., 2018.

[2] Muta et al., A. Limulus Factor C AN ENDOTOXIN-SENSITIVE SERINE PROTEASE ZYMOGEN., The Journal of Biological Chemistry 266, 1991 .

[3] Mizumura et al., Genetic engineering approach to develop next-generation reagents for endotoxin quantification, Innate Immunity 23, 2017.

CONFIRMATION METHODS AND DATA

A) DNA Sequencing

Isolation of genomic DNA from *Leishmania tarentolae* stored working cell bank clone (2022) derived from a 2012 lot was followed by preparation and amplification of the DNA material that allowed for sequencing by an external sequencing service. This study (2023) allowed for the confirmation that the complete construct was present and identical compared to both the DNA sequence from 2012 and as compared to the reference AA sequence of rFC identical to that given by Muta et al. 1991 [2] (Uniprot-ID: P28175).

B) SDS PAGE & Western blot

The Western blot can provide an indirect proof for the presence of the 5^{th} Sushi domain which represents a crucial structure within Factor C protein and is therefore one indicator for the correctly folded zymogen. That is because during activation of Factor C a tripartite protein is formed that is solely connected via two disulfide bonds of which both are formed by the 5^{th} Sushi domain. In its mature form but before activation one disulfide bond is already connecting H- and L-chain (see Figure 2).

At this stage, one can easily verify the presence of the domain by performing a Western blot under reducing and non-reducing conditions. The ≈110 kDa band under non-reducing conditions reveal the full-length Factor C protein with H- and L-chain still connected (Figure 3a). After the reducing condition has broken down the disulfide bond two new bands with sizes ≈70 kDa and ≈40 kDa appear corresponding to H- and L-chain, respectively. A control with BSA shows that it does not interfere with the rFC detecting antibody (see Figure 3 (b)).

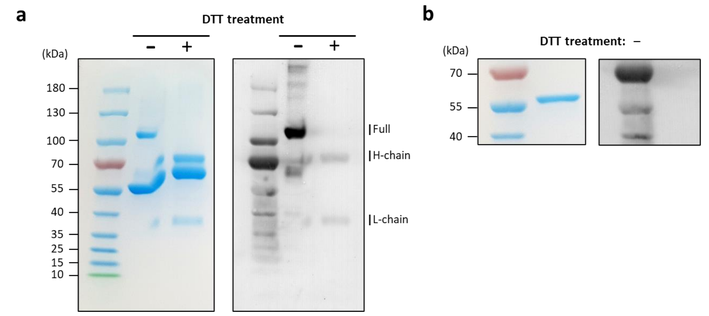

Figure 3. Coomassie-stained SDS-PAGE and Western blot of bulk rFC protein. (a) Left: Comassie-stained SDS-PAGE of bulk rFC sample under non-reducing (-DTT) and reducing (+DTT) conditions. The protein standard indicates a single rFC band at size ≈110 kDa for the sample without DTT treatment and two rFC bands at sizes ≈70 kDa and ≈40 kDa with DTT treatment. A strong BSA band (5 times excess over rFC) is visible at size ≈55 kDa and ≈65 kDa without and with DTT treatment, respectively. Right: Western blot of bulk rFC sample using a polyclonal anti-rFC antibody. The ≈110 kDa, ≈70 kDa and ≈40 kDa bands seen on SDS-PAGE exhibit binding of anti-rFC antibody thereby proving their identity as full-length (Full), H-chain and L-chain of the rFC protein. (b) Left: Coomassie-stained SDS-Page of BSA sample without DTT treatment exhibits a band at size ≈55 kDa. Right: The same BSA sample shows no binding of anti-rFC antibody. The BSA control shows that BSA does not interfere with antibody binding.

C) Historical comparison of lots using Coomassie-stained SDS-PAGE & Western blot

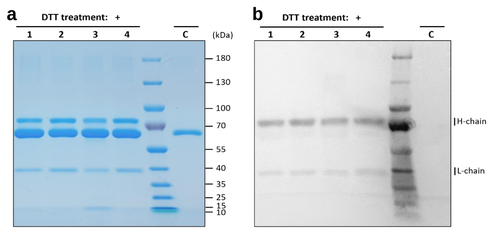

Figure 4. Coomassie-stained SDS-PAGE and Western blot of historical bulk rFC lots. (a), (b) Coomassie-stained SDS-PAGE and Western blot of the same bulk rFC lots with DTT treatment reveals separated H- and L-chain. Lane 1: 2018, 2: 2019, 3: 2020, 4: 2021, C: BSA control. The BSA control shows that BSA does not interfere with antibody binding.

CONFIRMATION METHODS AND DATA

D) Mass spectrometry analysis

i. MALDI-TOF MS of intact protein

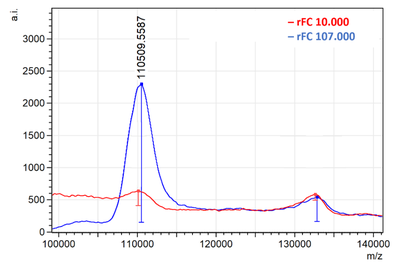

Figure 5. MALDI-TOF MS reveals full-length rFC protein. Ion gating blanking (rFC 107.000) was necessary because BSA intensity hides bulk rFC signal (rFC 10.000). In this manner the full length rFC protein (≈110 kDa) was detected in the sample.

ii. Sequence Coverage using enzymatic digestions by combined LC-QTOF and MALDI-TOF MS results.

A 100% sequence recovery is rarely achieved with this technique, due to some peptide not well digested and/or that can't be ionized by MS process. Nevertheless, ~80% coverage allows for the **confirmation that all Sushi domains are fully present.** The previous results with intact MALDI-TOF MS, already showed that rFC sequence is complete, as it was measured at 110kDa, as expected (which agrees with published studies by Muta et al. [2].

E) Glycosylation analysis of bulk rFC

i. PNGase F treatment

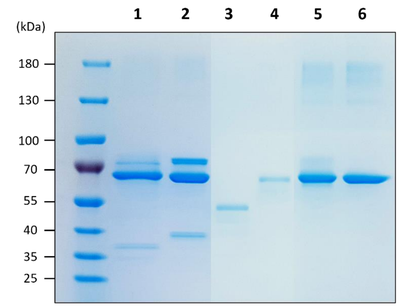

Figure 6.. Reducing, Coomassie-stained SDS-PAGE of rFC with and without PNGase F treatment. Lane 1, 2 : rFC treated and not treated with PNGase F, respectively, shows clear reduction in size with treatment indicating glycosylation removal [3]. Lane 3, 4: Fetuin (0.2 mg/ml) treated and not treated with PNGase, respectively shows clear size reduction with treatment (positive control). Lane 5, 6: BSA treated and not treated with PNGase F, respectively, shows no effect of treatment (negative control). All bands originated from the same SDS-PAGE but were merged differently for reasons of clarity.

ii. Lectin binding ability of historical bulk rFC

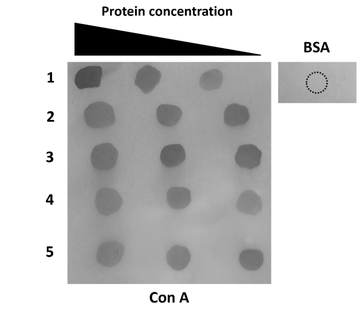

Figure 7. Dot Blot of historical bulk rFC protein lots reveals glycosylation. Fetuin as positive control (row 1) and rFC bulk (row 2-5: 2018, 2019, 2020, 2021) as sample were dotted in three different concentrations, treated with a mannose binding lectin (Con A, [3]) followed by readout via alkaline phosphatase substrate turnover. The strong signal over all protein concentrations indicates the presence of the sugar residue in bulk rFC protein across bulk rFC lots. Additionally, BSA was dotted as negative control (dotted lines) as it harbors no glycosylation and showed no binding of the lectin.

CONCLUSION

Given the critical nature of endotoxin testing as a quality attribute of parenteral drug product testing and the expectation of rigorous regulatory review, a thorough characterization of recombinant Factor C as a quality control test reagent should put test user's concerns to rest. Furthermore, test users should insist on detailed characterization of all current offerings.