

D19

A comparison of *Limulus* Factor C expressed with and without terminal sialic acid glycosylation

Purpose:

Our purpose was to compare the two forms of Factor C, with and without the (α -2,3)-linked terminal sialic acid, and determine their respective biological activities. We expressed the *Limulus* Factor C gene in two different host cell lines derived from HEK 293 cells. The first cell line was derived from the Thermo Fisher Freestyle 293 system, which produces expressed proteins fully glycosylated with terminal sialic acid. The second cell line was derived from the HEK 293 GnTI- host cell. This cell line lacks the enzymes necessary for addition of the terminal sialic acid. Specifically, GnTI- cells lack N-acetylglucosaminyltransferase, and are thus unable to synthesize complex N-glycans.

Methods:

Preparation of *Limulus* Factor C (rFC) derived from HEK293 cells and HEK293 GnTI- cells:

The *Limulus* Factor C gene was cloned into both HEK293 cells and HEK293 GnTI- cells. Cultures of each were grown in Thermo Fisher Freestyle 293 medium according to manufacturer's instructions. Expressed Factor C from both lines were collected from the medium and concentrated by tangential flow filtration (TFF) for use in all experiments.

Gel electrophoresis:

Standard SDS polyacrylamide gel electrophoresis was performed, then stained for protein with SYPRO Orange. Fetuin, a glycoprotein, was included as a positive control for sialic acid.

Western Blotting:

The gel was transferred to a nitrocellulose (NC) membrane by electroblotting and stained for sialic acid content by reaction with the sialic acid binding lectin from *Maackia Amurensis* using the following protocol:

Materials:

NC membrane from electroblotting		
Biotinylated Maackia Amurensis Lectin II	Vector Lab	1 mg/ml
Avidin-Horse Radish Peroxidase (HRP)	BioRad	1:1000
HRP Conjugate Substrate Kit	BioRad	
1% Casein / 1x TBS		
Blocker	BioRad	
TBS	BioRad	
TTBS	BioRad	

Procedure:

Blocking:	Rinse NC membrane with TBS Add 1% Casein / 1x TBS	5 min x 2 shake 1 hr / room temp.
1 st Reaction:	Prepare Biotin-Lectin solution (400 uL TBS) Rinse NC membrane with TTBS	shake 1 hr / room temp.
2 nd Reaction:	Add 20 uL Avidin-HRP in 20 mL TBS (1:1000) Rinse NC membrane with TTBS Rinse NC membrane with TBS	shake 1 hr / room temp. 10 min. x 3 10 min. x 2
Color Development:	Mix from HRP Conjugate Substrate Kit HRP development buffer 1x 20 mL Reagent A, 0.12 mL Reagent B, 4.0 mL After color develops, rinse w/ clean water, photograph	

Preparation of recombinant reagents using recombinant Factor C (rFC) derived from HEK293 cells (HEK293) and HEK293 GnTI- cells (GnTI-):

Recombinant Factor B (rFB), recombinant Proclotting Enzyme (rPCE) and the chromogenic substrate Ac-Ile-Glu-Gly Arg-pNA were added to standard formulation excipients for *Limulus* amebocyte lysate (LAL), containing 0.1 M Tris-HCl buffer, pH 8.0. Then, rFC from HEK293 or GnTI- was added to the solution to achieve two common mixtures of functional recombinant LAL, one containing rFC from HEK293 and one containing rFC from the GnTI- strain lacking terminal sialic acid glycosylation.

Establishment of standard curves:

Standard curves were established using US Reference Standard Endotoxin (RSE) at 0, 0.025, 0.05, and 0.1 EU/mL. RSE dilutions (0.1 mL) were mixed with the same volume of rLAL on a microplate, and the absorbance change rates (mAbs/min) of the reaction mixtures were measured at 405 nm after 30 minutes reaction time.

Measurement of endotoxin in salt solutions:

Endotoxin (RSE) was added to salt solutions at 0.05 EU/mL. Salt solutions used were 1.25% (214 mM) NaCl, 2.5% (428 mM) NaCl, 16 mM MgSO₄, 2.5 mM CaCl₂, 21 mM sodium citrate, and 52 mM sodium hydrogen carbonate.

The residual activity was calculated by dividing the average absorbance change (mAbs/min) measured in the salt solution by the absorbance change (mAbs/min) measured for the reference 0.05 EU/mL endotoxin without salt.

Results:

Table 1: Order of samples run on SDS Gel:

Lane	Sample	Load
1	Molecular Weight Markers	
2	M21219-32 HEK293 GnTI- <i>Limulus</i> Factor C no sialic acid	2.7ug
3	M212199-28 HEK293 <i>Limulus</i> Factor C with sialic acid	3.0 ug
4	Fetuin (positive control for sialic acid)	10 ug

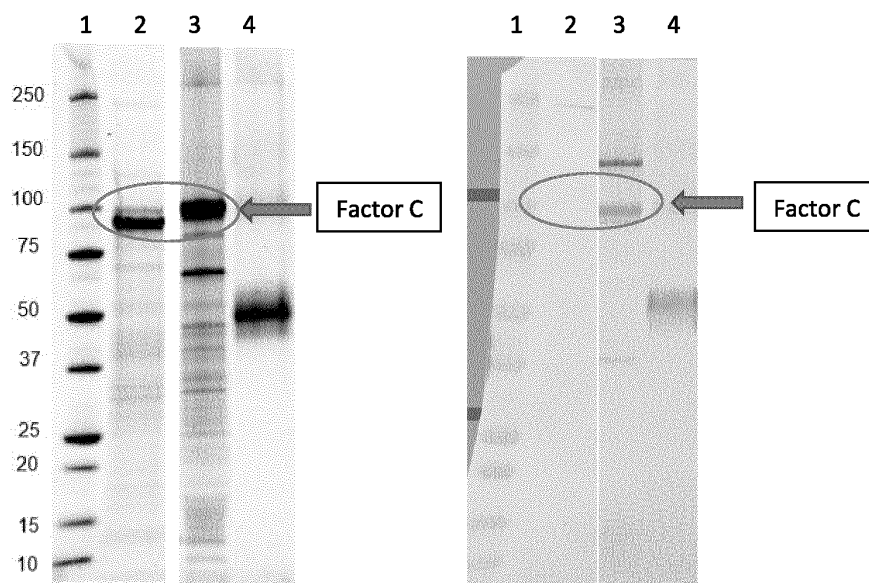


Figure 1 Image of SYPRO Orange stained gel

Figure 2 Image of sialic acid specific stained Western blot

Terminal Sialic Acid content of Factor C from HEK293 vs HEK 293 GnTI-:

Factor C produced by the HEK 293 clone (lane 3) was the only significant sialic acid staining other than the fetuin positive control (Lane 4). No sialic acid binding was observed in the GnTI- strains where Factor C migrates (lane 2.)

Measurement of endotoxin in NaCl, MgSO₄, and CaCl₂:

Good dose-responses were observed with both HEK293 and GnTI- rFCs (Figure 3). Table 2 shows the absorbance change rates for each endotoxin concentration. Reproducibility was also good (CV < 5.3%).

Figure 4 shows the salt effects on the residual activity (endotoxin recovery) of spiked endotoxin. There was no significant difference between the residual activities of rLALs using rFCs expressed in HEK293 and GnTI- cells in any of the salt solutions.

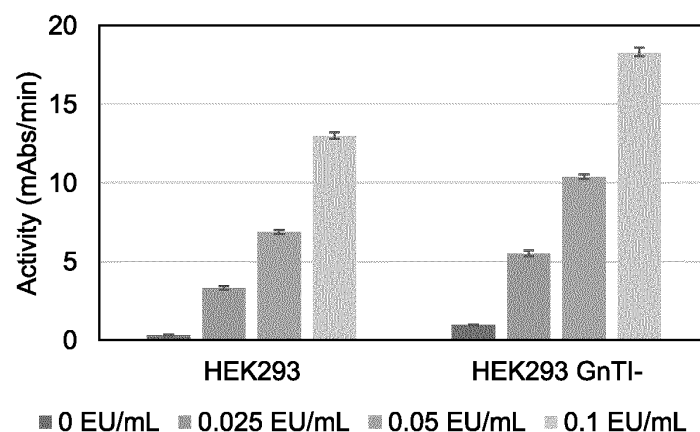
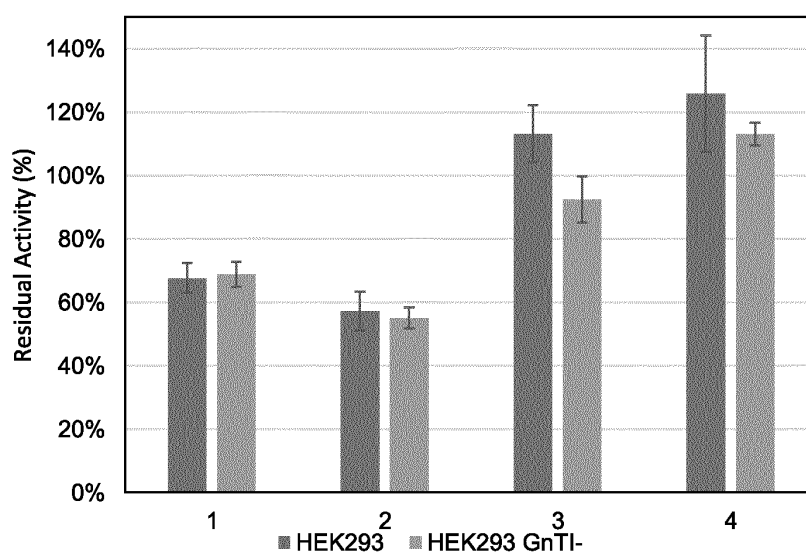


Figure 3 Standard curves of rLAL using HEK293 and GnTI- rFCs.



1- 1.25% (214 mM) NaCl; 2- 2.5% (428 mM) NaCl; 3- 16 mM MgSO₄; 4- 2.5 mM CaCl₂.

Figure 4 Residual activity (endotoxin recovery) of endotoxin in salt solutions. (n = 4)
Spiked endotoxin: 0.05 EU/mL. rLALs using HEK293 and GnTI- rFCs were used.

Measurement of endotoxin in sodium citrate, and sodium hydrogen carbonate:

Good dose-responses were observed with both HEK293 and GnTI- rFCs (Figure 5).

Figure 6 shows the salts effects on the residual activity (endotoxin recovery) of spiked endotoxin. There was no significant difference between the residual activity of rLALs using rFCs expressed in HEK293 and GnTI- cells in the two salt solutions.

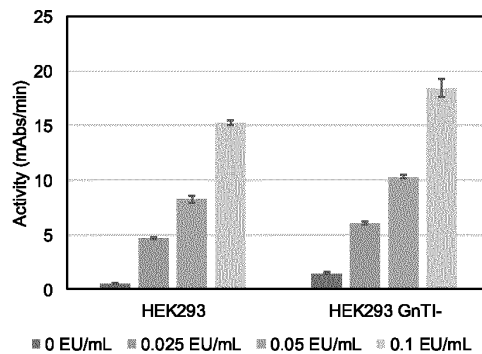
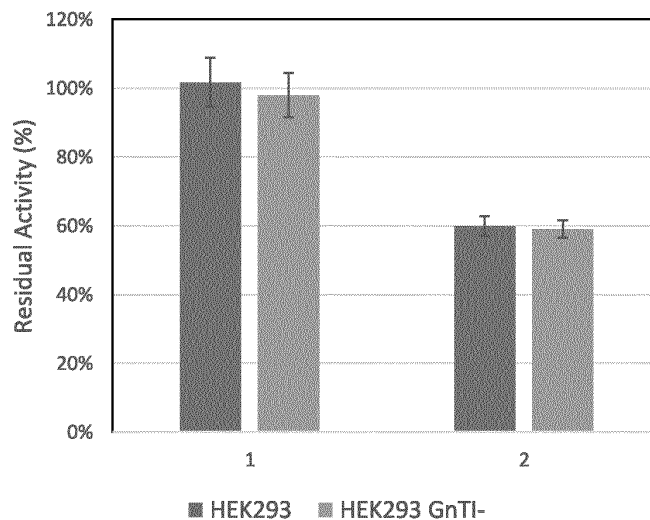


Figure 5 Standard curves of rLAL using HEK293 and GnTI- rFCs. (n = 2)



1- 21 mM sodium citrate; 2- 52 mM sodium hydrogen carbonate.

Figure 6 Residual activity (endotoxin recovery) of endotoxin in salt solutions. (n = 2)
Spiked endotoxin: 0.05 EU/mL. rLALs using HEK293 and GnTI- rFCs were used.

Discussion /Conclusions:

Recombinant factor C was produced using HEK293 cells and HEK 293 GnTI- cells. The difference between these rFCs is the terminal sialic acids. The rFC produced using HEK293 cells contains (α -2,3)-linked terminal sialic acid, whereas the rFC produced using HEK293 GnTI- cells lacks this modification.

Equivalent biological activity was observed in Factor C produced by either strain HEK293 or GnTI-, regardless of sialic acid content.

There was further no significant difference in the residual activities between rLALs using HEK293 and GNTI-rFCs. These results show that the presence of a (α -2,3)-linked terminal sialic acid has no effect on the residual activity of a *Limulus* Factor C. A *Limulus* Factor C containing (α -2,3)-linked terminal sialic acid did not exhibit a higher residual activity as compared with a *Limulus* Factor C lacking this modification.

Addendum:

Raw Data for figures 1 and 2: The SDS polyacrylamide gel and membrane blot of that gel contained samples not relevant to this experiment. Images of the gel and blot were cropped to place the two samples of Factor C adjacent to each other for clarity as lanes 2 and 3. The original images are shown below with the lane numbers used in the figures.

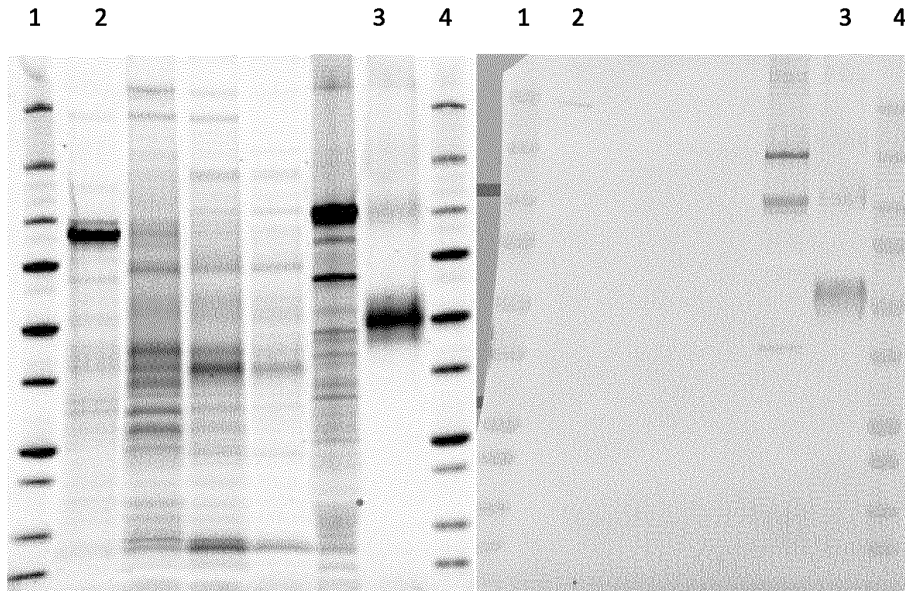


Figure 1 raw image

Figure 2 raw image