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DENV-Protease Cleavage of cGAS

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DENV-PROTEASE CLEAVAGE OF CGAS

By

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Table Contents

Abstract	4
Abbreviations	5
Introduction	5-7
Methods	7-11
Results	11-13
Discussion	13-15
cGAS sequence analysis	15-17
Future Directions	17
Acknowledgements	17-18
Figures	19-27
References	28

Abstract

Dengue virus (DENV) is a single-stranded RNA virus that is transmitted via mosquitos in tropical and subtropical regions. DENV is a significant public health threat, causing severe infections in humans. The virus causes potentially life-threatening symptoms in some people and has a particularly interesting evasion method to avoid the host's immune system. DENV targets a particular immune pathway called the cGAS-STING pathway to prevent or suppress type I interferon production, allowing it to persist in the host for a longer period. DENV protease NS2B3 is a critical component of the virus's replication complex and plays a crucial role in the viral lifecycle. The two components of this protease are NS2B and NS3. NS2B is a non-structural protein that acts as a cofactor for the NS3 protein, which is a serine protease. The NS2B protein helps to activate the NS3 protein by binding to it and creating a catalytic site for the protease activity. Together, NS2B and NS3 play a crucial role in the cleavage of the viral polyprotein into individual proteins that are necessary for viral replication. NS3, in addition to its role as a protease, also has helicase activity which is essential for the unwinding of viral RNA during replication. The NS3 protein is also involved in the formation of the viral replication complex and is important for the evasion of the host immune response. NS2B and NS3 are attractive targets for the development of antiviral drugs because of their essential role in the replication of flaviviruses. Previous research shows that human cGAS (hcGAS) is targeted for degradation by the NS2B3 protease complex of the virus. However, it remains unclear whether NS2B3 interacts with and cleaves mouse cGAS (mcGAS). In this experiment I purified mcGAS and conducted a cleavage assay to investigate the interaction between NS2B3 and mcGAS. I hypothesized that under ideal conditions for DENV that resemble a human's bodily temperature,

NS2B3 cleaves mcGAS in similar relative locations to those in hcGAS. Our results demonstrate that mcGAS is cleaved in a similar manner to hcGAS in the presence of NS2B3, suggesting that this mechanism of immune evasion is conserved between human and mouse hosts. These findings provide insights into the interaction between DENV and the host immune system and suggest that targeting this interaction could be a potential therapeutic strategy for DENV infections. Future studies should investigate the functional consequences of mcGAS cleavage and explore the potential of targeting this interaction for the development of novel antiviral therapies.

Abbreviations

cGAMP (cyclic GMP-AMP), cGAS (cyclic GMP-AMP synthase), DENV (Dengue virus), SEC (size exclusion chromatography), ADE (antibody-dependent enhancement), STING (Stimulator of interferon genes protein), SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Introduction

Dengue virus is a single-stranded RNA virus that is typically spread by mosquitos of the genus *Aedes* in tropical and subtropical regions (Dengue Viruses). It is expected that roughly four billion people live in areas where Dengue virus is present and is the leading arthropod-borne virus in the world with nearly 300 million cases annually (Ferrari et al.). Dengue viral infection may cause symptoms that may include nausea, vomiting, rash, and aches. In more severe

infections, symptoms such as abdominal pain, presence of blood in bodily excrements, and fatal hemorrhagic fever may occur (Dengue). Dengue viral infection is typically caused by four very closely related dengue viruses that are members of the Flavivirus genus. The virus functions by attaching to a skin cell where it is engulfed and enters the cell via an endosome. Once specific conditions are met that indicate that the endosome has entered the cell cytoplasm, the viral genome is released. It is then allowed to hijack the cell to use for its own replication (Dengue Viruses). Dengue viral infection is unique in the way that the body responds to the different DENV serotypes. After the host's immune system creates antibodies for that specific serotype, protection is granted for that strain of Dengue virus. In the case of infection by another of the serotypes, a phenomenon called antibody-dependent enhancement (ADE) can occur. This occurs when antibodies from the previous serotype bind to Dengue virus but are unable to properly neutralize the virus. This viral strain surrounded with non-neutralizing antibodies are then consumed by phagocytic cells, although this results in further viral replication. It is not uncommon for the more severe cases to be associated with ADE (Ferrari et al.). This potential for continuous reinfection makes DENV particularly serious.

The viral genome of the Dengue virus contains ten genes that are translated similarly into ten different proteins. Three of these proteins are responsible for structure, being capsid, envelope, and membrane proteins, while seven of the proteins are important for other functions such as replication and evasion (Dengue Viruses). Two of these specific proteins, NS2B and NS3, form a complex called NS2B3. This protease is believed to be capable of degrading a core component called cyclic GMP-AMP synthase (cGAS) in one of our immune pathways. This pathway is the cGAS-STING pathway, and it is responsible for detecting cytosolic double stranded DNA. It

then produces the second messenger molecule cyclic GMP-AMP (cGAMP), activates the STING protein in endoplasmic reticulum, leads to type 1 interferon gene activation, and begins the immune response. (Decout et al.). As NS2B3 targets cGAS for lysosomal degradation, this pathway is inhibited, similarly resulting in the absence of type 1 interferon production (Aguirre et al.).

Previous research by Ding, et al. suggested that mcGAS may not be a target for cleavage by NS2B3. The sequences of mcGAS and hcGAS have some striking similarities however, suggesting it may still be capable of binding to mcGAS and cleaving it. This study was performed to delve deeper into this interaction in vitro to detect cleavage.

Methods

Transformation

Competent cells were allowed to thaw. 20 μ L of BL21 (DE3) RIPL cells were carefully moved to a 5mL Eppendorf tube, followed by the addition of 1 μ L of the mcGAS plasmid. The mixture was allowed to rest for 20 minutes to facilitate optimal contact between the plasmid and the cells. Heat shock was then performed by immersing the mixture in a 42-degree Celsius water bath to enable the cell membranes to take in the plasmid. After heat shock, the mixture was promptly placed on ice for 2 minutes to facilitate the closure of the plasma membrane, following the entry of the plasmid into the cells. Next, 100 μ L of SOC medium were added to the mixture, which was then incubated at 37 degrees Celsius for one hour. Following the incubation, a 50mg/mL

kanamycin plate was used, and the mixture was evenly spread on it. The plate was then inverted and incubated overnight at 37 degrees Celsius to allow for optimal growth of transformed cells.

Cell cultures

Following incubation, a single colony was picked with a pipette tip and transferred into a cell culture medium in a 250 mL flask composed of 100 mL LB treated with 100 μ L kanamycin. The culture was incubated at 37 degrees Celsius until the following day. Kanamycin was added to 1L and 500 mL flasks (adding 1000 μ L and 500 μ L respectively). 10 mL of primary culture was added to the 1 L flasks and 5 mL of primary culture was added to the 500 mL flasks. These flasks were incubated at 37 degrees Celsius for approximately 4-5 hours until an optical density (OD) of approximately 0.8-1.0 was reached. Once an optimal OD was achieved, 0.4 M IPTG was added (400 μ L to the 1 L flasks, 200 μ L to the 500 mL flasks) to induce the bacterial cells. The flasks were then incubated at 20 degrees Celsius until the following day.

Pelleting down cells

The cell cultures were centrifuged at 4000 revolutions per minute (RPM) for 20 minutes at 4 degrees Celsius using a JLA 8.1 rotor. Once centrifugation was completed, 10 mL of lysis buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 20 mM imidazole, 5 mM β -mercaptoethanol, and 10% glycerol) was used to aid in removing the pellet for each 4L of culture pellet. This was stored in 50 mL Falcon tubes at -80 degrees Celsius. These previous steps were repeated until a suitable amount of pellet was obtained.

Column preparation

Ni-NTA affinity chromatography was used to purify His-MBP tagged mcGAS. was used to obtain elute fractions from supernatant following sonication and centrifugation. Elution buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 300 mM imidazole, and 5 mM β -mercaptoethanol) was first added to the column, followed by lysis buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 20 mM imidazole, 5 mM β -mercaptoethanol, and 10% glycerol). After the lysis buffer was added, supernatant followed, and the product was collected.

Sonication & Purification preparation

The pellet was thawed in room temperature water and vortexed until the pellet was liquid. Aliquots of 4 mL of pellet were added to 140 mL beakers on ice along with approximately 80 mL of additional lysis buffer. Six rounds of sonication (1 minute of sonication, 4 minutes of resting) were performed. Once sonication was completed, the mixture was centrifuged at 18,000 revolutions per minute (RPM) for 1 hour at 4 degrees Celsius using a JA 25.50 rotor. The supernatant is then collected and used in the Ni-Reverse column.

Ni-NTA affinity chromatography

The supernatant from the previous step was loaded onto the Ni-NTA gravity column. The column was then washed with the first wash buffer (50 mM sodium phosphate pH 7.4, 1M NaCl, 30 mM imidazole, and 5 mM β -mercaptoethanol) and a second wash buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 30 mM imidazole, and 5 mM β -mercaptoethanol). The elution buffer was then added and 25 mL of elute fractions were collected. An SDS-PAGE gel was run with the following fractions in order: 4 μ L of supernatant, flow through, wash 1, wash 2. 2 μ L protein ladder marker. 10 μ L of elute fractions 1-6.

Heparin column

The elute fractions from the previous step showing our protein were then loaded onto a heparin column for purification. The column had a 300 mM – 1 M NaCl linear gradient from buffer A (20 mM Tris·HCl pH 8.0, 300 mM NaCl) to buffer B (20 mM Tris·HCl pH 8.0, 1 M NaCl).

Fractions indicating the highest concentration of our protein were then used to proceed with the next step.

Size exclusion chromatography

The protein was subjected to size-exclusion chromatography for further purification. The column was a HiLoad 16/600 column and was first equilibrated with the running buffer (20 mM Tris·HCl pH 7.4, 300 mM NaCl, and 5% glycerol and 5 mM betamercaptoethanol). The sample was then loaded onto the column and eluted fractions were collected. A 20% SDS-PAGE gel was run with the fractions showing eluted protein to determine which fractions should be used for the cleavage assay. The gel was run at 180 V for approximately 60 minutes. These fractions showing the presence of mcGAS protein were concentrated and had concentration and absorbance measured to ensure that there is enough pure protein to be used in the cleavage assay. All the previous steps until this point were also repeated for hcGAS as well to compare to mcGAS following the cleavage assay.

Cleavage assay

Using the elute fractions for both mcGAS and hcGAS, the following systems were made:

- mcGAS without enzyme: 20 μ M mcGAS, 20 mM Tris-HCl 100 mM NaCl buffer (pH 7.4)
- mcGAS with enzyme: 20 μ M mcGAS, 2 μ M NS2B3, 20 mM Tris-HCl 100 mM NaCl buffer (pH 7.4)
- hcGAS without enzyme: 20 μ M hcGAS, 20 mM Tris-HCl 100 mM NaCl buffer (pH 7.4)
- hcGAS with enzyme: 20 μ M hcGAS, 2 μ M NS2B3, 20 mM Tris-HCl 100 mM NaCl buffer (pH 7.4)

These systems were incubated for three hours at 37 degrees Celsius and samples with the NS2B3 protease were taken at three-hour increments- 0 h, 1 h, 2 h, and 3 h. The samples without NS2B3 had a sample taken only after the three-hour period as there is no enzyme acting on the protein. These samples were run on a 20% SDS-PAGE gel.

Results

Transformation

The presence of evenly spread bacterial colonies on the kanamycin agar plate indicated the successful growth of the BL21 (DE3) RIPL bacterial cells. This step has no clear indicator of a successful transformation although the steps following transformation indicate the presence of cGAS such as running SDS-PAGE gels. It is worth noting that mcGAS tended to have fewer colonies than that of previous constructs I have worked with, as well as hcGAS.

Cell cultures

Growth of bacterial cells can be observed in the primary and secondary cultures due to opacity of the LB medium. The flasks were carefully monitored until an optimal OD was reached, indicating that there were sufficient bacterial colonies to proceed with the next steps.

Pelleting down cells

After centrifugation, the cell pellets were analyzed to ensure their physical consistency before being removed from the jars for collection and storage. Only once did I encounter cell pellets that appeared to be unusable. In this instance, the pellet appeared to indicate that the cells had been lysed, and the pellet was slimy and not firm. Typically, the pellet is expected to be firm and adhere to the sides of the jars.

Protein purification, Ni-Reverse column

An SDS-PAGE gel was run with the different collected fractions (supernatant, wash 1, wash 2, and elution) to ensure the presence of cGAS. If a suitable amount of protein was determined to be present in the elution fractions, the next steps would commence. This was indicated by the intensity of the bands on the gel following staining and de-staining. Figure 1 shows elute fractions with a strong presence of mcGAS protein. This is an indicator that the tagged mcGAS protein bound to the Ni-beads and was released into the elute fractions.

Heparin column

Ni elute fractions containing high protein content were subjected to heparin chromatography. The flowthrough fractions did not show the presence of mcGAS, indicating that it had bound to

the column. The mcGAS was later eluted from the column during the washing process, using a linear gradient of NaCl concentrations ranging from 300 mM to 1 M.

Size exclusion chromatography

Like the previous step, an SDS-PAGE gel was run to indicate the presence of cGAS. mcGAS eluted at around 72mL and I proceeded with the cleavage assay due to the presence of a suitable amount of protein.

Cleavage assay

As seen in Figure 6, I conducted a cleavage assay to assess the interaction between mcGAS and NS2B3. Specifically, I incubated purified mcGAS with or without NS2B3 and then analyzed the samples by SDS-PAGE to visualize any cleavage products. The bands on the gel following the assay indicated that mcGAS had been cleaved in the presence of NS2B3 by the presence of a new band towards the bottom of the gel. This major cleavage product band only appeared in the systems which also included NS2B3 and were absent in the systems without it, indicating that NS2B3 cleaved both mcGAS. Similarly, in Figure 7 it is apparent that hcGAS was also cleaved, although only when exposed to the WT NS2B3 and not with the mutant NS2B3S135A.

Discussion

Our results from purifying mcGAS and performing a cleavage assay three different times indicate that mcGAS is targeted and cleaved by DENV-NS2B3. This took a particularly long time due to the nature of mcGAS. My experience with mcGAS has shown that the protein is less

stable than hcGAS and more difficult to purify. Our protein yield was consistently lower in the case of mcGAS as opposed to hcGAS. This meant that it often took several weeks to purify and run cleavage assays for mcGAS. It also was not uncommon for there to not be enough viable protein following purification, forcing me to restart the purification. mcGAS sometimes also produced fewer colonies following transformation, which is something I have not experienced working with any other constructs. This is likely due to plasmid instability of mcGAS, causing transformation success rate to be lower than other constructs.

Multiple rounds of purification and cleavage assays were fundamental to ensuring the upmost integrity of our results. These results were particularly novel as we had previously run a cleavage assay that did not produce any major cleavage products prior to this Honors in the Major thesis. Despite this, it is important to understand that many things can influence cleavage between NS2B3 and mcGAS.

It is possible that the conditions of the cleavage assay were different. For example, we may have used different concentrations of the proteins, different buffers, or different temperatures. These differences could have affected the ability of NS2B3 to cleave cGAS. Different strains of mcGAS may have been used between our experiment and other studies due to possible mutations. Different strains of mcGAS can have different sensitivities to cleavage by NS2B3. The sensitivity of the assay that we used previously might be different.

It is possible that the DENV-NS2B3 protease that we used was more active than the protease that was used previously. This could be due to a mutation in the NS2B3 gene, or it could be due to

other factors that make the NS2B3 protein more efficient at cleaving cGAS. It is possible that there is a difference in the way that we treated the cells before and after the cleavage assay. This could include differences in the culture conditions, the transfection protocol, or the treatment with NS2B3.

cGAS sequence analysis

Using Clustal Omega, the two sequences have a similarity of 82.8%. This indicates that the two sequences are likely homologous and likely have similar functions. Overall, the two sequences have high similarity, and the two protein sequences share several identical and similar amino acid sequences. This high similarity is often indicative of similar function or evolutionary origin.

Human and mouse cGAS likely diverged relatively recently over evolutionary time, resulting in some differences in their genetic sequences. The genetic differences between human and mouse cGAS can also lead to some differences in their functions and regulation, despite sharing a similar overall protein structure and activity. Furthermore, cGAS may have evolved differently in different species due to differences in the types of pathogens they encounter or their immune responses.

The fact that both human and mouse cGAS are cleaved at the same sites by DENV-NS2B3 suggests that these cleavage sites may be highly conserved across different species. The conserved nature of these cleavage sites may reflect the importance of these regions in the overall structure and function of the cGAS protein, hence why NS2B3 would be targeting them.

It is also possible that the cleavage sites in cGAS are recognized by the DENV-NS2B3 protease due to their structural and chemical properties, such as local conformation or specific amino acid residues, rather than specific sequence conservation. In this case, the protease may be able to cleave similar sites in different species because they share similar properties in those regions. It is likely that they are both cleaved due to a combination of sequence conservation and structural / chemical properties.

When looking at Figure 8, it is apparent that there are evident similarities between the two sequences of mcGAS and hcGAS. Despite some differences in the beginning of each of the sequences, the middle and end portions of mcGAS and hcGAS share lots of genetic similarities. It is likely that some of these similarities are the reason that the two proteins share relative cleavage sites. Figure 8 also shows an identity of 57.26%, which is described by the presence of amino acids in corresponding positions between the aligned sequences.

The sequences from Figure 9 have been run in InterProScan and the results are as follows:

Mouse cGAS signal peptide: 26 amino acids

Mouse cGAS nucleotidyltransferase domain: 367 amino acids

Mouse cGAS DNA-binding domain: 149 amino acids

Human cGAS signal peptide: 30 amino acids

Human cGAS nucleotidyltransferase domain: 367 amino acids

Human cGAS DNA-binding domain: 149 amino acids

Cleavage sites are also indicated by highlights within the sequences. It is apparent that some of these cleavage sites are not only similar in amino acid sequence, but also in the relative position. It also seems that some of the cleavage sites for one protein are also present in the other protein, albeit not as a cleavage site. This similarity between the two sequences is one reason why I believe that NS2B3 is theoretically capable of interacting and cleaving both mcGAS as well as hcGAS.

Future Directions

Future directions for this experiment include purifying cGAS without any tag to ensure that this has no effect on the interaction with NS2B3 in a cleavage assay. This paper outlines the process we have taken to suggest that NS2B3 cleaves mcGAS, although further experimentation can be done. Mass spectrometry can be used to identify the sites of cleavage on the cGAS protein as well as to identify purity. X-ray crystallography would also be a reasonable direction in the future. This would allow us to visualize the 3-D structure of the complex formed by NS2B3 and cGAS to better understand cleavage and binding sites as well as understanding the differences between how NS2B3 interacts with mcGAS and hcGAS.

Acknowledgements

I would like to thank the Dr. Yin lab for everything that they have done for me over the past four years. I feel as though I have learned and matured significantly during my time working in this

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Figure 1: SDS-PAGE gel showing Ni-NTA affinity chromatography products.

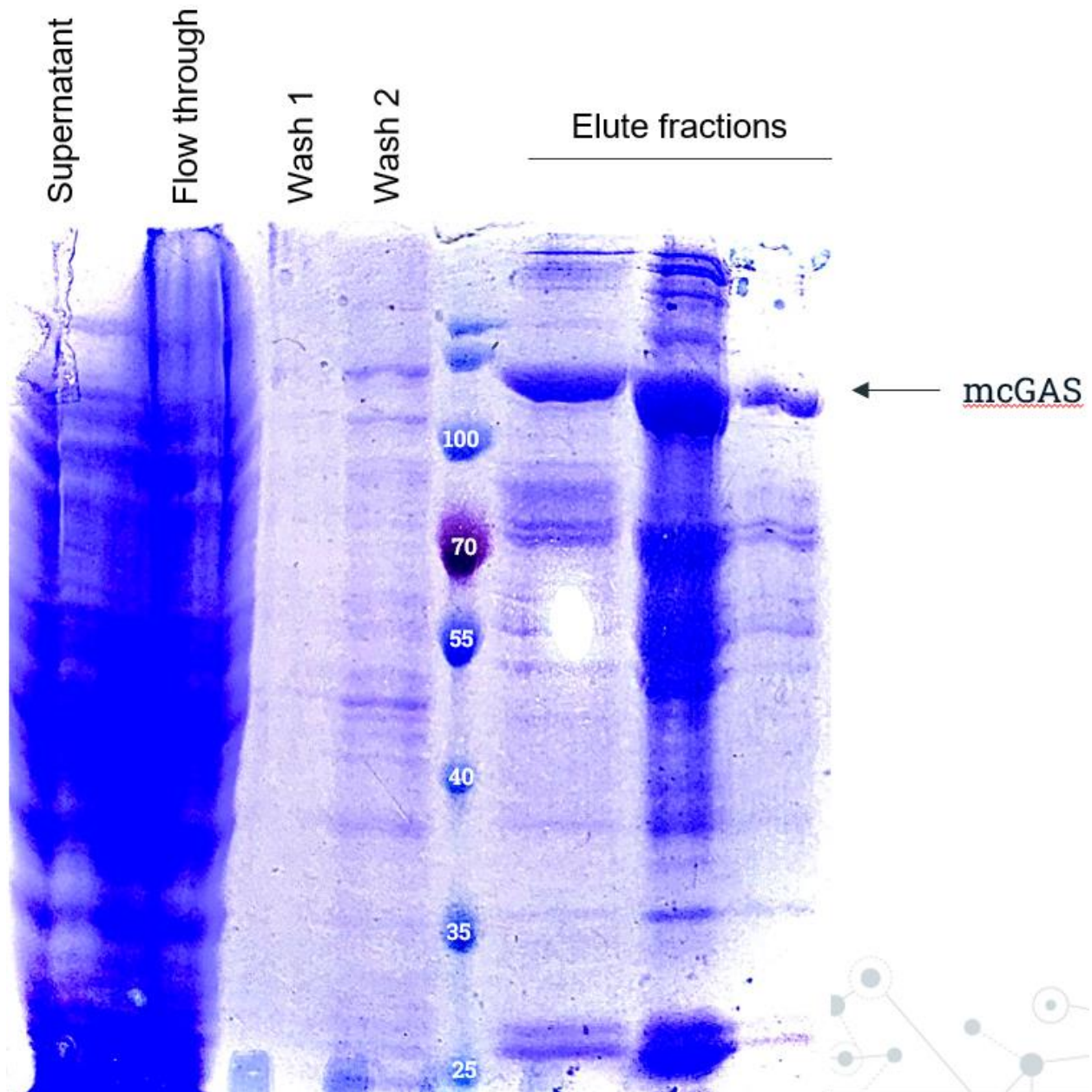


Figure 1 shows the products of Ni-NTA affinity chromatography. Elute fractions deemed most usable for Heparin column are indicated by the presence of a mcGAS band indicated by the arrow above. mcGAS was not present in the wash steps as it was bonded to the Ni-NTA gravity column due to the HIS-MBP tag. mcGAS left the column via elution buffer in the fractions.

Figure 2: SDS-PAGE gel showing Heparin column products.

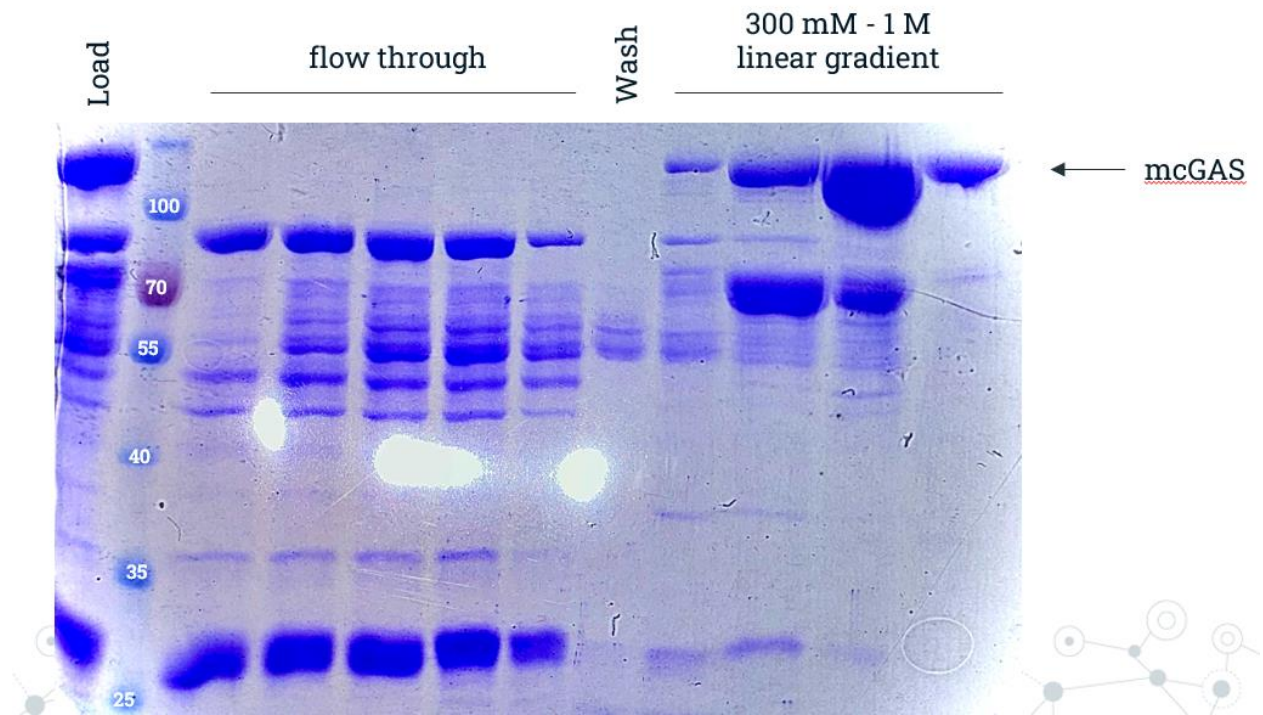


Figure 2 shows the products of the Heparin column. mcGAS does not appear to be present until the linear gradient released mcGAS in the elute fractions. This is due to mcGAS binding to the ion exchange column. These fractions were later used for Size exclusion chromatography.

Figure 3: Heparin profile

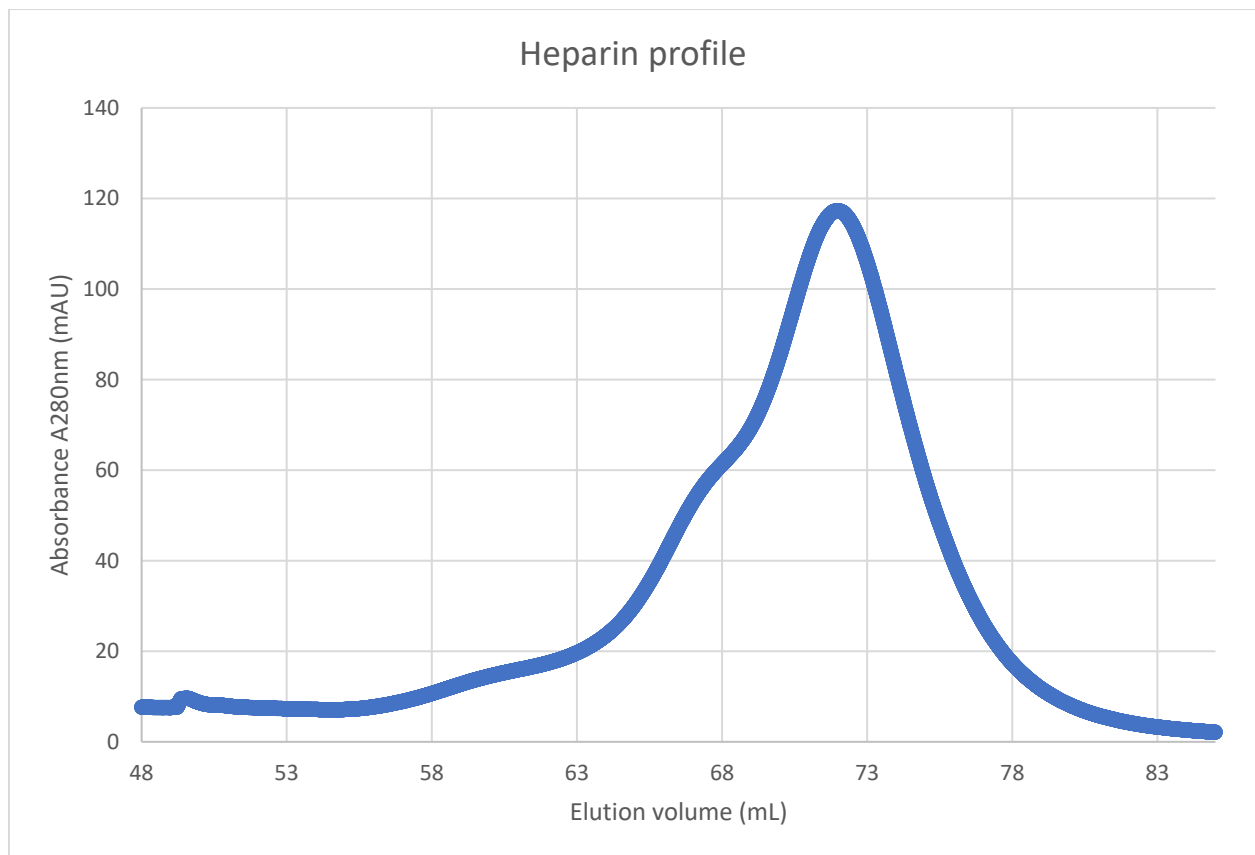


Figure 3 shows the Heparin profile produced from mcGAS running in the AKTA 1 machine.

Figure 4: SDS-PAGE gel showing Size exclusion chromatography products.

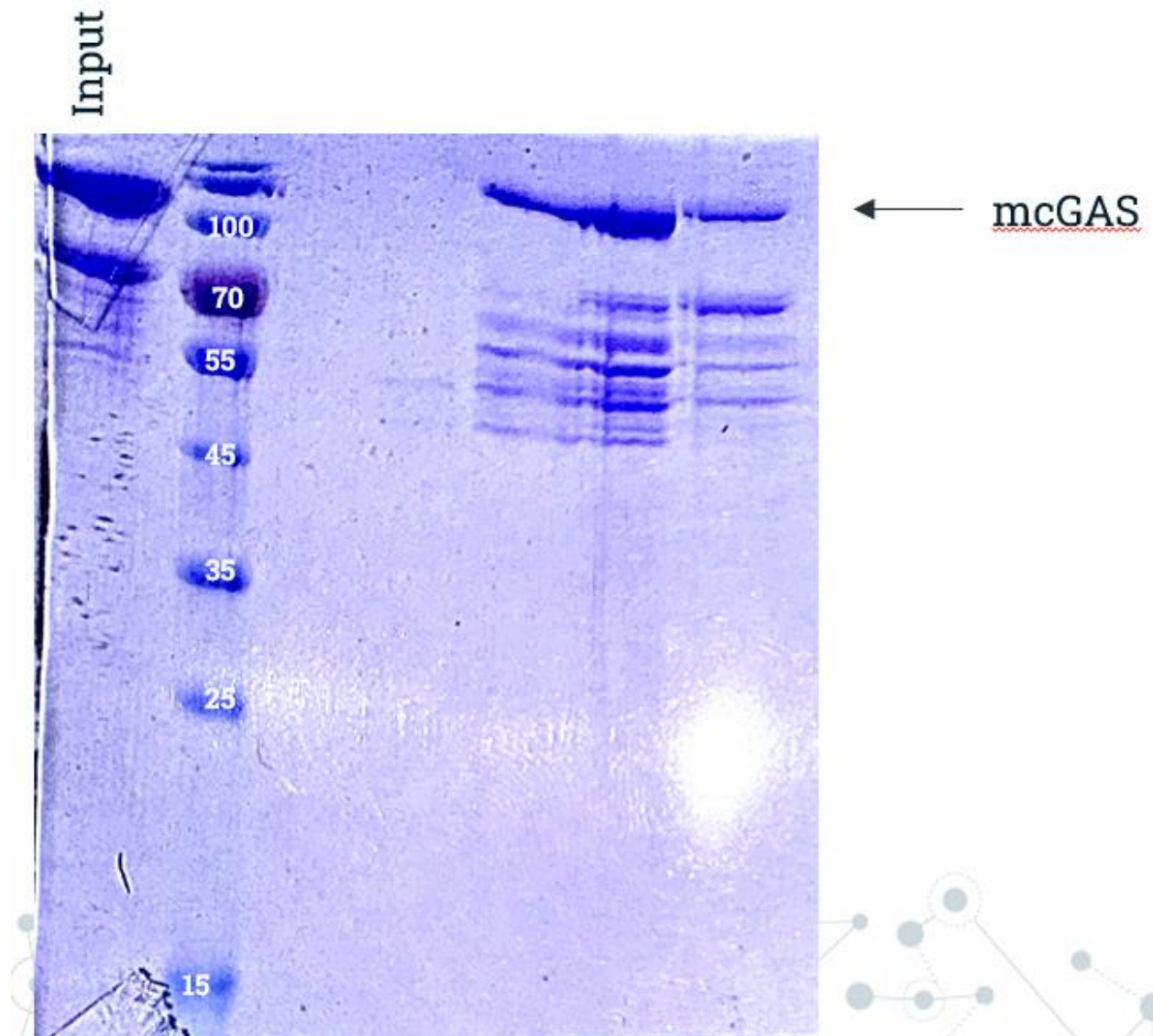


Figure 4 shows the products of size exclusion chromatography. Pure mcGAS protein is indicated by the arrow. These fractions were pooled to be used for cleavage assays. Bands under the mcGAS band are a result of mcGAS fragments from degradation of the protein. This is due to the instability associated with mcGAS.

Figure 5: Size exclusion chromatography profile

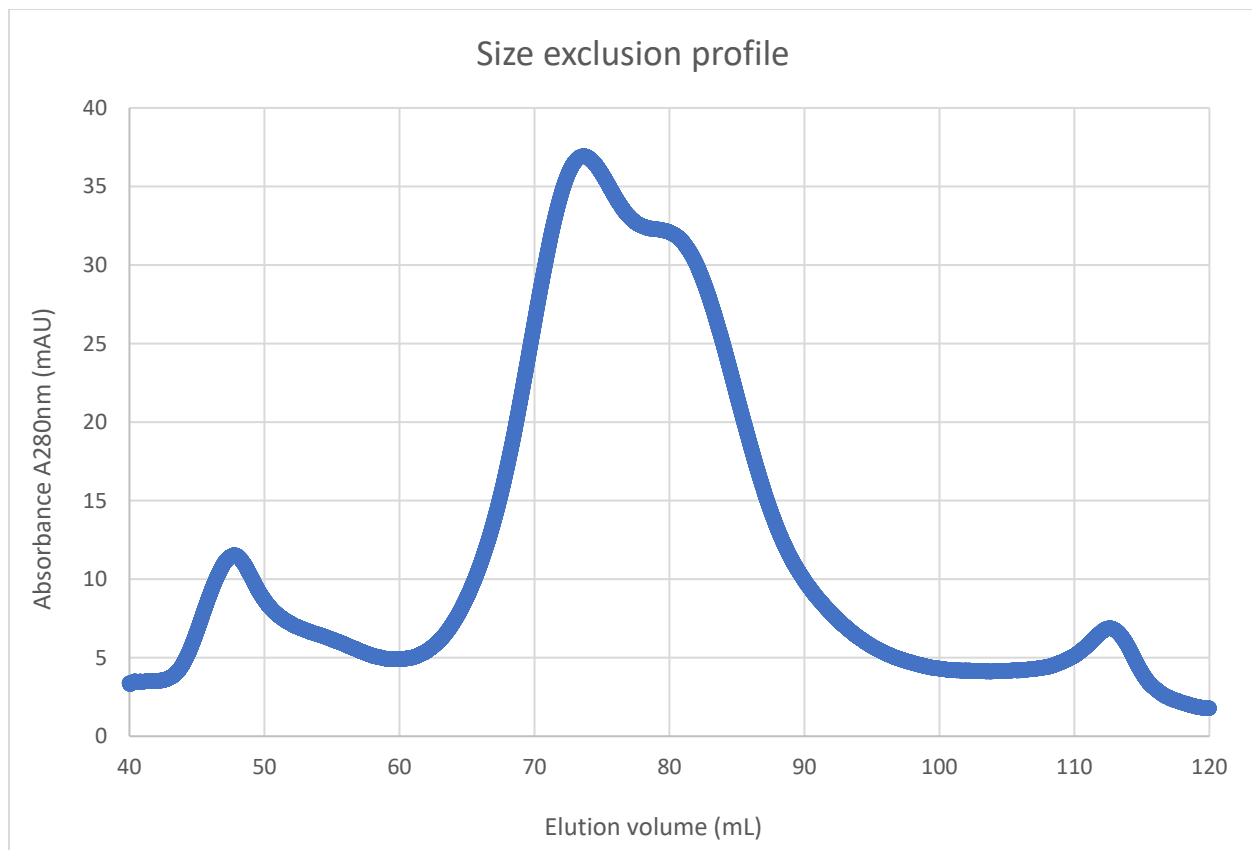


Figure 5 shows the Heparin profile produced from mcGAS running in the AKTA 2 machine.

Figure 6: DENV-NS2B3 protease cleaves mcGAS in vitro

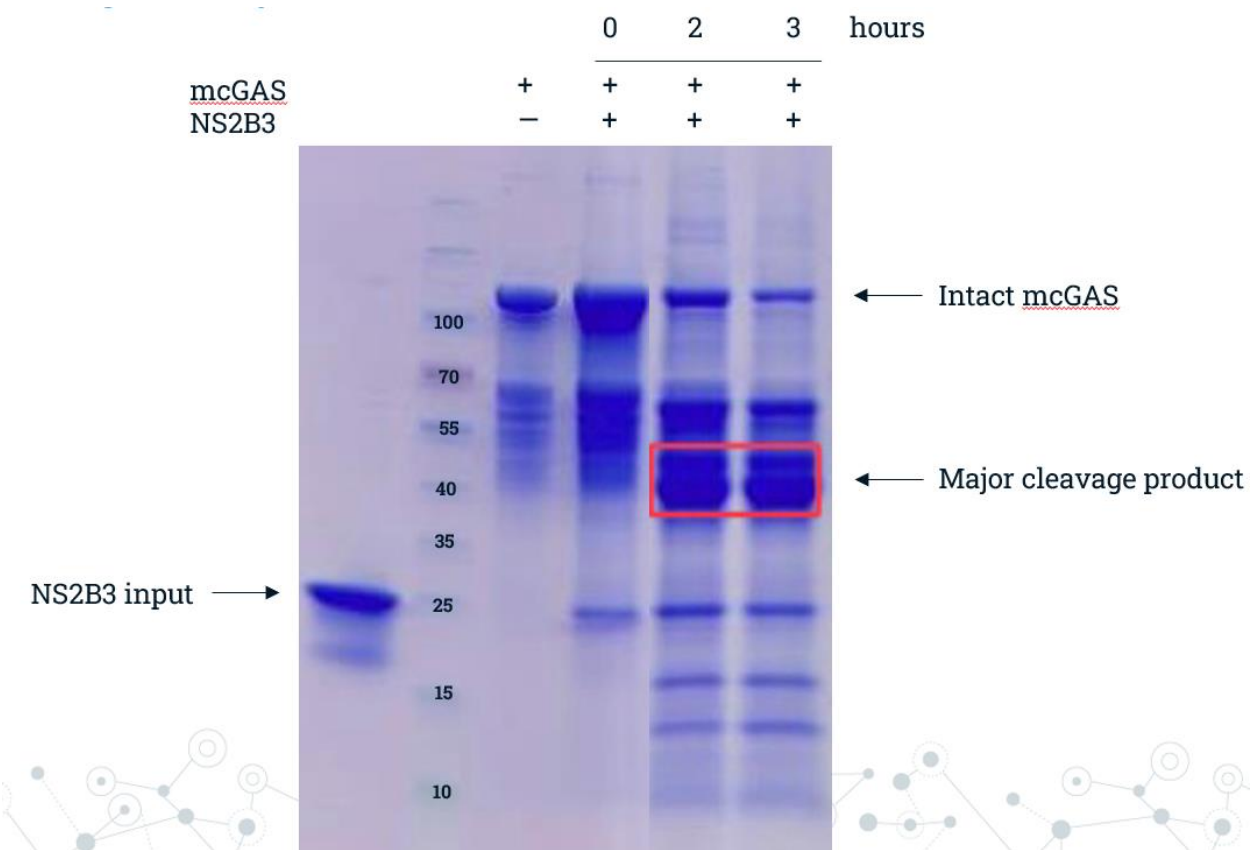


Figure 6 shows the NS2B3 protease, mcGAS protein, and the systems from the cleavage assay. Systems from the cleavage assay had samples taken at different time points (0hr-3hr). Hour 1 is omitted due to a loading issue. Major cleavage products are boxed in red.

Figure 7: DENV-NS2B3 protease cleaves hcGAS in vitro

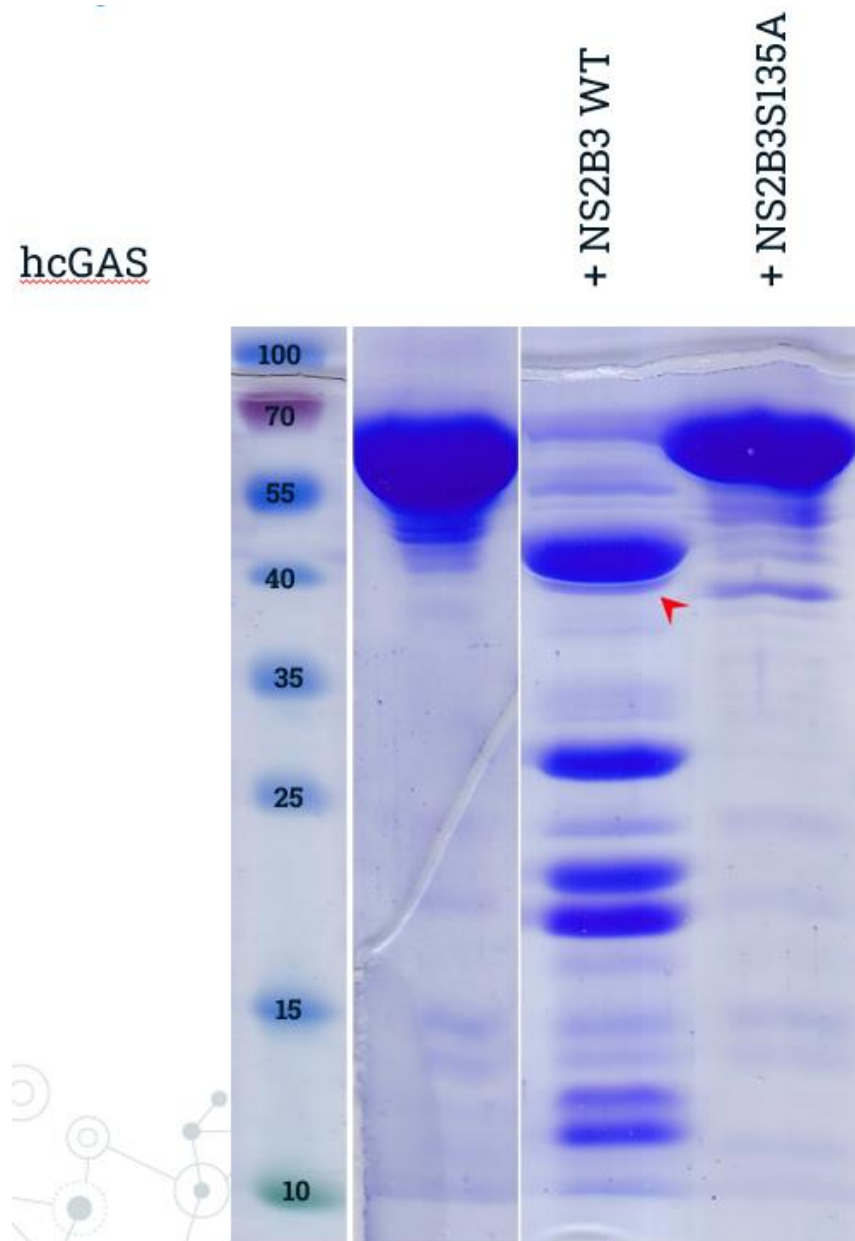


Figure 7 shows the hcGAS protein and the systems from the cleavage assay. Systems from the cleavage assay were exposed to either no protease, NS2B3 WT, or a mutated NS2B3S135A. Each of these samples followed 3 hours of incubation after addition of the protease. Major cleavage product is pointed to.

Figure 8: LalnView sequence alignment of mcGAS and hcGAS.

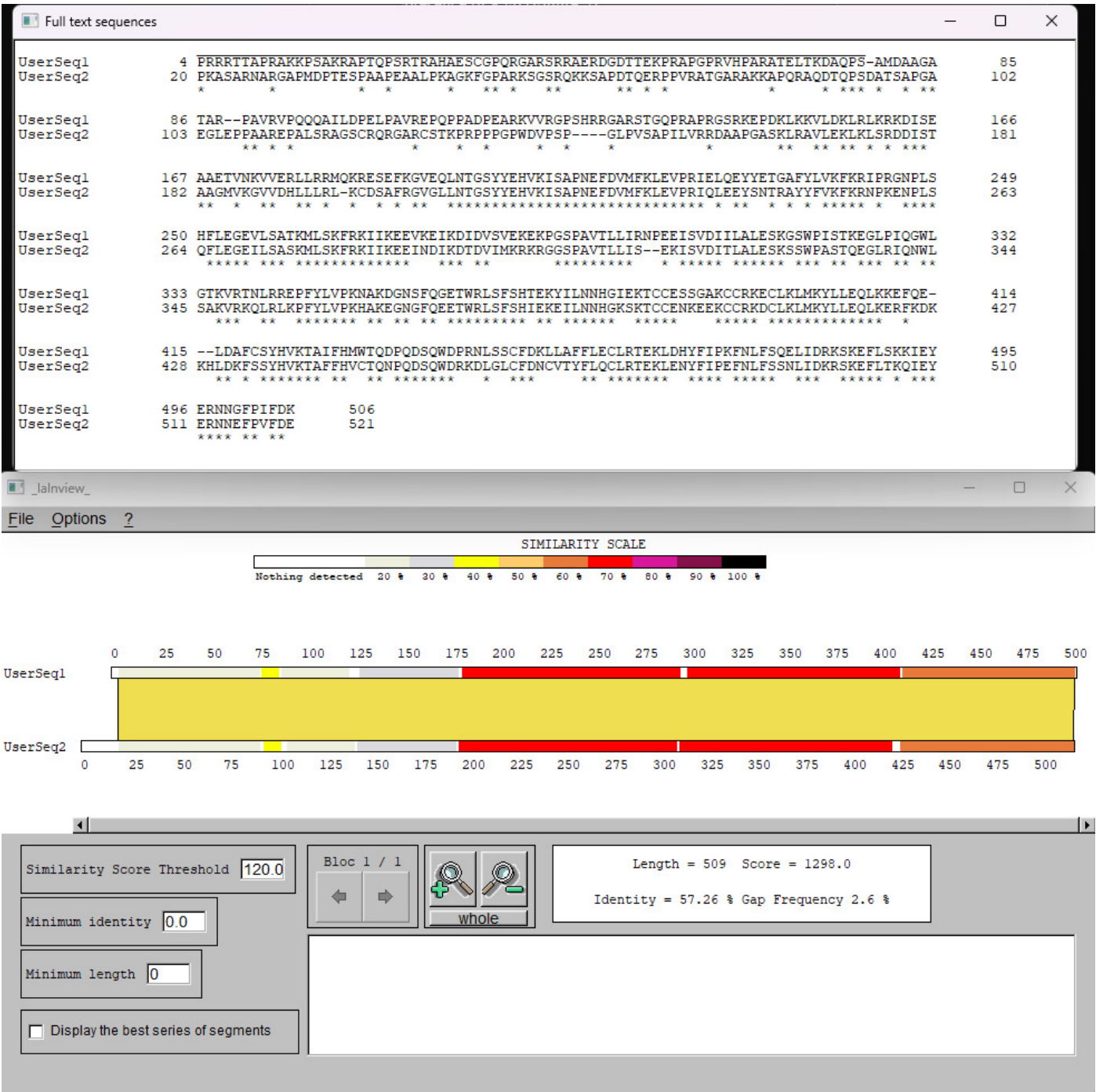


Figure 8 shows the similar residues between mcGAS (sequence 1) and hcGAS (sequence 2) lined up to indicate similarities in the sequences. A similarity scale is also shown corresponding to the relative positions on the sequences.

Figure 9: mcGAS and hcGAS sequences and cleavage sites when interacting with NS2B3



Figure 9 shows the sequences of both mcGAS and hcGAS. Cleavage sites are highlighted. Some similar residues are underlined as well. Some of these underlined residues also appear highlighted as they can be cleavage sites in one of the proteins.

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