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The Cloning and Purification of Sig-Lec 9

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ABSTRACT

To introduce and express a protein of interest in the selected host, cloning, transforming, and purifying procedures are performed. Cloning allows production of identical copies of the gene and insert it into a plasmid vector called pET28a(+). This protects the protein's genetic material from the host's degradation mechanism. Next, the vector and protein are transformed into a bacterial cell line, or expression host, to propagate and produce the target protein, Siglec-9. Siglec-9 was transformed into BL21(DE3) and Shuffle T7 Express cell lines for expression purposes. After liters of bacterial culture were grown, it underwent a purification process. The goal is to isolate the protein of interest and test for expression with protein electrophoresis and further analysis if needed. The objective is to clone, transform, and purify Siglec-9 for further experimentation.

INTRODUCTION

Sialic acid-binding Immunoglobulin-like lectin-9 (Siglec-9) are transmembrane proteins that serve as immunomodulatory receptors, which are found on several immune cells (neutrophils, NK cells, B cells, T lymphocytes). They play a role in activating and inhibiting immune responses by interacting with sialoglycans, sialic acid sugar-carrying sugars, which are aberrantly expressed on cancer cells (Choi et al). The binding between Siglec-9 and sialoglycans allows the cancer cell to avoid immunodetection, which promotes cancer metastasis and inhibits T cell activity (Śledzińska et al). Therefore, Siglec-9 has gained attention as a target site for immunotherapies. The goal is to reverse immune suppression in cancer by focusing on sialic acid-Siglec interactions. Using pre-clinical studies conducted, immunotherapies are tailored to block the receptor binding between Siglec-9 and its respective ligand. Comparing the results from studies conducted on mice using Siglec-E (mice homolog of Siglec-9) in myeloid cells, it shows that the binding of Siglec-E and sialoglycans caused apoptosis of neutrophils. Yet, the deficiency of Siglec-E allowed an increased immune response from neutrophils and lead to the killing of tumor cells (Adams et al). Ultimately, the objective is to progress these therapies to the clinical trial stage as the in *vitro* and in *vivo* stages display promising results.

MATERIALS AND METHODS

Siglec-9 and the plasmid vector pET28a(+) into the cell were transformed into *E.Coli* cell line, BL21 (DE3). The bacteria was heat shocked (85°C, 30 sec) and placed on ice to get the recombinant vector inside the expression host to allow the gene on the plasmid to be expressed. Next, Bacterial liquid culture and measured OD to ensure a suitable density of bacteria containing the protein.

Afterwards, a sample was taken from the bacterial culture and spread onto an agar plate containing kanamycin (KAN) to obtain isolated colonies that contained the plasmid coded for antibiotic resistance and Sig-lec 9 in BL21(DE3). Two plates were grown, labeled with colony 1 and colony 5. The colony 1 plate was selected due to more growth, and it was grown at 37°C with 1L LB media and 1mL of KAN.

The purification process included centrifuging to obtain a cell pellet at (5000rpm, 15 min, 4°C). From there, TBS buffer was used to scrape the pellet into a beaker to sonicate it for (2.5 min, 5 sec on, 10 sec off , 40% Amp). Next, the lysate was centrifuged again (30 min, 12 urpm, 4°C). During that time, 1mL of Ni-NTA and the wash and elution buffers for purification using manufacturer's protocols (IMAC). The supernatant obtained from the second centrifugation process was added to Ni-NTA and allowed to bind for 45 minutes. It was then run through a chromatopgraphy column, and FT, W1-W2, and E1-3 are collected.

Lastly, 14% SDS page was ran to check for protein expression. If the gel image is clean and there is only a band at the protein's molecular weight, the stock can be frozen. If not, further analysis, such as Western Blots, are required.

RESULTS AND DISCUSSION

Once cloning, transforming, and purifying processes were completed successfully, 14% SDS pages were ran to confirm protein expression. Siglec-9 is 15.3 kDA and the goal is to have a singular band running horizontally through the gel at that molecular weight to ensure our protein expressed. From the first purification conducted with Siglec-9 transformed into BL21 (DE3), protein expression was minimal as seen Figure 1 below. The presence of unwanted proteins and little protein expression meant further immunoanalysis needed to be done to confirm that Siglec-9 did indeed express in the cell line.

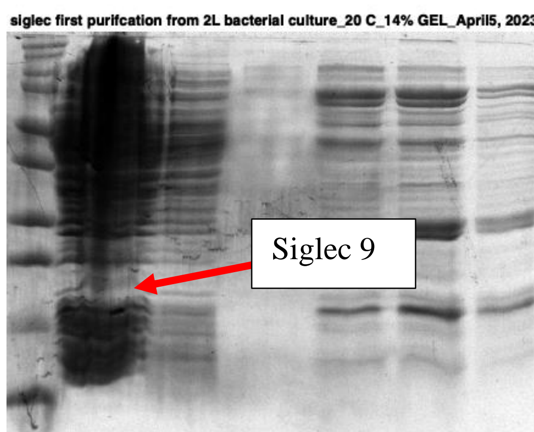

Figure 1: 14% SDS page ran on Siglec-9 FT, W1-2, and E1-3, but bands are present at 15.3 kDA.

In order to confirm Siglec-9 expressed, a Western blot was performed. As seen in Figure 2, Siglec-9 did express, but it still did not explain why the protein expressed so little.

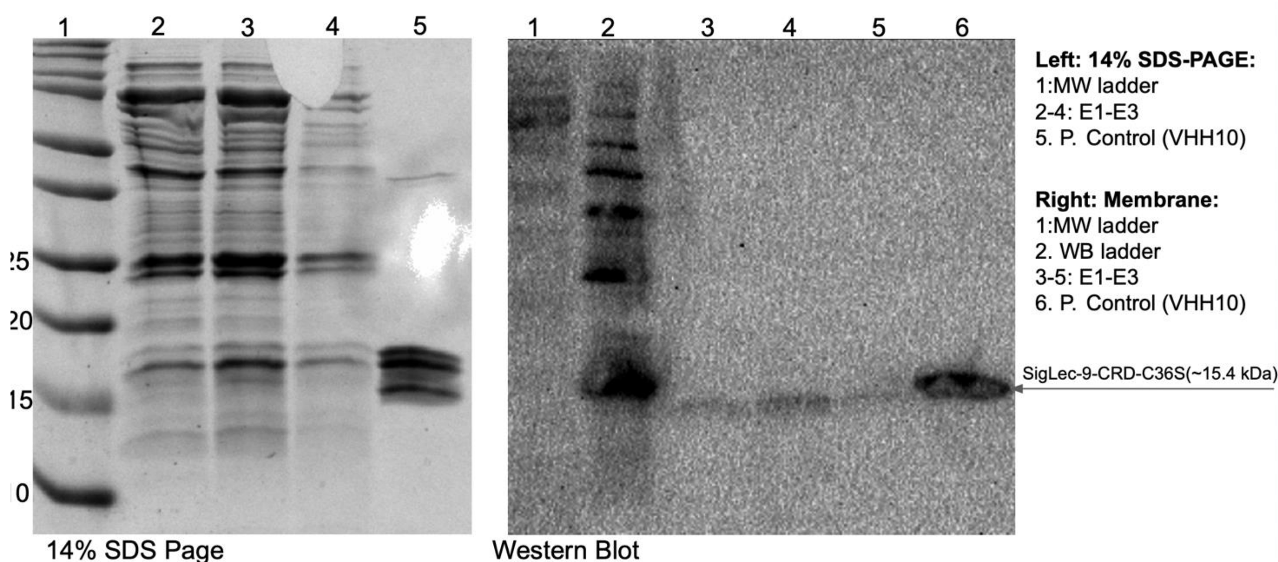

Figure 2: This was a 14% SDS page ran as a control and a Western blot performed.

It may have been the cell line as there were only a few colonies that grew when Siglec-9 pET28a(+) was transformed with BL21(DE3). As a result, Siglec-9 pET28a(+) was transformed into Shuffle T7 Express to see if it would express better. The same methods were repeated for Shuffle T7 Express, except this time a colony from C5 was selected rather than C1.

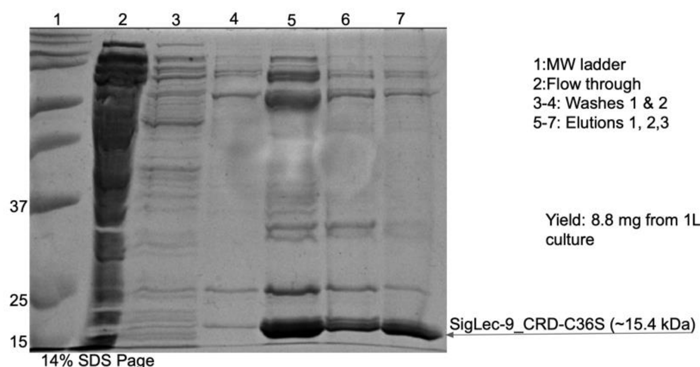

Figure 3: 14% SDS page ran on Siglec-9 from 1L bacterial culture of Shuffle T7 Express after second purification.

As seen in Figure 3 above, Siglec-9 expressed significantly more in the Shuffle T7 Express cell line. However, the gel image is still not clean which implies that the protein is not purified to its fullest extent. Therefore, size exclusion chromatography (SEC) was performed to clean the protein further. Figure 4 shows the fractions A16-A38 which were shown in the second peak of the SEC data to potentially contain the protein. The images are significantly cleaner, however the appearance of two bands indicates our protein and His-tag are falling apart.

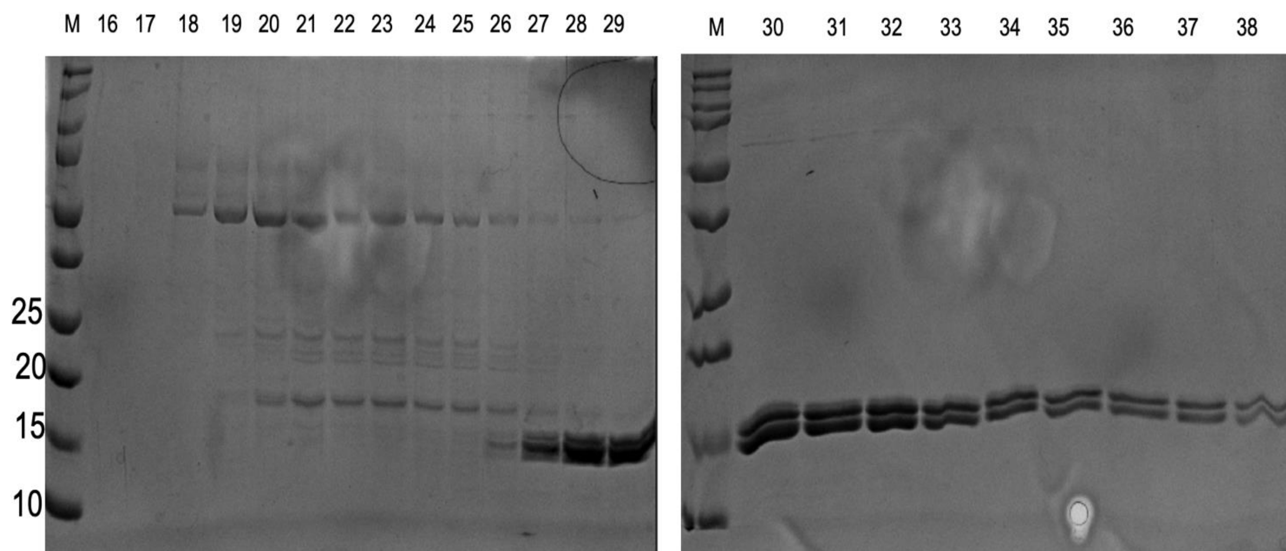

Figure 4: 14% SDS page on Fractions A16-38.

Currently, Siglec-9 was purified again and performed SEC once more to obtain the most updated results. The two red arrows on Figure 5 indicate Fractions 21 and 22, which show the least number of additional proteins and a high concentration of Siglec-9 expressed. Therefore, these two fractions will be re-ran and the following consecutive fractions.

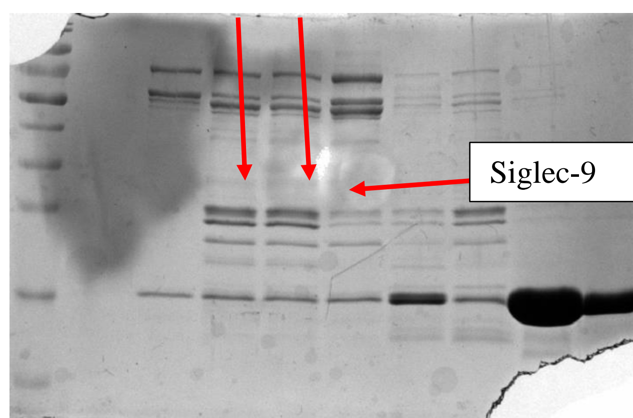

Figure 5: 14% SDS Page ran on May 25th, 2023, after SEC with Fractions A14-22

Once the purest possible form of Siglec-9 was obtained depending on SEC data and SDS pages, future work includes crystallizing Siglec-9 with its sugar, sialic acid, to study the protein's structure and perform further experimentation.

There is currently a paper available that has shown Siglec-7 successfully binding to the sugar and the goal is to replicate the method and tailor it to Siglec-9.

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