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Optimizing expression, refolding and purification of VEGFR1D2

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Abstract

The process of angiogenesis is important in tumor growth. During this process, the VEGF family acts as a pro-angiogenic factor. VGB is a new compound that inhibits the effect of VEGF in angiogenesis and therefore is a promising compound in the therapy for cancer. To study the interaction of peptide VGB with VEGFR1, crystallization of the extracellular binding domain $VEGFR1_{D2}$ is necessary. During this experiment, the process of expression, refolding and purification of the protein was optimized to reach a sufficient concentration of $VEGFR1_{D2}$. It was found that an increase in the volume of the culture helped to increase the concentration. Also, TEV protease was used to minimize the precipitation of the protein due to the hydrophobic His-tag. This process could be further optimized in further research.

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Introduction

Angiogenesis is a very important process in tumor growth. The formation of new blood vessels is necessary for the blood supply to the cells in tumors. (1) The process of angiogenesis is activated by endothelial cell activation and survival, after which the basement membrane is degraded. Then endothelial cell proliferation and migration takes place, to form a tube, which becomes the new blood vessel. Finally, this new blood vessel will mature. As shown in the figure, the vascular endothelial growth factor (VEGF) family is important in multiple steps of the angiogenesis process. (2)

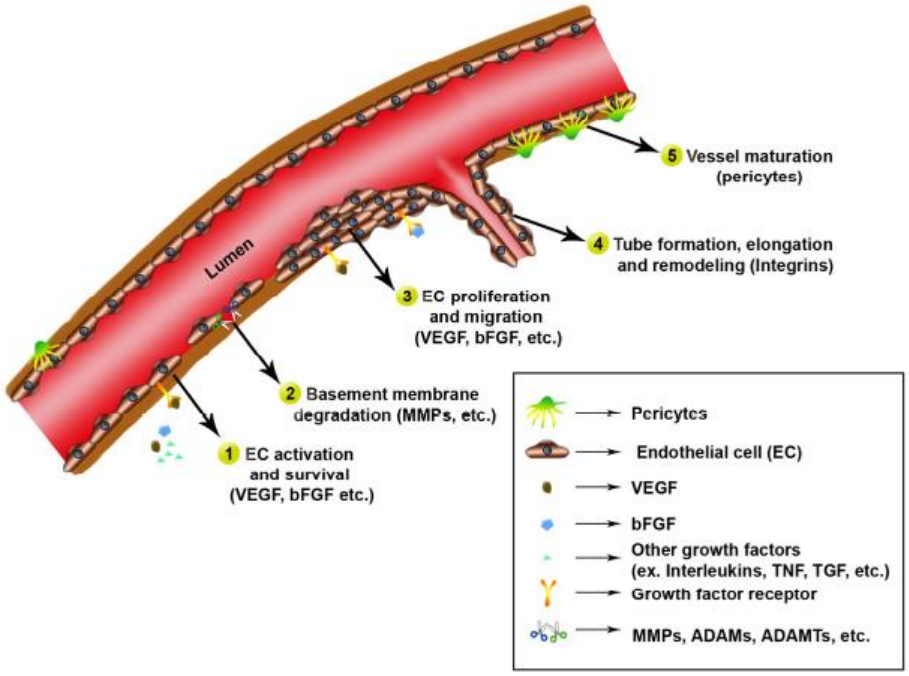

Figure 1: A schematic depiction of the process of angiogenesis. (2)

In the process of angiogenesis VEGF is a pro-angiogenic factor, which will cause an increase in the formation of new blood vessels. This factor, among other pro-angiogenic factors, is upregulated in cancer, causing an increase in blood supply to the tumor. (3,4)

In mammals, the VEGF family consists of VEGF A to D and the platelet growth factor (PlGF). These ligands can activate the VEGF receptors. These are VEGFR-1, -2 and -3. (5) The activation of these receptors will lead to the dimerization of the receptor monomers, after which a downstream signaling cascade is activated. (2) In the process of angiogenesis, mainly VEGF-A and VEGF-B are deemed very important factors. (5,6)

VEGF-A is seen as the most important pro-angiogenic factor of the VEGF family. VEGF-A can act via both VEGFR-1 and VEGFR-2. (5) Mainly the activation of VEGFR-2 by VEGF-A causes the activation of the angiogenic process. (7,8) VEGF-B lately was also found to be a very important factor in the process of angiogenesis, mainly in that of metastasis of tumors via the VEGFR-1 receptor. (6) Therefore these two factors are very important in the process of angiogenesis and metastasis of tumors.

To inhibit angiogenesis multiple compounds have been developed to act on the VEGF/VEGFR interaction. In these studies, it became clear that inhibiting the effect of both VEGF-A on receptors VEGFR-1 and VEGFR-2 as well as the effect of VEGF-B (and PlGF) on VEGFR-1 are important. This is because the compounds that interact with both of these interactions, such as the VEGF trap aflibercept, inhibited VEGF-A induced migration of endothelial cells more potently than drugs acting on only one of the receptors or factors. (9)

A new compound that was developed is peptide VGB ($_2$HN-CIKPHQGQHICNDE-COOH). This peptide is based on the interaction of VEGF-A with both VEGFR-1 and VEGFR-2. This leads to the peptide being able to bind both receptors since it contains the interaction sites of both receptors. Therefore the peptide can also block both receptors. The blocking of the receptors will inhibit the downstream signaling for the process of angiogenesis. (10)

Previous research by A. Sadre Momtaz et al. has indicated that treatment with VGB causes inhibition of the growth of the tumor. This has been studied in mice. (10) Next to inhibiting tumor growth, downstream factors for metastasis of tumors also are decreased during treatment with VGB. (11)

To study the exact interaction of VGB with the receptors, the interaction between VGB and the extracellular domain of VEGFR-1, VEGFR1$_{D2}$, should be studied. This is necessary for further development and optimization of the binding of the compound. To be able to study the interaction of the peptide with VEGFR-1, the protein VEGFR1$_{D2}$ should be crystallized. For crystallization, however, a sufficient concentration of the protein is necessary. (10) Therefore the main goal of this research was to optimize the process of expression, purification and refolding to reach to this concentration. This was done by changing multiple aspects of the methods that were used.

Materials & methods

Procedure used for transformation

For the transformation, RIL bacteria were transformed with pETM11-VEGFR1$_{D2}$. (12) First, the cells were defrosted, after which 50 µL of the cell suspension was added to 1 µL of DNA in a sterile environment. The mixture then was incubated for 30 minutes on ice. After 30 minutes, the cell suspension was heat shocked by reaching 42 °C for 1 minute. After heat shock, the suspension was put back on ice for about 5 minutes. Then, 450 µL of fresh LB medium was added to the mixture to reach a total volume of around 500 µL. The mixture containing the fresh LB medium then was incubated in a Minitron incubator at a speed of 180 rpm, at 37°C for 1 hour.

After incubation for 1 hour, the suspension was centrifuged for 2 minutes. The cells were resuspended (50-60 µL) and spread over an agar plate (20 mL) containing 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. This plate then was incubated overnight at 37 °C. (12)

Procedure used for the preculture

For the formation of a preculture, LB medium was used. This medium contained 25 g of LB powder per liter. The 25 g of LB powder consisted of 10 g tryptone, 10 g NaCl and 5 g yeast extract. For each liter of media, 20 mL of preculture was made, consisting of the same ratio of LB powder to volume. To both the preculture and media, antibiotics were added in a final concentration of 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. The preculture then was incubated overnight at 180 rpm, 37 °C. (12)

The induction

After the overnight incubation of the preculture, the preculture was divided over the liters of media (20 mL preculture per liter). The liters of media contained kanamycin and chloramphenicol in a final concentration of 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. To each liter 20 mL of the preculture was added. The media were then incubated at 180 rpm, 37 °C for 2-3 hours until an OD_{600} around 0.65-0.7 was reached. Then, IPTG was added to each liter with a final concentration of 0.7 mM. After the induction, with IPTG, the cultures were incubated at 180 rpm, 37 °C for 4 hours. After the incubation, the pellets were collected by centrifugation at 5000 rpm, 4°C for 30 minutes. (12) The pellets then were kept in the freezer until further use.

Washing and lysis of the pellets

The collected pellets were washed using a lysis buffer containing 50 mM Tris-HCl with a pH of 8. The first time the pellets were dissolved in 30 mL per liter of the buffer together with a pipet point of DNAse. After dissolving the pellets, the sample was sonicated, after which it was centrifuged for 15 minutes at a temperature of 4°C and a speed of 15.000 rpm. The then obtained pellet was washed multiple times (4 to 6), using 30 mL per liter of culture of the same buffer. After each time centrifuging a sample of both the pellet and supernatant was taken for SDS-PAGE analysis. (12)

After completely washing the pellets, they were dissolved in a second lysis buffer (50 mM Tris-HCl, 10 mM imidazole and 8 M urea, pH = 8). The pellet was incubated at 4°C on a roller overnight. (12)

Purification

For the purification of the protein 2 methods were used, Ni^{2+}-NTA and size exclusion chromatography (SEC). The use of 2 purification methods is to obtain the protein in a higher level of purity, which is necessary since the final product will be used in crystallography to determine structural properties. (13)

Ni^{2+}-NTA

For Ni^{2+}-NTA 3 different buffers were made. These are given in table 1.

Table 1: The different compounds and the concentrations of the compounds that were used in the three different buffers for Ni^{2+}-NTA purification.

Compound	Buffer A	Buffer B	Buffer C
Tris-HCl	50 mM	50 mM	50 mM
Imidazole	10 mM	10 mM	300 mM
NaCl	300 mM	300 mM	300 mM
Urea	8 M	-	-

The first buffer, buffer A, was used to equilibrate the column. After equilibration, the supernatant (centrifuging 30 minutes, 18.000 rpm, 4°C) was added onto the column. Then the supernatant together with the resin was incubated for 1 hour on a roller at 4°C. (12)

After the incubation, the sample with the resin was loaded onto the column. The column then was washed using the second buffer, buffer B, together with buffer A. Using both buffers a gradient in the urea concentration was made to reach to concentrations of urea of 8M, 6 M, 4 M, 2 M, 1 M, 0.5 M, 0.25 M and 0 M (table 2). The gradient was used to refold the protein. (12)

Table 2: The different concentrations of urea used in the washing of the column with the amount of buffer A and buffer B that should be added to reach to a final volume of 10 mL.

Concentration	Buffer A (mL)	Buffer B (mL)
8 M	10	0
6 M	7.5	2.5
4 M	5	5
2 M	2.5	7.5
1 M	1.25	8.75
0.5 M	0.625	9.375
0.25 M	0.312	9.688
0 M	0	10

After the washing steps, the elution of the protein was initiated by using the third buffer, buffer C. During each step, 1 mL of the buffer was added onto the column. This 1 mL was then collected in an Eppendorf cup. Using Bradford assay, the fractions in which a high concentration of protein was found, were combined and then incubated with glutathione. For the incubation of glutathione, a final concentration in the sample was reached of 3 mM reduced glutathione and 0.3 mM oxidized glutathione. (12) After incubation, the sample was concentrated until 1 mL using a concentrator with a MWCO of 5,000. This sample then was further purified, using size exclusion chromatography (SEC).

Size exclusion chromatography (SEC)

For SEC a buffer was used containing 50 mM Tris-HCl and 250 mM NaCl with a pH of 7. After washing the system, the column was equilibrated. For this, the maximum pressure was 0.5 MPa and the minimum pressure 0.04 MPa, with a flow rate of 1.6 mL/min. The system was then equilibrated for 80 minutes. After the equilibration of the column, the sample was injected onto the column and collected.

After SEC, the sample was concentrated again with a 10 kDa MWCO Amicon Ultra 0.5 mL centrifugal filter (Sigma Aldrich). For the measurements of the concentration, the absorption of the sample was measured against the buffer. This was done using BioDrop.

SDS procedure

For the preparation of the gels first, the running gel was made with the amounts of the different compounds that are given in table 3. The amounts are given for 1 gel.

Table 3: The different compounds used in the running gel 17.5% (volume 5.95 mL).

Compound	17.5 % Acrylamide running gel (5.95 mL)
1.5 M Tris-base	1.45 mL
Acrylamide (40%)	2.19 mL
UP water	2.19 mL
10% SDS	58 µL
10% APS	58 µL
TEMED	5.8 µL

After preparing the running gel, isopropanol was added on top of the gel. After the gel was fully set (around 30 minutes), the isopropanol was removed and the stacking gel was added. The different compounds and the amounts for the stacking gel are given in table 4.

Table 4: The different compounds used for the stacking gel 5% (volume 2 mL).

Compound	5% Acrylamide stacking gel (2 mL)
0.5 M Tris-base	0.5 mL
Acrylamide (40%)	0.25 mL
UP water	1.46 mL
10% SDS	20 µL
10% APS	20 µL
TEMED	5 µL

After pouring the stacking gel onto the running gel, a comb was added to create 10 or 15 wells in the gel. This was left until the gel was completely set (around 30 minutes). The gel was then put in a holder, with either another gel or a glass on the other side. The space in between the glasses was filled with fresh running buffer. The rest of the system was filled with used running buffer. After the running buffer was added, the samples were loaded onto the gel. For this 25 µL of the sample was mixed with 10 µL SDS buffer. From each sample and the marker around 10 µL was loaded after boiling the sample for 5-10 minutes. Next, the gel was run until the front reached the bottom of the gel, at 200 V and 120 mA.

Changes during every round

During each round that was done, changes were made to the method described above. During the first round, the procedure described was followed. After this round, the volume of culture was increased from 2 L to 4 L. This change was used in all rounds that followed (round 2 to 5). Before the fourth round was started the Ni^{2+}-NTA was recharged. However, during the last round, TEV protease was used. To use TEV, the sample that was collected after Ni^{2+}-NTA was incubated with TEV as well as glutathione. This incubation was tested at different temperatures (room temperature (around 20 °C) and 4 °C) for 3 hours. The sample at 4 °C was put on a roller. The sample at room temperature was mixed every 20 minutes. During the incubation with TEV, first, the ratio given in the paper by Rosella Di Stasi et al. (12) was followed since this was the last round. This ratio was 1:35 M (protease : substrate). During the first 2 hours, this ratio was followed, however, during the last hour, a concentration of 10% protease was reached.

Results

A total of 5 rounds have been completed. The concentrations that were reached after Ni^{2+}-NTA during each of the rounds are shown in table 5.

Table 5: The final concentrations, volumes and mass after Ni^{2+}-NTA for each round.

Round	Concentration (mg/mL)	Volume (mL)	Total mass (mg)
1	1.37	1	1.37
2	2.20	1.5	3.30
3	2.72	1.5	4.09
4	1.14	1.25	1.43
5	2.74	0.9	2.47

Next, the sample was concentrated (1 mL) and injected onto an S75 column for size exclusion chromatography. The samples were collected after SEC and concentrated. For each round, the final concentrations reached after concentrating are given in the table below (table 6).

Table 6: The final concentrations, volumes and mass after size exclusion chromatography for each round.

Round	Concentration (mg/mL)	Volume (µL)	Total mass (mg)
1	1.04	100	0.10
2	1.99	220	0.44
3	5.33	130	0.69
4	1.93	100	0.19
5	1.35	200	0.27

As indicated in the table, during the third round a concentration higher than the other rounds was reached. Therefore the focus will be on this round and the fifth round. The fifth round included incubation with TEV protease.

To investigate the process of purification during the different rounds, samples were taken for SDS-PAGE analysis. Also, a chromatogram from the size exclusion chromatography was obtained as can be seen in the results of round 3 and 5.

Round 3

The figure below (figure 2) shows the SDS-PAGE of the pellet and supernatant. The samples were taken during the washing of the pellets. The marker is shown on the left.

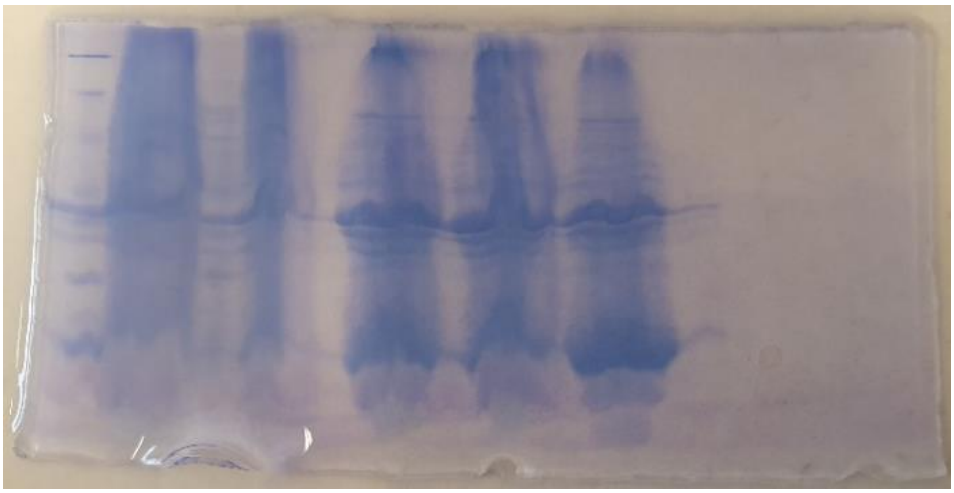

Figure 2: SDS-PAGE of the pellet and supernatant after each step of washing the pellets.

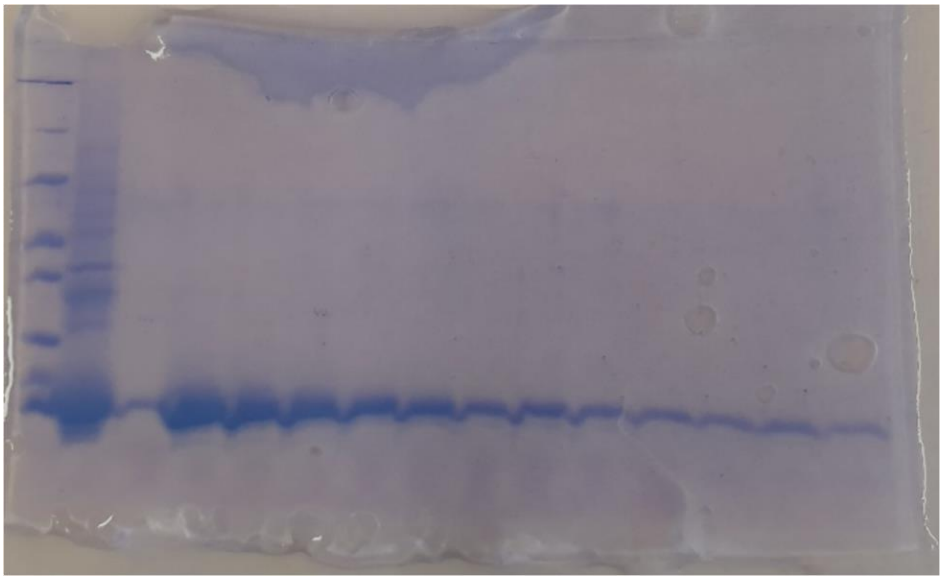

Figure 3: SDS-PAGE of the fractions after Ni^{2+}-NTA, with on the left the marker, with next to that a sample of before Ni^{2+}-NTA.

The SDS-PAGE in figure 3 shows the marker on the left, with next to that a sample before Ni^{2+}-NTA and each of the fractions that were obtained after Ni^{2+}-NTA. As indicated before, after Ni^{2+}-NTA a final concentration of 2.72 mg/mL was reached with a volume of 1.5 mL. The different measurements during the concentrating of the sample are given in appendix 2. The results of the Bradford assay that was used to determine the fractions being used, is given in appendix 1 figure 12. In this round fractions 2 to 14 were used resulting in a volume of 12.5 mL.

Shown in figure 4 is the SDS-PAGE of the different fractions after SEC. Here the marker is again on the left with next to that a sample of before SEC. Here a final concentration of 5.33 mg/mL with a volume of 130 µL was obtained. The different measurements during concentrating are again shown in appendix 2.

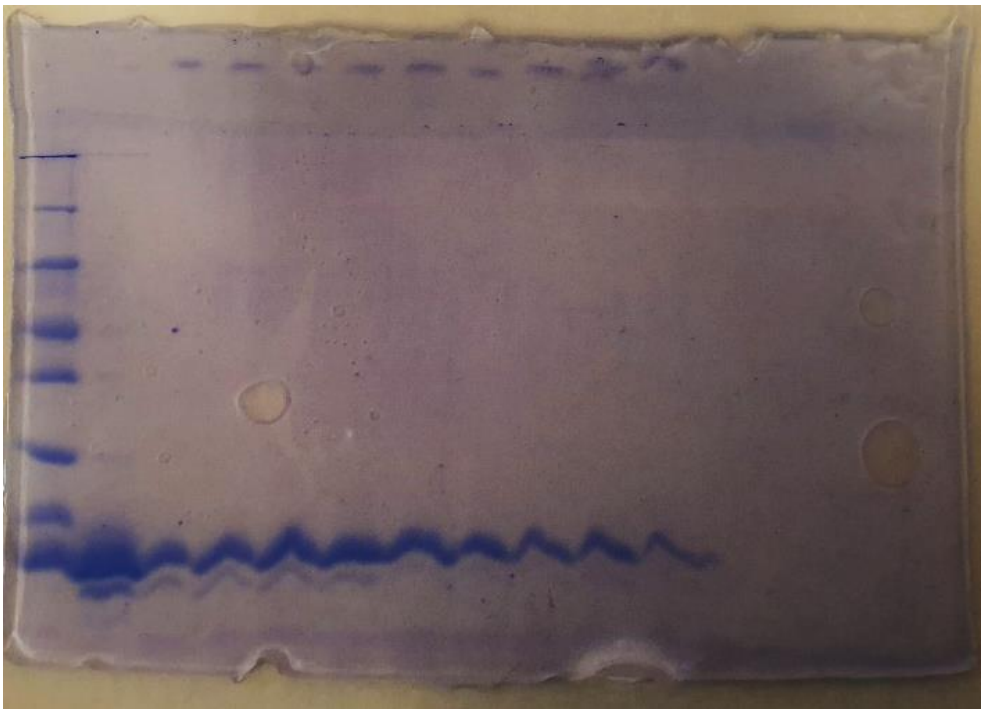

Figure 4: SDS-PAGE of the fractions after size exclusion chromatography, with the marker on the left and next to that the sample of before SEC.

The chromatogram of the third round shows 2 peaks, as is shown in figure 5. The peak is that of VEGFR1$_{D2}$ with the corresponding fractions A82 to A90. These fractions together had a total volume of 8 mL.

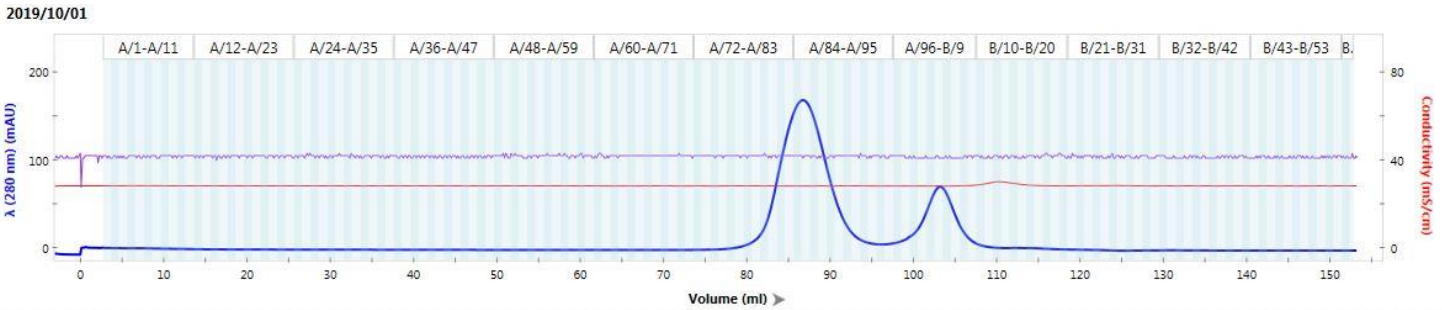

Figure 5: Size exclusion chromatogram. With the protein of interest, VEGFR1$_{D2}$, from A82-A90. Peak 1: 168 mAU, 0.72 CV and slope = 406.1 mAU/CV. Peak 2: 69 mAU, 0.85 CV and slope = 753.99 mAU/CV.

Round 5

For the fifth round, the SDS-PAGE of the supernatant and the pellets during the washing of the pellets is shown in figure 6. Here the marker is shown on the left.

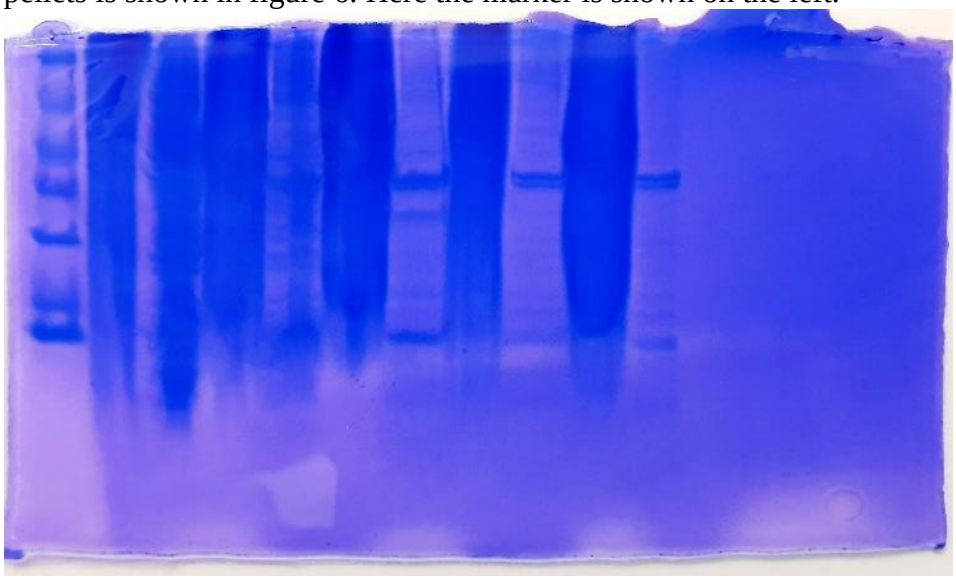

Figure 6: SDS-PAGE of the pellet and supernatant after each step of washing the pellets.

Figure 7 shows the SDS-PAGE of the supernatant and pellets. Here the marker is shown on the left with a sample of before Ni^{2+}-NTA next to that. The other samples are from the different fractions that were obtained during the elution steps of Ni^{2+}-NTA. After collecting the fractions, the sample was incubated with glutathione and TEV. After the incubation, a final concentration of 2.74 mg/mL in a total volume of 0.9 mL was reached before SEC. This concentration was reached from a starting volume of 9 mL. The fractions used, are fractions 2 to 11 (appendix 1, figure 13). The measurements during concentrating are given in appendix 3 (table 9).

For the incubation with TEV, the molar concentration of the protein was calculated. To reach 1:35 M, 59 µL of stock was added. Then another 30 µL was added to reach 10% of the concentration for the last hour. Now a concentration of 0.9 mg/mL was reached.

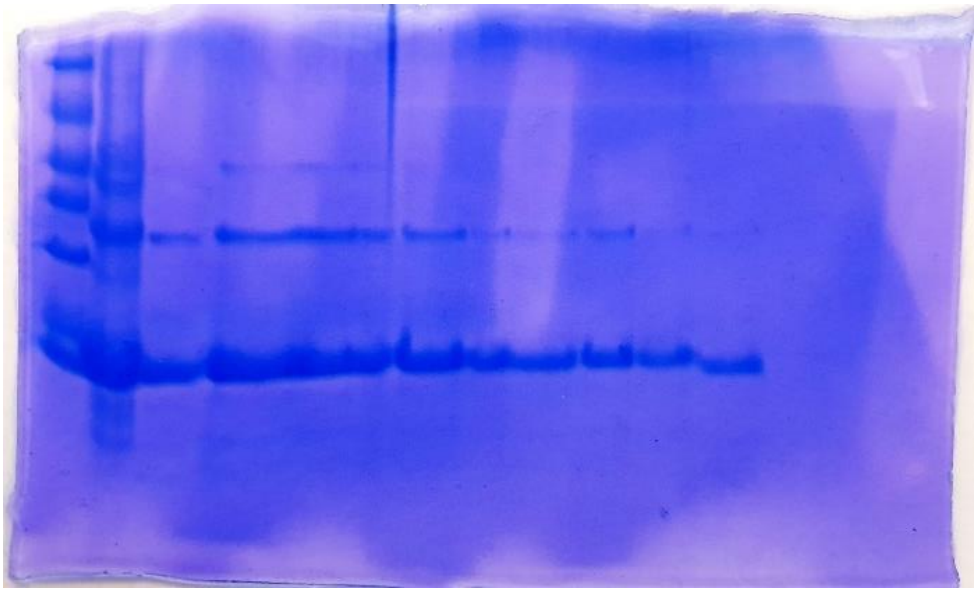

Figure 7: SDS-PAGE of the fractions after Ni^{2+}-NTA, with on the left the marker, with next to that a sample of before Ni2+-NTA.

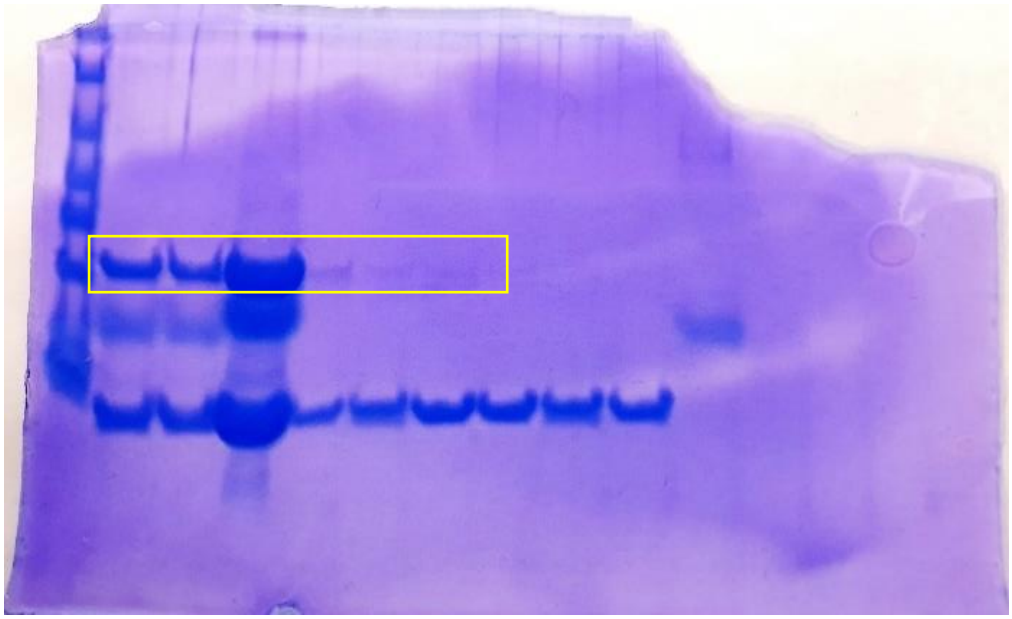

Figure 8: SDS-PAGE of the fractions after size exclusion chromatography. Here the marker is shown on the left. Next to the marker first a sample after TEV incubation at 4°C, then at room temperature and both samples together. After that the samples of the second peak from the chromatogram are shown. Completely on the right is a sample form the third peak.

The SDS-PAGE above (figure 8) shows the marker on the left. Next to the marker is the sample before SEC form the sample that was incubated at 4°C. Then the sample incubated at room temperature and a sample of the two samples combined. Completely on the right is a sample from the third peak in the chromatogram. In between these samples are the different fractions that were collected from peak 2 (A85-A90). After concentrating the collected fractions, a final concentration of 1.35 mg/mL with a volume of 200 µL was reached. The gel shows that the band of $VEGFR1_{D2}$ (the lowest bands) are slightly lower compared to the marker than in the samples before SEC (figure 7). The measurements during the concentrating of peak 2 are given in appendix 3, table 10. For the third peak concentrating was also started, but not completed. The concentrating is also given in appendix 3 (table 11).

In the gel (figure 8) the samples before SEC show 3 bands instead of 2 bands. The middle band is the band that is also there before SEC during the other rounds (figure 4). However, the top band normally is not there. This band shows TEV, also in the first samples of after SEC, which is indicated by the yellow rectangle.

Before the sample was injected onto the column for SEC, the sample at 4°C and room temperature were combined after short centrifugation. This showed that the room temperature sample had more precipitation (figure 9). Also, the fraction had a higher starting concentration than the sample at 4°C. For the sample at room temperature, a concentration of 0.66 mg/mL in a volume of 5.3 mL was measured. The sample at 4°C, which was 4.3 mL, had a concentration of 0.44 mg/mL.

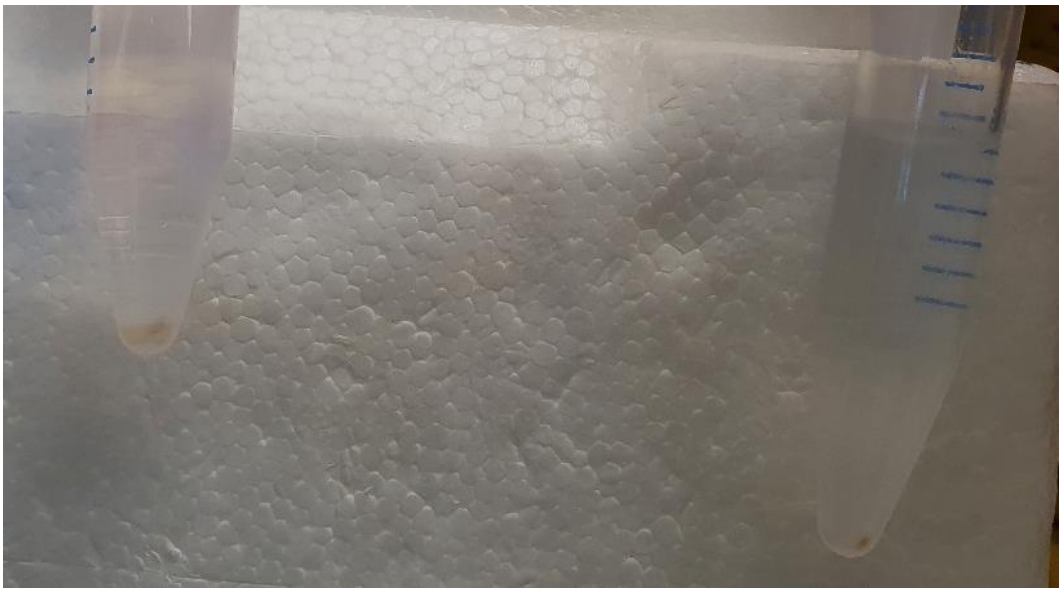

Figure 9: The difference in precipitation between the sample incubated at 4°C (right) and at room temperature (left).

The chromatogram of the fifth round shows 3 peaks (figure 10). With the first two peaks being close to one another. For the concentrating, the second peak from A85 to A90 with a total volume of 6 mL was used.

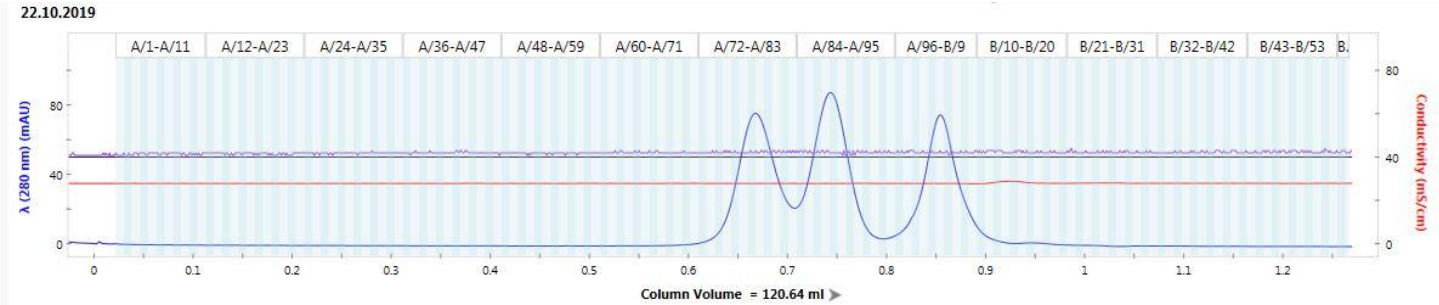

Figure 10: Size exclusion chromatogram. With the protein of interest, VEGFR1D2, from A85-A90. Peak 1: 73 mAU, 0.72 CV, slope = 1056.77 mAU/CV. Peak 2: 86 mAU, 0.79 mAU, slope = 1037.19 mAU/CV. Peak 3: 65 mAU, 0.85 CV, slope = 2594.88 mAU/CV.

Discussion

For further studies regarding the binding of peptide VGB to $VEGFR1_{D2}$, the protein should be obtained in sufficient concentrations. This is necessary for crystallizing the protein. (10)

During the third round, a higher concentration was reached as is shown in table 6. This indicates that the changing of the volume of culture from 2L to 4L could lead to an increase in concentration. This increase, however, was not consistent as can be seen in the results of round 2 and 4. For round 2 however, the sample could have been further concentrated, since the volume was almost double that of round 3. This could have led to a higher concentration.

Figure 11: Sequence of the pETM-11 vector with the TEV site and His-tag shown.(14)

Since the His-tag of the protein, which is necessary for the Ni^{2+}-NTA binding, contains many hydrophobic amino acids, the protein can leave the solution and thereby form a precipitation (14). This can be seen in figure 11. To reduce this precipitation on the membrane of the concentrator, TEV protease was added during the fifth round to cleavage the His-tag after Ni^{2+}-NTA. In the results of round 5 after the incubation with TEV at different temperatures, a difference in precipitation was observed. The sample that was incubated at room temperature showed more precipitation than the sample that was incubated at 4°C. However, TEV is less reactive at 4°C, while this temperature is favorable for the stability of the protein. (15) Also, TEV is found to be less active with a high salt concentration. (16)

Another observation in the results of the fifth round was the presence of an extra peak in the chromatogram. This peak is most likely the presence of TEV. The presence can also be seen in the after SEC gel. In this gel, an extra band was also observed in the before SEC samples, which was not there in the after Ni^{2+}-NTA gel. The upper band on the gel indicates the presence of TEV in the sample, with a molecular weight of TEV of 28.4 kDa (17). The presence of a small amount of TEV left in the samples after SEC is caused by the overlap of the peaks.

Also, the gel shows that TEV incubation should be optimized since the TEV cleavage reaction was not completely performed. For optimization of TEV incubation, the incubation time, concentration and temperature should be considered. The cleavage reaction could be followed by using SDS-PAGE.

Another part of the process that could be looked at is the refolding process on the Ni^{2+}-NTA resin. During this experiment, only one protocol for the refolding of the protein was used. Other methods could be tried for the refolding of the protein. One example of a method that could be used is the use of a chaperone to increase the refolding efficiency. (18) Another example is the repetition of the denaturing and refolding of the protein. Optimizing the process of refolding could help improve the refolding efficiency and thereby help increase the concentration of protein. During the refolding also aggregation of the protein can take place, which was present in the form of a second peak and band in the results. (19) This is also a process that should be further examined.

Furthermore, during these experiments, a concentrator with a MWCO of 5,000 was used. Using a concentrator with a smaller MWCO, such as 3,000, could also help improve the concentration.

Lastly, the process of transformation could also be optimized or changed during future research, so that fresh bacteria are used for preculture and a higher concentration could be reached.

Conclusion

Overall a concentration at which crystallization could be tried was reached once. Also information in regards to the incubation with TEV was obtained. This process, however, should be further optimized during further research. Therefore the goal of optimizing the expression, refolding and purification processes to reach a concentration sufficient for crystallization was partially reached. However, the outcome has not been consistent. Also, the processes contain many more steps that could be looked at for further improvement of the process.

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Appendix 1: Bradford assays of round 3 and round 5

Figure 12: Bradford assay after Ni^{2+}-NTA to identify the fractions from the elution that contain the protein in round 3.

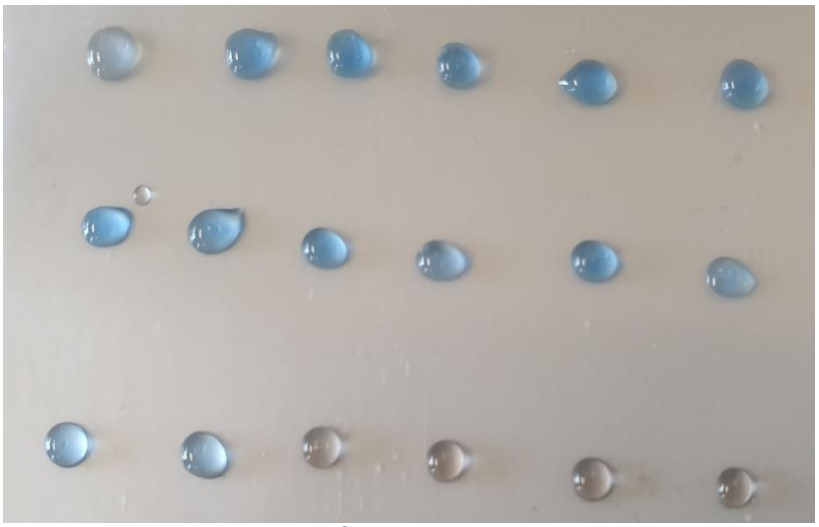

Figure 13: Bradford assay after Ni^{2+}-NTA to identify the fractions from the elution that contain the protein in round 5.

Appendix 2: Concentrating results before and after SEC, round 3

Table 7: The measurements per step of concentrating after Ni^{2+}-NTA. In between the before last and last measurement shown in the table, the concentrating was continued, however no samples were taken. The leakage of the membrane in the concentrator was followed using Bradford assay.

Absorption	Concentration (mg/mL)	Volume (mL)	Total mass (mg)
0.021	0.41	10	4.06
0.029	0.56	8.5	4.76
0.031	0.60	7.5	4.49
0.041	0.79	6	4.75
0.141	2.72	1.5	4.09

Table 8: Measurements during the different steps of concentrating after size exclusion chromatography. Two of the measurements belong together as two small concentrators were used.

Measurement	Absorption	Concentration (mg/mL)	Volume (µL)	Total mass (mg)
1	0.050	0.97	175	0.17
1	0.046	0.89	150	0.13
2	0.047	0.91	180	0.16
2	0.052	1.00	200	0.20
3	0.064	1.24	160	0.20
3	0.066	1.28	200	0.26
4	0.071	1.37	190	0.26
4	0.104	2.01	200	0.40
5	0.071	1.37	190	0.26
5	0.100	1.93	200	0.39
6	0.144	2.78	175	0.49
6	0.181	3.50	150	0.52
7	0.161	3.11	180	0.56
7	0.062	1.20	180	0.22
8	0.110	2.13	175	0.37
8	0.235	4.54	140	0.64
9	0.195	3.77	160	0.60
9	0.151	2.92	190	0.55
10	0.134	2.59	190	0.49
10	0.182	3.52	180	0.63
11	0.233	4.50	175	0.79
11	0.176	3.40	190	0.65
12	0.279	5.39	140	0.75
12	0.299	5.78	140	0.81
Final	0.276	5.33	130	0.69

Appendix 3: Concentrating results before and after SEC, round 5

Table 9: The measurements per step of concentrating after Ni^{2+}-NTA.

Absorption	Concentration (mg/mL)	Volume (mL)	Total mass (mg)
0.024	0.46	5.5	2.55
0.021	0.41	5	2.03
0.068	1.31	2.25	2.96
0.142	2.74	0.90	2.47

Table 10: Measurements during the different steps of concentrating the second peak after size exclusion chromatography. Two of the measurements belong together as two small concentrators were used.

Measurement	Absorption	Concentration (mg/mL)	Volume (µL)	Total mass (mg)
1	0.036	0.69	100	0.069
1	0.036	0.69	100	0.069
2	0.050	0.96	100	0.096
2	0.050	0.96	100	0.096
3	0.064	1.23	100	0.123
3	0.054	1.04	100	0.104
4	0.082	1.58	100	0.16
4	0.065	1.25	100	0.13
5	0.085	1.64	100	0.16
5	0.058	1.12	100	0.11
6	0.085	1.64	100	0.16
6	0.058	1.12	100	0.11

Table 11: Measurements during the different steps of concentrating the third peak after size exclusion chromatography. Two of the measurements belong together as two small concentrators were used.

Measurement	Absorption	Concentration (mg/mL)	Volume (µL)	Total mass (mg)
Start	0.003	0.06	5300	0.31
1	0.002	0.04	60	0.002
1	0.001	0.02	60	0.001
2	0.000	0	100	0
2	0.004	0.08	100	0.008
3	0.042	0.81	110	0.09
3	0.000	0	120	0