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Abstract

With a 5-year survival rate of approximately 10 %, glioblastoma multiforme (GBM) is the most common and aggressive brain tumor which replicates rapidly and invades surrounding healthy cells. Numerous approaches have been made towards the cure of this disease such as chemotherapy, surgery or radiotherapy, but the curing chance could not be significantly improved over the last decades. A novel, promising strategy is the use of oncolytic viruses as they can selectively induce cell death in malignant cells. One of those oncolytic viruses, the parvovirus H-1, has shown promising results of selectively killing glioblastomas in rat models and is currently investigated as therapeutic agents in patients for the first time. The major parvovirus H-1 protein, the non-structural protein 1 (NS1), is a multidomain protein which plays crucial roles in interacting and interfering with host cellular events, thereby inducing cell death in tumor cells. The C-terminal domain of NS1 functions in transactivation of the p38 promoter of the viral genome which implies an essential role in progeny virus production. However, the precise structural and functional basis for transactivation is unknown so far. Therefore, we have expressed and partly purified the parvovirus H-1 NS1 C-terminal domain for the purpose of structural characterization.

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Pongpawan Sethanant

LIST OF FIGURES

LIST OF TABLES

CONTENTS

INTRODUCTION

1.1 Background

Glioblastoma multiforme (GBM) is a life-threatening disease which is the most common and most aggressive primary brain tumor. They arise from astrocytes, a type of star-shaped glial cells which then becomes malignant called astrocytomas. Classified as Grade IV astrocytoma, these cells have abnormal shape and grow rapidly. These malignant cells often arise in the cerebrum and are able to invade surrounding healthy tissues in wide areas or even the spine but rarely spread to other parts of the body. Surprisingly, research has shown that men are more frequently diagnosed with GBM than women with a ratio of 1.6 to 1 [1, 2].

1.1.1 Cancer Therapy

There are many approaches to cancer therapy such as chemotherapy, radiology and surgical resection. However, these methods are unspecific to cancer cells leading to the damage of normal surrounding cells. On the other hand, oncolytic viruses show high specificity to cancer cells, thereby inducing cell death.. That is to say, oncolytic viruses specifically kill cancer cells. Moreover, strong evidence has shown that the oncolytic viruses can also stimulate host anti-tumor immune response [3, 4]. Another big advantage of this approach is that normal cells will not be affected by the activities of the oncolytic viruses. Many types of oncolytic viruses are used nowadays such as adenoviruses, Herpes Simplex Virus-1 (HSV-1) and parvoviruses. Ongoing research shows promising results of its ability to induce cell death in cancer cells *in vitro*. Due to its success *in vitro*, clinical trials are also being carried out to test its efficiency in humans.

1.1.2 Oncolytic virus selectivity

At the normal state, normal cells are at rest and do not replicate whereas cancer cells replicate continuously. On the other hand, viruses cannot replicate autonomously but require the host cell's replication machinery such as DNA polymerase for its genome replication. Therefore, oncolytic viruses are able to multiply in replicating cancer cells. In contrast, oncolytic viruses cannot replicate in normal cells because normal cells are at rest and do not replicate. In conclusion, the oncolytic viruses can only replicate in cancer cells but not normal cells.

1.1.3 Parvoviruses

Parvoviruses are amongst one of the most actively studied oncolytic viruses and also belong to the smallest viruses known so far. Their genome is a single-stranded DNA with an approximate size of 5 kb packaged into a 25 nm-diameter viral particle. They are non-enveloped, icosahedral and replicate in the nucleus. Moreover, they do not integrate into the host chromosomes [5]. Due to its small genome, it can only encode a total of 7 proteins which are 2 capsid proteins (VP1 and VP2) and 5 non-structural proteins (NS1, 3 spliced NS2 forms and SAT) [6]. Of these, one of the most extensively studied parvoviral proteins, the NS1 protein has been shown to induce cancer cell death by interfering with cellular events. Also, this protein was found to induce death to cells resistant to cisplatin and/or TRAIL even when there is an overexpression of Bcl-2 [7].

1.1.4 Parvovirus H-1 NS1 protein

The NS1 protein is a major regulatory protein of 672 amino acids. This protein comprises of three domains, the DNA-binding N-terminal domain, helicase domain and C-terminal transactivation domain. In contrary to its small size, it plays several roles such as regulation of viral genome replication, interaction with nucleic acids and proteins and exerting enzymatic activities [8] thus interfering with numerous host cellular events as shown in Fig.1. The interference of the NS1 protein

with host cellular proteins leads to apoptosis, lysosomal-like programmed cell death, necrosis or cytolysis [8].

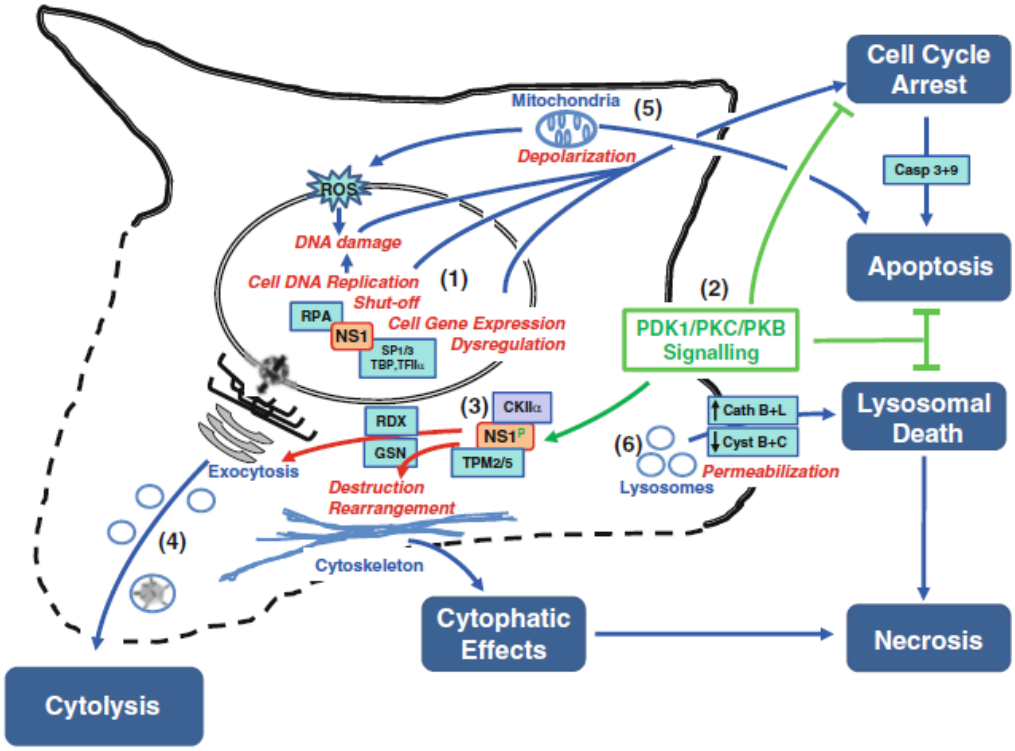

Fig. 1 Interference of NS1 protein with numerous cellular events. The scheme here impressively demonstrates the variability of NS1 in functions leading to oncolysis. [8]

1.1.5 Parvovirus NS1 C-terminal domain

The parvovirus NS1 C-terminal transactivation domain (aa 545-672) is an acidic domain involved in transactivation of the P38 promoter which regulates the expression of the late parvoviral capsid proteins VP1 and VP2.

Typically, the P38 promoter regulates the expression of the viral capsid genes. But for engineered parvoviruses, the capsid genes are replaced with therapeutic transgenes e.g. toxins and cytokines/chemokines that stimulate host anti-cancer immunity (Fig.2). Moreover, the therapeutic transgenes are constructed behind the strong p38 promoter that can be induced by the C-terminal transactivation domain of the NS1 protein.

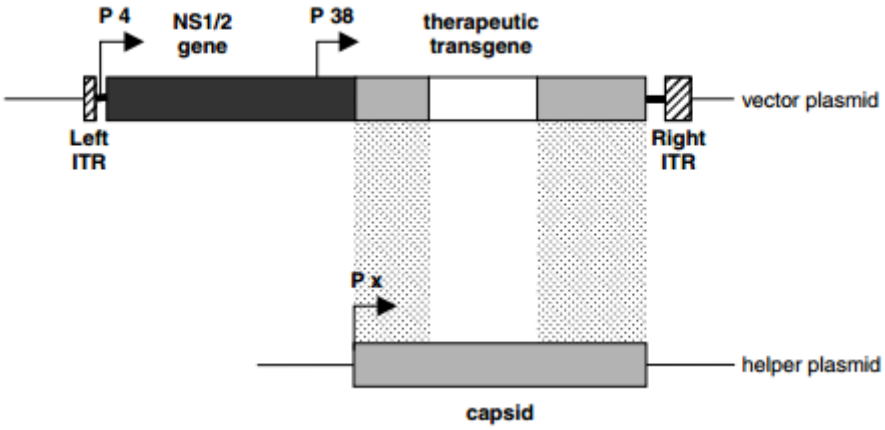

Fig. 2 Genome organization of parvovirus H-1 [9]

1.2 Objective

To express and purify the C-terminal domain of parvovirus H-1 NS1 protein.

1.3 Scopes of work

1.3.1 Express and purify the parvovirus H-1 NS1 C-terminal domain by using *E. coli* system.

1.3.2 Construction of pFastBac vectors for expressing parvovirus H-1 NS1 C-terminal domain in Sf9 cells.

MATERIALS AND METHODS

2.1 Bacterial strain

2.1.1 *Escherichia coli* BL21 DE3

2.1.2 *Escherichia coli* XL10-Gold

2.2 Cloning vectors

2.2.1 pRSETA (Invitrogen)

2.2.2 pFastBac (Invitrogen)

2.3 Restriction Enzymes

2.3.1 *Sfo*I

2.3.2 *Dpn*I

2.4 Expression trial of parvovirus H-1 NS1 C-terminal domain

The parvovirus H-1 NS1 C-terminal domain (amino acid 543-672) was previously constructed in-frame into pRSETA and was verified by sequencing. Then, the plasmid was transformed into *E. coli* BL21 DE3 for expression trial. Freshly transformed competent cells containing the NS1 C-terminal gene in pRSETA were inoculated in 5 ml of Luria Bertani broth with 100 µg/ml ampicillin. The preculture was incubated overnight at 37ºC. Then, 1% of preculture was added to 50 ml of LB with 100 µg/ml ampicillin. The culture was grown until OD_{600} reached 0.6 and protein production was induced by the addition of 1mM IPTG. Samples were collected every hour for four hours. The amount of samples that were collected were up to the equation:

$$Amount\ of\ sample\ (ml) = \frac{Initial\ OD_{600}\ after\ induction}{Current\ OD_{600}}$$

After the samples were collected, 50 µl of sample buffer were added and 10 µl of sample was loaded onto 12% Acrylamide gels and SDS-PAGE was performed at 300V, 25mA.

2.5 Large scale production of parvovirus H-1 NS1 C-terminal domain

After transformation of pRSETA into *E.coli* BL21 DE3, the transformants were directly poured into 200 ml Luria Bertani broth with 100 µg/mL ampicillin to make a preculture followed by incubation at 37ºC overnight with shaking at 180 rpm. After overnight incubation, 40 ml of preculture were added to four 5L flasks with 1L Luria Bertani broth with 100 µl ampicillin. The culture was grown at 37ºC with shaking until OD600 reached 0.6. Then, 1mM IPTG was added directly to the culture to induce protein production. After 4 hours of induction, the cells were collected by centrifugation at 4000 x g for 30 minutes. The cells were resuspended in Buffer 1 (50 mM Tris pH 7.5, 500 mM NaCl and 10 mM Imidazole).

2.6 Protein purification by affinity chromatography

After cell resuspension in Buffer 1, the cells were lysed by ultrasonication on ice for 8 minutes twice. The cell lysates were subjected to centrifugation at 12,000 x g for 30 minutes at 4ºC and the supernatant was collected for purification.

The Ni-NTA column was washed with 1 column volume of dH_20 and 2 column volumes of Buffer 1 prior to addition of the supernatant. After the addition of supernatant to the column, the flow rate of the column was approximately 1 drop/sec. Then, the column was washed with Buffer 2 (50 mM Tris pH 7.5, 400 mM NaCl and 50 mM imidazole). Finally, a stepwise elution with different imidazole concentrations was performed starting from 50 mM, 100 mM, 150 mM, 200 mM and 500 mM twice. To check for protein existence, 10 µl from each step was mixed with 15 µl of sample buffer followed by SDS-PAGE analysis.

2.7 pFastBac vector construction

The parvovirus H-1 NS1 C-terminal gene will be constructed into pFastBac, a donor plasmid used in the baculovirus expression system. This system is used to express the target protein in high amounts in insect cells for *in vivo* crystallography screenings. To establish an easy and less time-consuming method, the T/A cloning system was brought to use.

The T/A cloning system involves an A-tailed gene, which can be obtained from a normal PCR reaction by use of *Taq* polymerase. On the other hand, the T-tailed plasmid must be manually constructed. In this experiment, five pFastBac constructs that contain different localization signals were used which includes pFastBac (no localization signal), pFastBac-ER (ER), pFastBac-ER-SKL (ER and peroxisomes), pFastBac-ER-KDEL (ER and ER retaining signal) and pFastBac-SKL (peroxisomes).

2.7.1 NS1 C-terminal gene amplification

The NS1 C-terminal gene was amplified by using gene-specific primers (Appendix) by using C-terminal-containing pRSETA as a template. The gene was amplified by PCR with the following steps (Appendix). In the amplification step, the *Taq* polymerase without proofreading adds a single adenine at the end of each gene. After amplification, the PCR solution was further digested with *Dpn*I to digest the remaining pRSETA plasmid. *Dpn*I was then inactivated by heating the solution to 55ºC for 10 minutes. The amplified PCR product was analyzed by agarose gel electrophoresis and was checked for protein concentration and purity via Nanodrop™.

2.7.2 T-tailing

The five pFastBac constructs were linearized with a blunt-end restriction enzyme, *Sfo*I. Then, the plasmid is subjected to agarose gel electrophoresis for analysis. The gel slice containing the linearized plasmid was excised and purified with a commercial PCR cleanup kit according to the manufacturer's instructions (Thermo Scientific). Next, a single thymine was added to the blunt ends of the

linearized plasmids by PCR. In this step, dTTP was added instead of dNTP to the PCR reaction. Therefore, only a single thymine could be added to the blunt ends of each side of the linearized plasmids. Finally, the plasmids were purified with PCR cleanup kit and the purity and concentration was measured by Nanodrop™.

2.7.3 Ligation

The two components, amplified NS1 C-terminal domain gene and the linearized pFastBac constructs were ligated at a ratio of 5:1 at room temperature for 30 minutes.

2.7.4 Transformation

The ligation solution was transferred into 50 µl of competent *E. coli* XL10-Gold cells. The solution was placed on ice for 10 minutes, then heat shocked at 42ºC for 45 seconds and incubated on ice for another 15 minutes. Then, 500 µl of LB media was added to the solution followed by incubation at 37ºC with shaking for 15 minutes. The cells were collected by centrifugation at 10,000 x g and 400 µl of the solution was discarded. The rest of the solution was mixed thoroughly and plated onto LB agar plates containing ampicillin.

2.7.5 Verification of positive colonies

The colonies that grew on the LB agar plates were verified by Colony PCR as shown in Fig.10.

RESULTS

3.1 Protein expression and purification of the parvovirus H-1 NS1 protein C-terminal domain by *E. coli* system

3.1.1 Parvovirus H-1 NS1 C-terminal domain expression trial at 37ºC

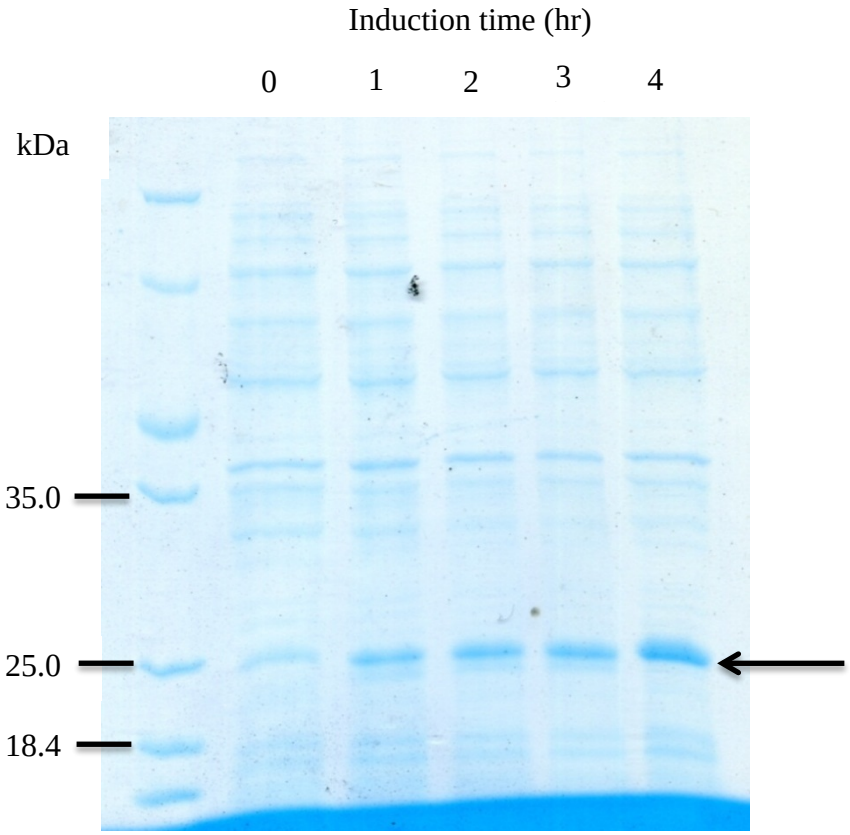

Fig. 3 Test expression of Parvovivrus H-1 NS1 C-terminal domain at 37ºC. A clear increase of a protein band around 25 kDa is visible, fitting to the expected molecular weight of the target protein.

Parvovirus H-1 NS1 C-terminal domain was tested for expression levels in *E. coli* BL21 DE3 cells after collection of cell pellets every hour for four hours. Fig.3 showed that NS1 C-terminal domain was overexpressed in an increasing manner after induction with 1 mM IPTG as marked with an arrow.

3.1.2 Test purification and solubility test of parvovirus H-1 NS1 C-terminal domain

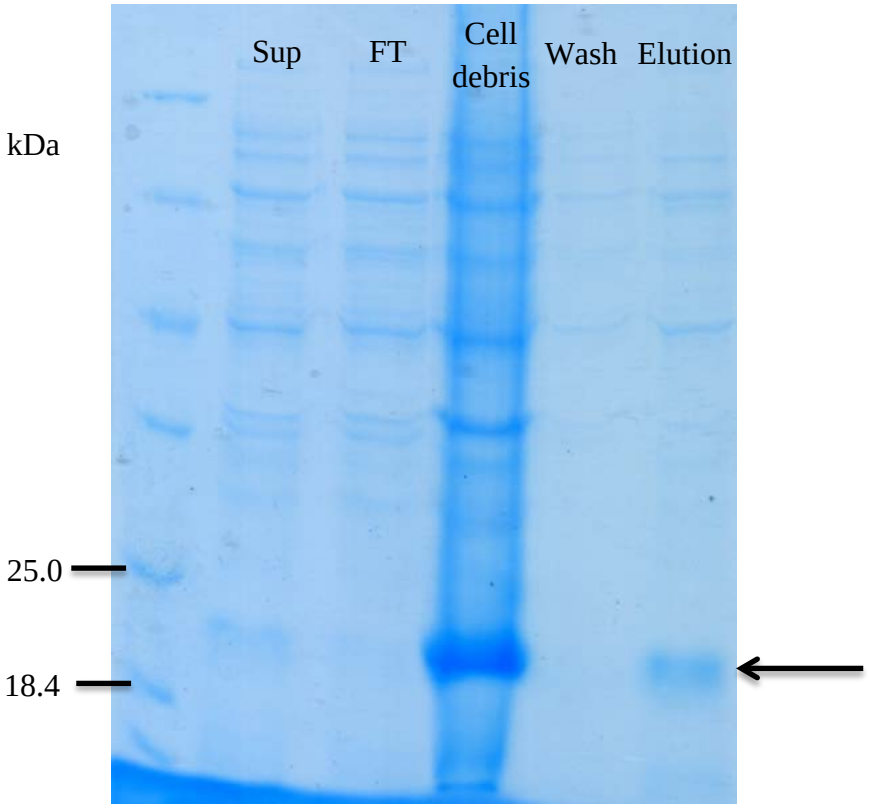

Fig. 4 Test purification and solubility test of parvovirus H-1 NS1 C-terminal domain

Fig.4 showed that the NS1 C-terminal domain protein was partly soluble when comparing the cell debris and elution column. However, it could be purified by affinity chromatography as seen in the elution column. To improve the purity of the protein for further analysis, the protein was repurified by size exclusion chromatography.

3.1.3 Large batch expression

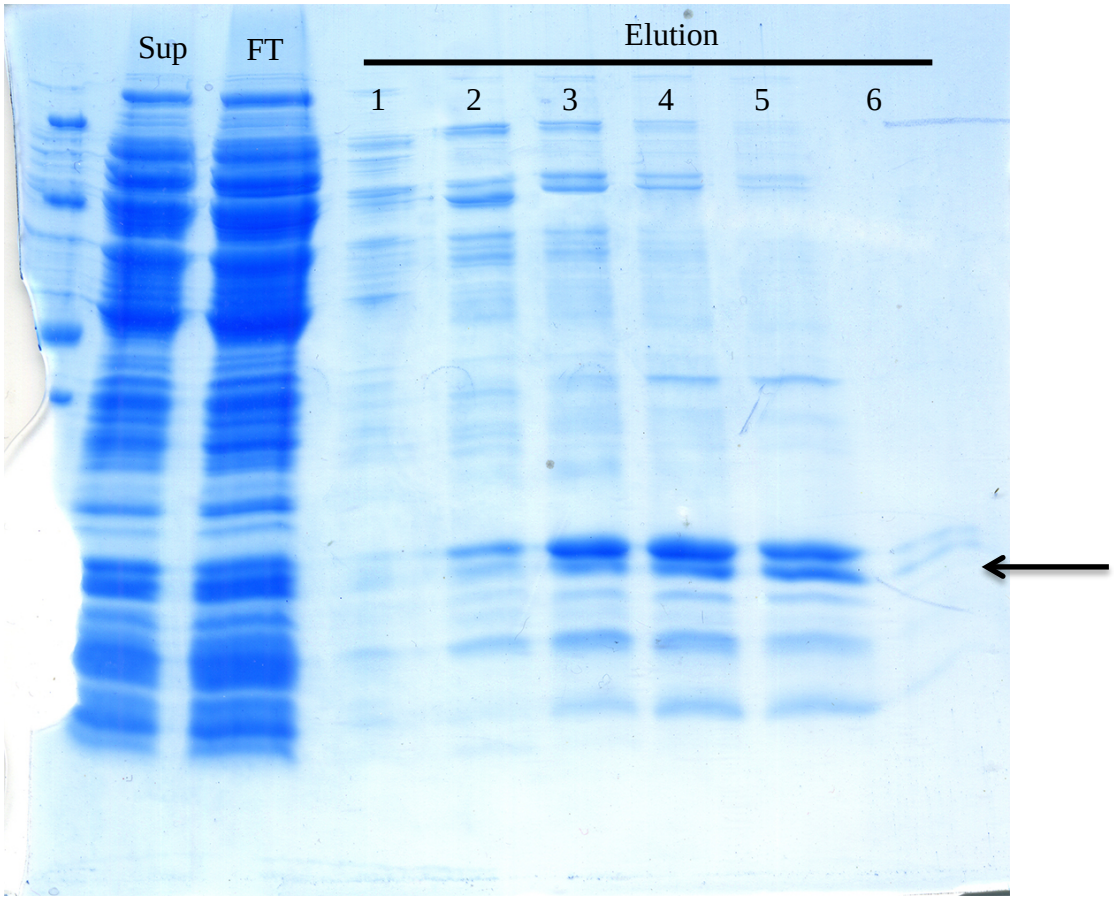

Fig. 5 Large scale production (4 L) of parvovirus H-1 NS1 C-terminal domain

After a successful test expression was carried out at 37ºC, protein production was up-scaled to a volume of 4L. Fig.5 showed results from an SDS-PAGE after purification with affinity chromatography. The proteins were then eluted with a stepwise gradient of 50 mM, 100 mM, 150 mM, 200 mM, 500 mM and 500 mM imidazole, respectively. The figure above showed that the parvovirus NS1 C-terminal domain can be eluted starting at the lowest concentration of 100 mM imidazole.

3.1.4 Purification by size-exclusion chromatography

From Fig.3, since the eluted proteins were not pure enough for primary screening such as DLS and CD spectrometry, the protein was subjected to size exclusion chromatography.

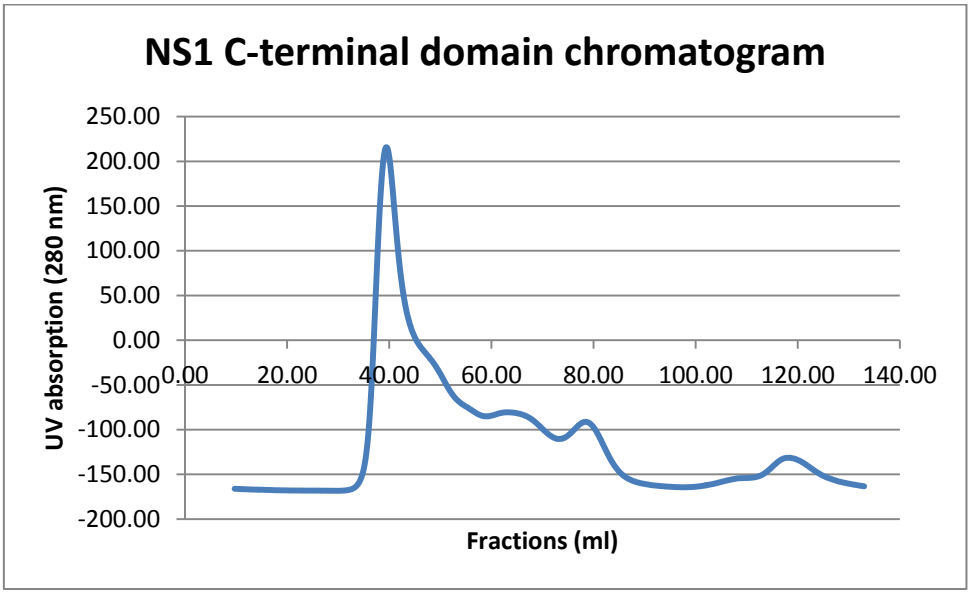

Fig. 6 NS1 C-terminal domain chromatogram

Table 1 Size exclusion chromatography fractions for SDS-PAGE

Fractions	ml	Fractions	ml
B11	38.08	C2	62.08
B10	40.08	C3	64.08
B9	42.08	C4	66.08
B8	44.08	C5	68.08
B7	46.08	C8	74.08
B6	48.08	C9	76.08
B3	54.08	C10	78.08
B2	56.08	C11	80.08
		C12	82.08

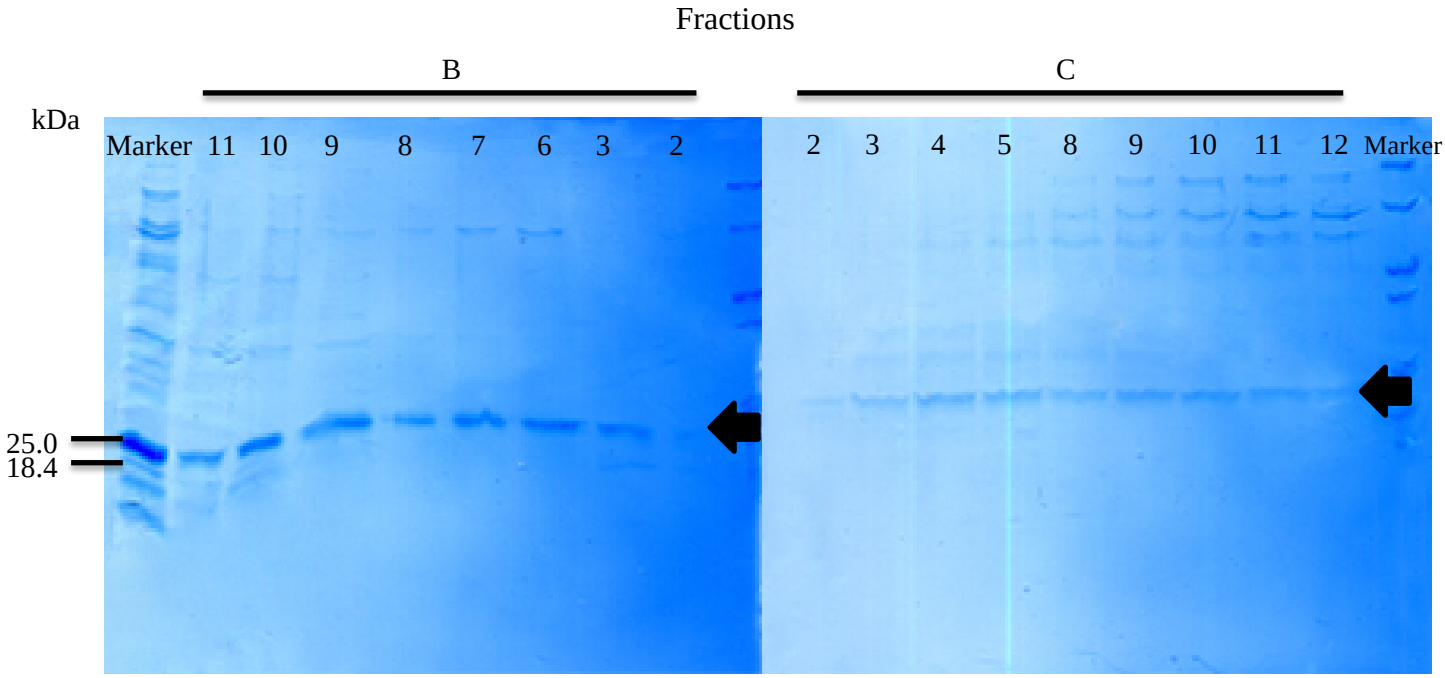

Fig. 7 SDS-PAGE from Size exclusion chromatography

After subjected to size exclusion chromatography, a chromatogram of the UV absorption at 280 nm was generated (Fig.6). Samples were collected from the fractions as written in Table 1. The samples were then subjected to SDS-PAGE for analysis. In Fig.7 it showed that the protein of interest was present in most of the fractions which indicates unspecific binding to the size exclusion column and requires optimization of buffer conditions.

3.2 Construction of parvovirus H-1 NS1 C-terminal domain into pFastBac

3.2.1 Gene amplification

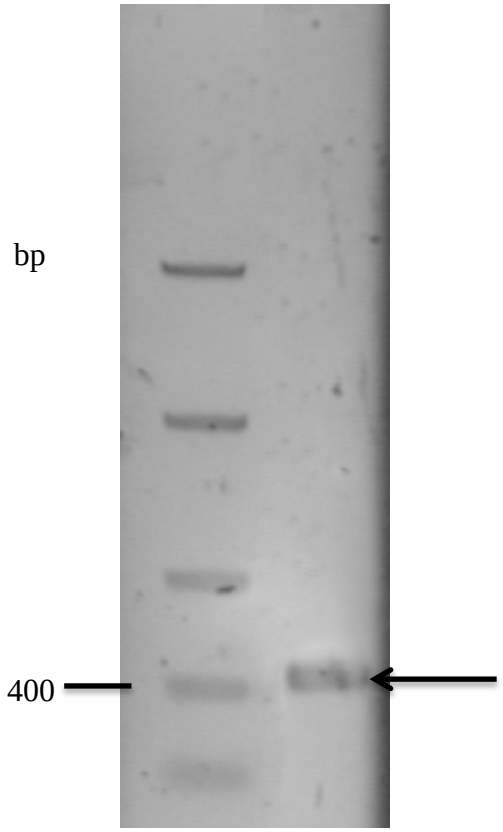

Fig. 8 Parvovirus H-1 NS1 C-terminal gene amplification

The parvoviral H-1 NS1 C-terminal gene was amplified by PCR by using gene specific primers (Appendix). The amplified gene was approximately 400 bp in size as indicated with arrows.

3.2.2 Plasmid digestion check

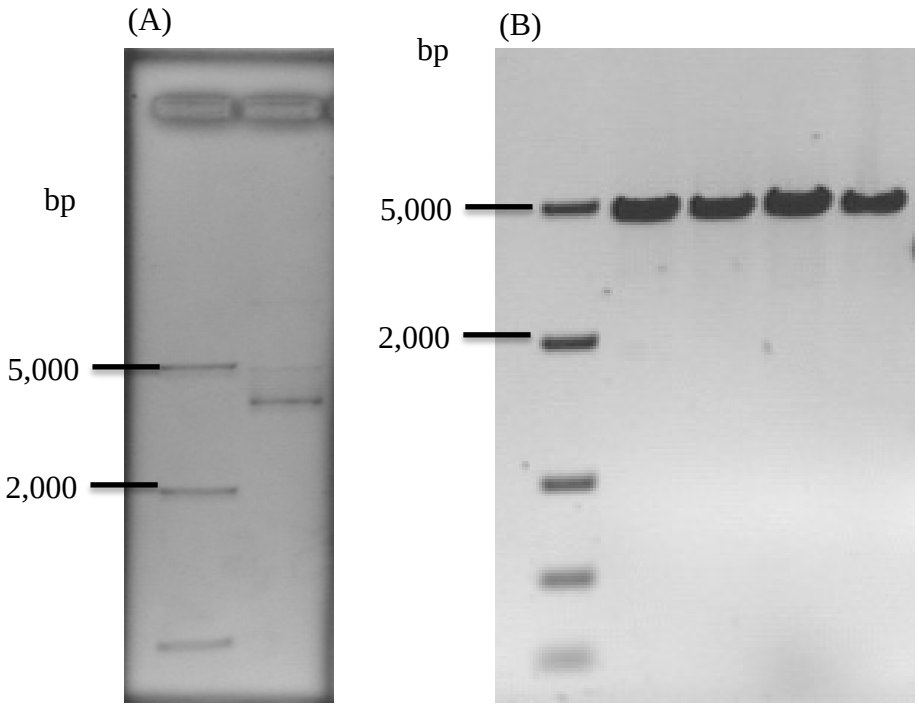

Fig. 9 Digestion of different pFastBac constructs. pFastBac digestion of (A) pFastBac, (B) pFastBac-SKL, pFastBac-ER, pFastBac-ER-KDEL, pFastBac-ER-SKL by *Sfo*I

Fig.9 shows the different plasmid constructs after linearization with *Sfo*I restriction enzyme. Then, the digested plasmids were analyzed by 1% agarose gel electrophoresis.

3.2.3 T-tailing

Table 2 Amount of colonies grown on LB+ampicillin plates

	Sample					Control				
	1	2	3	4	5	1	2	3	4	5
No. of colonies	/////		/	//	//	////	/	/	/	//

After the A-tailed gene was ligated with the T-tailed vector, the ligated plasmid was transformed into *E. coli* XL10-Gold to investigate the transformation efficiency. Table 2 compares the amount of colonies that were grown on sample and control plates (Control plates = Ligation solution lacking A-tailed genes). From the table it shows that in the sample and control plates showed approximately the same number of colonies.

3.2.4 Colony PCR

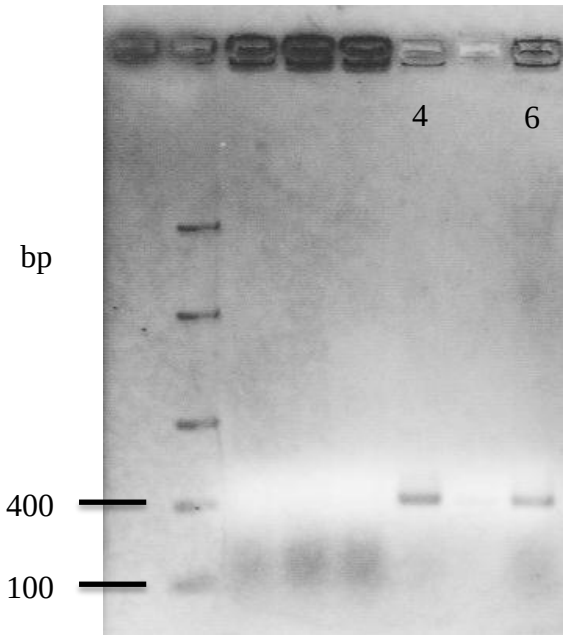

Fig. 10 Colony PCR of positive constructs

After transforming the pFastBac containing the NS1 C-terminal gene into *E. coli* XL10-Gold, the colonies were spread on LB agar with ampicillin. Then, six colonies were randomly picked for Colony PCR to check the insert gene. From Fig.10, only 2 colonies (Colony 4 and 6) were positive. Before proceeding to the next step, the colonies needed to be verified via sequencing.

DISCUSSIONS

The parvovirus H-1 NS1 C-terminal domain could be produced at high amounts when induced at 37ºC. In the test expression, high amounts of the target protein were found in the cell pellet which means that the protein was insoluble when expressed at 37ºC. To increase the solubility of the target protein, test expressions at different temperatures may be carried out. Lower temperatures such as 25ºC and 30ºC might increase the solubility of the protein due to the chaperones inside the *E. coli* cells might function better at lower temperatures to help the target protein fold properly.

For the large batch purification, the elution columns still have a high background because the Ni-NTA beads used in protein purification might interact non-specifically with other proteins in the solution. In elution fraction 1, high amount of background was removed after washing with Buffer 1 with an imidazole concentration of 50 mM. So, in future purifications, Buffer 1 with an imidazole concentration of 50 mM will be washed heavily before elution to increase the purity of the eluted protein. Moreover, the purified protein was not seen as a single band, but a double band. This might result from the degradation of the target protein.

After performing size exclusion chromatography, the target protein was found in every fraction. This might be because the protein is polydisperse, meaning that they are not in one single state all the time such as monomers or dimers. So, if the proteins form monomers, dimers and/or oligomers at the same time, the protein will be found in most of the fractions in the chromatogram. This showed that size exclusion chromatography might not be suitable in purification of the parvovirus H-1 NS1 C-terminal domain. Another possibility is that NS1 C-terminal domain might interact with the column. Therefore, to prevent this interaction, another type of column has to be used.

For pFastBac cloning, the efficiency of the normal *Taq* polymerase might not be high enough to yield a successful T-tailed pFastbac vector. Therefore, a terminal transferase might be more efficient in adding a thymine to the ends of the linearized plasmids. Moreover, only a single thymine has to be added. So, a ddTTP (2',3'-Dideoxythymidine-5'-Triphosphate, ddTTP) will be used for T-tailing of the pFastBac constructs.

If the cloning becomes successful, one disadvantage of the T/A cloning system is that the inserts inside the plasmids might be wrongly oriented. However, this problem can be overcome by checking the insert orientation by using 2 PCR reactions:

Reaction 1: Primers = Forward of plasmid, Reverse of gene

- If the results are positive it means that your insert is in correct orientation.

Reaction 2: Primers = Forward of gene, Reverse of plasmid

- If the results are positive it means that your insert is in wrong orientation.

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APPENDIX

PCR cycle steps used for parvovirus NS1 C-terminal domain amplification

Temperature (ºC)	Time	Cycles
95	30 s	
95	45 s	
55	45 s	30
72	1 min	
72	5 min	
16	∞	

Parvovirus H-1 NS1 C-terminal primer sequence

Forward: 5´- CAT ACC AAT CTA CCA TGG CTT GTT ACT - 3´ (27 bp)

Reverse: 5´- GCG TCC AAG GTC AGC TCC TCG - 3´ (21 bp)