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PURIFICATION, AND ACTIVITY OF HUMAN RHINOVIRUS 3C PROTEASE FUSED WITH N-TERMINAL GST-tag And C-terminal His-tag (GST-HRV3C-His) expressed in *Escherichia coli*

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ABSTRACT

The Human rhinovirus 3C protease (HRV3C) is one of the most effective enzymes for removing fusion tag in purification process. This protease is often produced as fusion form GST-HRV3C but there is no study about the fusion form: GST-HRV3C-His. In this study, researchers conducted the purification GST-HRV3C-His expressed in E. coli,checked the activity and investigated its application. GST-HRV3C-His could be purified using His-tag column with 86.6% purity and GST column with 96.87%. The specific activity of GST-HRV3C-His was demonstrated to be about 4500 U/mg and its application in the purification of another proteins carrying HRV3C-specific recognition sequence, LEVLFQ↓GP based on His-tag or GST-tag was also proved in this study.

Keywords: Human rhinovirus 3C protease, GST-HRV3C-His, purification

1. Introduction

Affinity purification and solubility enhancement tags are essential tools that help in the overexpression of recombinant proteins and keeping them soluble. Since some tags can alter the structural and functional integrity of some target proteins, they have to be removed by proteolytic cleavage. Therefore, some specific endoproteases are used to remove the fusion tag. Endoproteases with specific recognition sites include thrombin, enterokinase, tobacco etch virus (TEV) protease and the human rhinovirus 3C protease (HRV3C) (Waugh, 2011).

Up to now, like the TEV protease, the HRV3C protease has become one of the most effective agents for removing fusion tag. The HRV3C protease is a 20 kDa recombinant cysteine protease that is derived from the human rhinovirus which belongs to the small positive-strand RNA picornavirus (Wanga & Chen, 2007). This protease is highly specific for its recognition sequence, LEVLFQ↓GP. The major disadvantage of the HRV3C protease is that the glycine-proline after the scissile bond is an absolute necessity, which means that the product of most recombinant proteins with a native N-terminus is not possible. However, the advantage of the HRV3C protease is that it is reported to have up to 10x more activity at 4°C (Ullah et al., 2016).

Currently, the commercial HRV3C is often produced as a fusion form with GST-tag (called PreScission) and His-tag. In this study, GST tag is combined with HRV3C protein at N-terminus to increase the solubility of HRV3C and be used for purification but do not influence the cleavage activity of this protease (Walker et al., 1994). Furthermore, His-tag is the most common fusion tag to purify recombinant proteins. It is a polypeptide which has from 2 to 10 Histidine, usually 6 Histidine and have binding capacity with Nickel (Zhao, Li, & Liang, 2013). The His-tag is usually used for combining target proteins at either N-terminus or C-terminus but the fusion of His tag to N-terminus of protein has been proven to be the most effective thus far (Eschenfeldt et al., 2010). Therefore, in the commercial form, HRV3C protease is also fused with His-tag at N-terminus and there is no study about the fusion between C-terminal His-tag and HRV3C. Nevertheless, in some cases, the biological activities of other proteins were influenced by N-terminal His-tag (Eschenfeldt et al., 2010). In this study, we conducted the purification, and application of intracellular GST-HRV3C protease fused C-terminal His-tag in *E. coli* in order to have a specific evaluation of this fusion protease.

2. Materials and methods

2.1. Materials

The strains, plasmids, and primers used in this study are listed in Tables 1, 2 and 3, respectively. *Escherichia coli* strain OmniMAX (Invitrogen) was used for cloning experiments, *E. coli* BL21(DE3) to analyze expression of the protease fusion (His-GST-HRV3C). Cells were routinely grown in Luria broth (LB) at 37°C under aeration and shaking at 250 rpm; other conditions (IPTG concentration, temperature, time) for induction were surveyed in this study. Antibiotics (ampicillin) were added where appropriate (100 µg/mL).

Table 1. *Bacterial strains used in this study*

Strains	Genotype	Source/references
E. coli OmniMAX	F′ *proAB*+ *lacIq lacZΔM15* Tn*10*(Tet^R) Δ(*ccdAB*) *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80(*lacZ*)ΔM15 Δ(*lacZYA-argF*) U169 *endA1 recA1supE44 thi*-1 *gyrA96 relA1 tonA panD*; used for cloning	Invitrogen
E. coli BL21(DE3)	*F*$^-$ *omp*T *gal dcm lon hsd*S_B(*r*$_B^-$ *m*$_B^-$) λ(DE3 [*lac*I *lac*UV5-*T7p07 gen* 1 *ind*1 *sam*7 *nin*5]) [malB$^+$]$_{K-12}$(λ^S); used for expression	Invitrogen

Table 2. *Plasmids used in this study*

Plasmid's name	Description	Source/references
pGEX4T-HRV3C	Having a gene encoding for GST-HRV3C under control of P*tac* promoter; Basic vector for construction of pHT2081	Lab collection

pHT2081	Having a gene encoding for GST-HRV3C-His	This work
pHT01	Negative control	Lab collection

Table 3. *Oligonucleotides used in this study*

Oligonucleotide	Sequence 5' → 3"	Used for
ON2183	GTAGAGAAACAACATCATCATCATCAT CATTAAGAATTCATCGTGACTGACTGAC	Adding C-terminal His-tag to pGEX4T-HRV3C for constructing pHT2081
ON2184	GAATTCTTAATGATGATGATGATGATG TTGTTTCTCTACAAAATATTGTTTTTTA AG	Adding C-terminal His-tag to pGEX4T-HRV3C for constructing pHT2081
ON1444	GGCCAGATCTGTCGACCGTCTCCGGGA GCTG	Testing DNA sequence of pHT2081

2.2. Methods

2.2.1. Constructing recombinant plasmid

The target vector is formed by the site-directed mutagenesis using PCR method. The sequence coding 6 Histidine tag was added downstream the GST-HRV3C sequence of the original vector pGEX-4T-HRV3C by using a couple of specific primer pairs ON2183 / ON2184 (Table 3). PCR products were treated with *Dpn*I enzyme to remove the original plasmid (pGEX-4T-HRV3C), and then transformed into *E. coli* OmniMAX. The strains were screened and analyzed by DNA sequencing (Macrogen Inc., Korea) with primer ON1444 (Table3).

2.2.2. Transformation

The plasmid pHT2081 confirmed by DNA sequencing was transformed into the expression strain *E. coli* BL21(DE3) (Table 1) by heat shock method (Froger & Hall, 2007).

2.2.3. Analysis of the expression of GST-HRV3C-His protein

To identify the appropriate temperature for the expression of recombinant proteins, *E. coli* BL21(DE3) harboring pHT2081 were grown at 37°C until the optical density at 600 nm (OD_{600}) reached to 0.8. Then, the synthesis of proteins was induced by the addition of 0.5 mM IPTG. Cells were cultured at 3 temperature conditions (37°C, 30°C and 23°C) and then harvested by centrifugation. The amount of *E. coli* cells was equivalent to those present in 1 ml of growth culture with an OD_{600} of 6.

The cell pellet was added to 500 µl of lysis buffer (Tris-HCl pH 7.2, 15% sucrose) then sonicated to break the cells and centrifuged at 13000 x g for 5 min. Samples were taken just before centrifugation (representing the total amount of proteins) and after centrifugation (soluble proteins in the supernatants and insoluble proteins in the pellets). Each protein sample was analyzed by SDS-PAGE to identify which temperature promotes the highest amount of the soluble GST-HRV3C-His protein. Moreover, the IPTG

concentration level (0, 0.01, 0.05, 0.1, 0.5, 1 mM) and induction time (0, 2, 4, 6, 8, 10, and 12 h) were also tested.

2.2.4. Purification of GST-HRV3C-His proteins by HisTrap™ HP column

E. coli BL21(DE3) cells harboring pHT2081 were grown under optimal conditions and then harvested by centrifugation at 6000 x g for 20 min. The cells were resuspended in binding buffer (30 mM Tris-HCl, pH 8.0; 500 mM NaCl; 1mM DTT; 5% glycerol) and DNase I at 20 µg/ml. PMSF at 0.5 mM were added before sonication. Soluble proteins were obtained in supernatants after centrifugation at 19 000 x g and 4°C for 30 min. Then, proteins were clarified by membrane filtration (pore size 0.22 µm).

HisTrap™ HP column (GE Healthcare) was equilibrated with 30 ml of binding buffer. The clarified sample was pumped into the column with a flow rate of 2 ml/min. The recombinant proteins containing the His-tag bind to Ni-NTA present in the columns. By contrast, the other proteins which do not contain a His-tag were eluted from the columns by washing with 30 ml of binding buffer containing imidazole at 5 mM. After that, GST-HRV3C-His protein was eluted from the column with elution buffers (binding buffer containing various imidazole concentrations: 10, 20, 40, 60, 80, 100, 150 or 200 mM). The fractions were analyzed by SDS-PAGE.

2.2.5. Purification of GST-HRV3C-His proteins by GST Bulk Kit

As same as purification by HisTrap™ HP column (2.2.4), the sample was prepared and treated to have the soluble intracellular proteins sample. The GST columns were equilibrated with 10 ml of binding buffer. The clarified sample was flown through the column. The recombinant proteins containing the GST-tag bind to Glutathione in the column. In contrast, the other proteins which do not contain a GST-tag were eluted from the columns by washing with 10 ml of binding buffer containing reduced Glutathione at 0.1 mM. After that, GST-HRV3C-His protein was eluted from the column with elution buffer (binding buffer containing various imidazole concentrations (0.2, 0.5, 1, 2, 4, 6, 8, 10 mM). The fractions were analyzed by SDS-PAGE.

2.2.6. Determination of the concentration of protein

The concentration of proteins were determined based on the extinction coefficient of them by using Eppendorf Biophotometer (Watson & Veeraragavan, 2014).

2.2.7. Determination of the activity of HRV3C protease

The activity of HRV3C protease was determined by cutting reactions of BlysSN-His-HRV3C/CS -GFP carrying the specific recognition site between BsLysSN-His and GFP (received from Center of Bioscience and Biotechnology). This protein was dialyzed with cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0 at 25°C) and then mixed with GST-HRV3C-His proteases for 16 h at 4°C.

During this step, the concentrations of protease were surveyed using several levels of dilution. The SDS-PAGE method was used to determine the percentage of proteins being

cut. Based on these percentages, the activity of HRV3C protease was calculated based on the definition: One unit of HRV3C protease cleaves >95% of 100 µg of the control target protein at 4°C for 16 h.

2.2.8. Purification of GFP from a fusion protein

For purification of GFP, the cleavage sample was pumped and flew through a HisTrap^{TM} HP column. The target protein GFPs which had been cut by the HRV3C protease lost their His-tag. Therefore, they could not bind to Ni-NTA and were obtained in the flow-through solution. The others showing not reaction were bound to the His-Trap column.

2.2.9. SDS-PAGE method

Sample buffers 5xSDS-PAGE (25 µl) was added to 100 µl solution of proteins and heated at 95°C for 5 min, then centrifuged at 13 000 x g for 5 min. The supernatants were loaded into wells of an SDS-PAGE gel. The electrophoresis was running at the current of 25 mA for one gel. Gels were stained by soaking in SDS-PAGE stain solution (0.25 g Coomassie Brilliant Blue in 50 ml ethanol 50% and 50 ml glacial acid acetic) for 1 h. Transfer of the gel into the destaining solution (10% ethanol, 7.5 acid acetic , dH_2O) changed several times (Phan, Huynh, Truong, & Nguyen, 2017). SDS-PAGE gels were scanned and analyzed by AlphaEaseFC Software.

3. Results and discussions

3.1. Construction of plasmid pHT2081 carrying a gene coding for GST-HRV3C-His

Plasmid pHT2081 were constructed by site-directed mutagenesis using PCR, in which the DNA coding His-tag was added into the C-terminus of GST-HRV3C coding gene. The PCR product of the mutagenic PCR treated by *Dpn*I was transformed into *E. coli* OmniMAX. The transformants on LB-Amp agar were chosen to conduct DNA sequencing to prove their accuracy (data not shown)

Since the plasmid template pGEX4T-HRV3C is derived from pGEX4T, the GST-HRV3C-His coding gene is under control of P*tac* promoter. The expression of this fusion gene will be induced by addition of IPTG. The constructed plasmid was transformed into *E. coli* BL21(DE3) to check the expression.

3.2. Analyses the expression patterns of GST-HRV3C-His proteins under different conditions

First of all, *E. coli* BL21(DE3) strain harboring pHT2081 was incubated and induced at three different temperatures and analyzed the proteins produced under these conditions by SDS-PAGE. At 23°C, the most GST-HRV3C-His protein present in the soluble fraction if growth occurred at 23°C, while at 30°C, about 40% of the protein is present in the insoluble fraction (pellet sample) and at 37°C, more than half of target protein is also insoluble (Fig. 1).

Secondly, to find out the optical IPTG concentration, cells harboring pHT2081 was grown at 23°C. The result presented in Fig. 2A demonstrates that concentrations of 0.5 and 1 mM IPTG resulted in the highest expression levels of the target protease. Therefore, 0.5 mM of IPTG was chosen for the expression of GST-HRV3C-His in *E. coli* in further experiments.

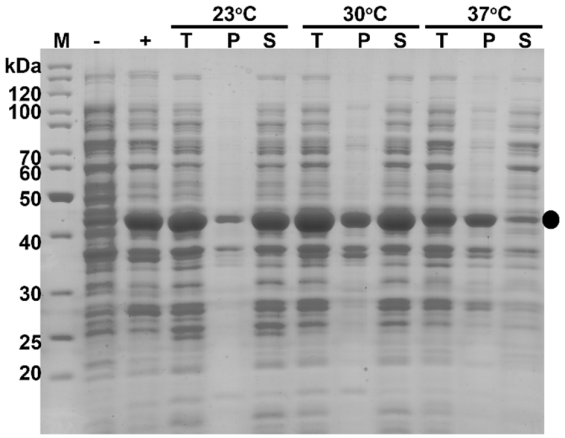

Figure 1. *SDS-PAGE analysis for the influence of temperature on the soluble expression of GST-HRV3C-His. The E. coli carrying plasmid pHT2081 were cultured at different temperatures (23°C, 30°C and 37°C). M: Marker. Black dot: showing the target fusion protein bands (GST-HRV3C-His). (-): negative sample from E. coli BL21(DE3) / pHT01. (+): positive sample from E. coli BL21(DE3) / pHT2081 at 23°C. T: total sample. P: pellet sample-insoluble fraction. S: soluble sample*

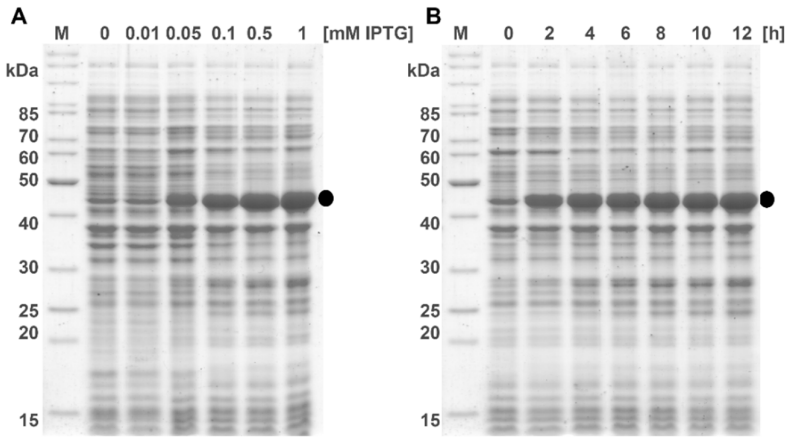

Figure 2. *SDS-PAGE analysis for the concentration of IPTG and incubation time. E. coli BL21(DE3) harboring pHT2081 was cultured at 23°C and in various conditions: IPTG concentration: 0, 0.01, 0.05, 0.1, 0.5 and 1 mM (A) or time/hours after induction (B). M: marker. Black dot, showing the target fusion protein bands (GST-HRV3C-His).*

In the third experiment, the cells harboring pHT2081 was grown at 23°C in the presence of 0.5 mM IPTG for up to 12 h. Samples were taken every two hours, and the amount of the recombinant proteins is visualized by SDS-PAGE. The percentage of the recombinant protein encoded by pHT2081 was almost identical at 12 h after induction (about 22.1 % total protein).

3.3. Purification of GST-HRV3C-His by HisTrap™ HP column

The *E. coli* BL21(DE3) strain harboring pHT2081 was cultured in two litter of the LB medium for the expression of HRV3C fusions at 37°C. When OD600 reached 0.8, added IPTG to 1 mM and reduced the temperature to 23°C and kept shaking for 12 hours. The cells were harvested for purification by using a standard protocol for the His-tag fusion (2.2.4). The fusion protein binding Histrap™ HP column was eluted by using buffers with various concentrations of Imidazole, 10, 20, 40, 60, 80, 100, 120, 160, 250, 500 mM, as shown on the Fig. 3. The protein samples were analyzed by SDS-PAGE.

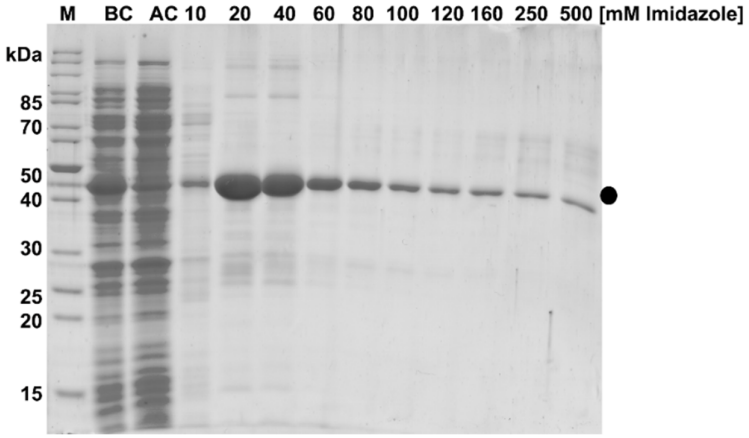

Figure 3. *Purification of GST-HRV3C-His under HisTrap™ HP column. BC, the samples before loading on the Ni-NTA column; AC, the samples collected after going through the Ni-NTA column. 10, 20, 40, 60, 80, 100, 120, 160, 250, 500 mM Imidazole: serial of concentration of Imidazole used for elution. Black dot, showing the target fusion protein bands (GST-HRV3C-His).*

The SDS-PAGE result of purifying GST-HRV3C-His (Fig. 3) demonstrates that there are extremely larger bands at 46.8 kDa (size of GST-HRV3C-His) in BC sample and all of the eluted samples. Especially at 20 mM and 40 mM Imidazole fraction, the target protease exists in highest amounts. Thus, we conclude that the target protein can bind to Ni-NTA present on the His-trap column. When the same experiment conducts with *E. coli* cells producing the GST-HRV3C-His protein, this protein can also bind to Ni-NTA and the highest amount of the His-GST-HRV3C protein could be eluted from 20 – 40 mM imidazole.

After the dialysis step of target protein with store buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0, glycerol 20%), the concentration of protein obtained is 3.08 mg/ml (with 86,6% purity)

3.4. Purification of GST-HRV3C-His by GST Bulk Kit

As same as 3.3 step, the *E. coli* BL21(DE3) cells harboring pHT2081 was cultured, harvested and sonicated to obtain the intracellular protein solution in order to purify by using a standard protocol for the GST-tag fusion (2.2.5). The fusion protein binding GST column having Glutathione was eluted by using buffers with various concentration of reduced Glutathione: 0.1, 0.2, 0.5, 1, 2, 4, 6, 8, 10 mM, as shown on the Fig. 3. The protein samples were analyzed by SDS-PAGE.

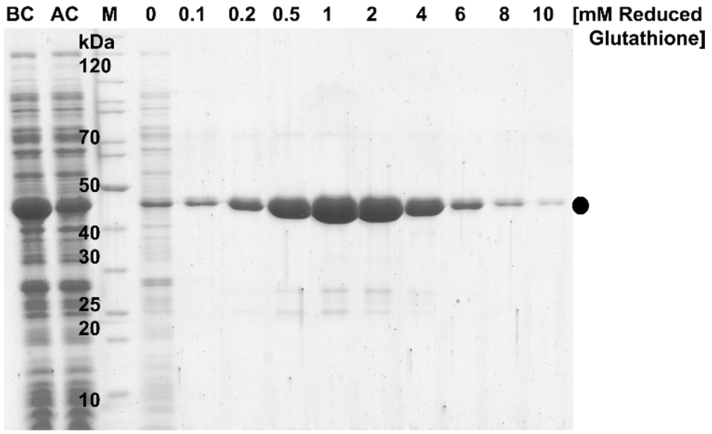

Figure 4. *Purification of GST-HRV3C-His by GST Bulk Kit. BC, the samples before loading on the GST column; AC, the samples collected after going through the GST column. 0.1, 0.2, 0.5, 1, 2, 4, 6, 8, 10 mM: serial of concentration of reduced Glutathione used for elution. Black dot, showing the target fusion protein bands (GST-HRV3C-His).*

The SDS-PAGE result of purifying GST-HRV3C-His (Fig. 4) demonstrates that there are extremely larger bands at 46.8 kDa (size of GST-HRV3C-His) in the sample before adding to the column (BC sample) and all of the eluted samples. Especially at 0.5, 1, 2, 4 mM reduced Glutathione fractions, the target protease exists in the highest amounts. Thus, we conclude that the target protein can bind to Glutathione presenting on the GST column. While the same experiment conducts with *E. coli* cells producing the GST-HRV3C-His protein, this protein can also bind to Glutathione and the highest amount of the His-GST-HRV3C protein could be eluted from 0.5 to 4 mM reduced Glutathione.

After dialysis the target protein with store buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0, glycerol 20%), the concentration of protein obtained is 3.12 mg/ml (with 96,8% purity) from the pool fraction of 0.2, 0.5, 1, 2, 4, 6 mM reduced gluthathione.

3.5. The activity of GST-HRV3C-His in cleavage of BlysSN-His-HRV3C/CS -GFP

The activity of the purified proteases was determined by carrying the cleavage reaction with the substrate BsLysSN-His-HRV3C/CS -GFP. The solutions containing 100 µg substrate were mixed for 16 h at 4°C with 10 µl of various HRV3C protease concentration, in which the ratio between protease and substrate was varied from 1/100 to 1/2000. Based on UNCUT bands on SDS-PAGE gel, AlphaEaseFC software was used to determine the minimum ratio between protease and substrate in which 95% substrate was cleaved.

In this experiment, the minimum ratio between protease and substrates is 1/450 (Fig.5A). It means that the lowest concentrations of GST-HRV3C-His corresponding to 1 Unit HRV3C protease is 0.22 µg and the specific activity of GST-HRV3C-His is greater than or equal to 4.5 U/µg (or 4500 U/mg).

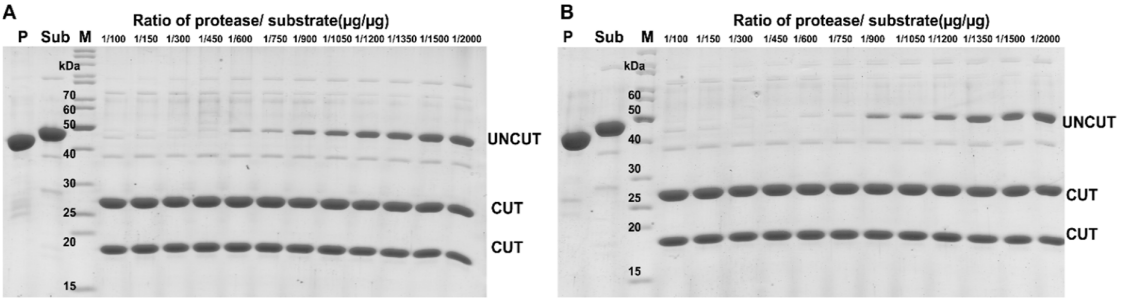

Figure 5. *Analysis of protease activities of GST-HRV3C-His and GST-HRV3C using 100 µg LysSN-6xHis-HRV3C/CS-GFP at 4°C in 16 h. Proteases (P): GST-HRV3C-His (A) and GST-HRV3C (B). Substrate (Sub): LysSN-6xHis-HRV3C/CS-GFP. M: marker. UNCUT: non-clevage substrate. CUT: products of cutting reaction from this substrate by the proteases.*

On the other hand, while GST-HRV3C (the commercial form of HRV3C) expressed in *E. coli* BL21(DE3)/pGEX4T-HRV3C system used for cleavage reaction with BsLysSN-His-HRV3C/CS -GFP, the minimum ratio between protease and substrates is 1/750 (Fig.5B). It means that the lowest concentrations of GST-HRV3C corresponding to 1 Unit HRV3C protease is 0.22 µg and the specific activity of GST-HRV3C is greater than or equal to 7.5 U/µg or (7500 U/mg). Because of the insignificant difference between the specific weights of GST-HRV3C-His and GST-HRV3C, those proteases have the approximate amounts of molecules while having the same volumes and concentrations. Therefore, we can conclude that the fusion between GST-HRV3C and C-terminal His-tag has lessened the activity of HRV3C. Nevertheless, when comparing with GST-HRV3C, GST-HRV3C-His have an advantage that it can be used to remove His-tag from other purified proteins.

3.6. Application of the HRV3C protease for the purification of GFP

The substrates BsLysSN-GFP (BsLysSN-HisTag-HRV3C/CS-GFP) protein was cleaved about more than 95% by the purified GST-HRV3C-His (Fig 6). The recombinant

GFP in the cleavage reaction was isolated from the flow-through after loading the mixtures consisting of the purification tag and the recombinant protein on the HisTrap™ HP column. The target proteins which had been cleaved by the HRV3C protease lost their His-tag so they could not bind to Ni-NTA and were obtained in the flow-through solution. All of the other proteins having the affinity with Nickel were retained in the column. Finally, GFP having 97.3% purity (Fig. 6) could be collected in the flow-through step. This results proved that the purified GST-HRV3C-His proteases can be applied to purify GFP from fusion form containing the HRV3C cleavage site.

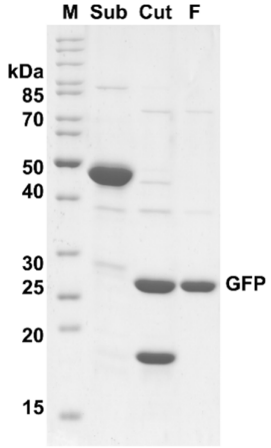

Figure 6. *Application of the enzyme for purification of GFP. The substrates (Sub) BsLysSN-6xHis-HRV3C/CS -GFP was cleaved by His-HRV3C (A, C) or His-GST-HRV3C (B, D) for 16 hours at 4°C. The mixture after cleavage (C) was loaded on Ni-NTA column. F, the flow-through after loading the mixture into the column containing the target protein GFP.*

4. Conclusions

In this studying, GST-HRV3C-His could be over-expressed in *E. coli*, reach at 22.1% of total protein. GST-HRV3C-His protease can be purified via both His-tag with 86.6% purity and GST-tag with 96.8% purity. This proves that GST-HRV3C-His has binding capacity with Ni-NTA and Glutathione. Moreover, the specific activity of GST-HRV3C-His is 4500 U/mg,

Therefore, this fusion protease can be used for removing His-tag and GST-tag of other HRVC3 fused proteins in purification process. A practical example was conducted with BsLysSN-6xHis-HRV3C/CS-GFP. After cleaving by GST-HRV3C-His protease, pure GFP was obtained with 97.3% purity. This achievement will be an essential milestone for the production and application of GST-HRV3C-His protease in purification.

5. Declarations

5.1. Ethics approval and consent to participate

Not applicable

5.2. Consent for publication

Not applicable

5.3. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The materials, the pHT vectors and bacterial strains are available via Center for Bioscience and Biotechnology or associated companies such as HTBioTec, Vietnam or MoBiTec, Germany**.**

5.4. Competing interests

The authors declare that they have no competing interests.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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TINH CHẾ VÀ HOẠT TÍNH CỦA HUMAN RHINOVIRUS 3C PROTEASE DUNG HỢP VỚI GST-tag Ở ĐẦU N VÀ His-Tag Ở ĐẦU C ĐƯỢC BIỂU HIỆN TRONG *Escherichia coli*

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TÓM TẮT

Human rhinovirus 3C protease (HRV3C) là một trong những công cụ phổ biến nhất để loại bỏ đuôi dung hợp trong quá trình tinh chế. Protease này thường được tạo ra ở dạng GST-HRV3C nhưng chưa có nghiên cứu nào về dạng dung hợp GST-HRV3C-His. Nghiên cứu này làm rõ quá trình tiến hành tinh chế GST-HRV3C-His được biểu hiện trên E. coli, kiểm tra hoạt tính và ứng dụng của nó. GST-HRV3C-His đã được thu nhận được với độ tinh sạch 86,6% (với cột tinh chế HisTrap) và 96,8% (với cột tinh chế GST). hoạt tính riêng của GST-HRV3C-His được xác định ở khoảng 4500 U/mg. protease này được ứng dụng trong quá trình tinh chế các protein khác mang trình tự nhận dạng đặc hiệu của HRV3C (LEVLFQ↓GP) dựa trên đuôi dung hợp His hoặc GST.

Từ khóa: Human rhinovirus 3C protease, GST-HRV3C-His, tinh sạch.