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Effect of Freeze-Thaw and Urea in Solubility of GPC3-Csub Protein Expressed in *Escherichia coli*

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Abstract

Glypican-3 is a protein encoded by the Glypican-3 gene located on human X chromosome (Xq26), composed of two subunits, a 40 kDa N-terminal subunit, and a 30 kDa C-terminal subunit. Glypican-3 is a currently potential target molecule for liver cancer treatments because of its over-expression and growth effects on hepatocellular carcinoma (HCC). This study examined the expression and purification of a C-terminal subunit of Glypican-3 protein (GPC3-Csub) due to its application in both diagnosis and therapy for hepatocellular carcinoma. The gene encoding for GPC3-Csub was successfully cloned into plasmid pET28a fused with an affinity tag composed of six consecutive histidine residues (His-tag). Recombinant protein GPC3-Csub was expressed in *Escherichia coli* BL21 (DE3) in the condition of adding 3% ethanol with IPTG induction. GPC3-Csub was extracted using repeated freeze-thaw cycles with lysozyme, and inclusion bodies were solubilized by 8M Urea, SDS 10% in pH 12. His-tag fused GPC3-Csub proteins allowed it to be purified by affinity chromatography method using the Nickel-nitrilotriacetic acid (Ni-NTA) column. High expression of GPC3-Csub was confirmed by Coomassie staining and western-blot. GPC3-Csub could be isolated with a Ni-NTA column and have a purity of about 90%.

Keywords

Glypican-3, Affinity Chromatography, Inclusion Body, Liver Cancer

1. Introduction

Liver cancer is one of the top 6 most common cancer deaths in the world, with about more than 900,000 new cases each year, most commonly in Africa and

Southeast Asia. About 80% of histologically diagnosed liver cancer is Hepatocellular carcinoma (HCC). Vietnam is a top 5 of the highest rates and the highest number of deaths from this disease, with an average of over 20,000 new cases of liver cancer detected and about 22,000 deaths each year.

Glypican-3 (GPC3) is a potential target molecule for liver cancer treatments since its expression significantly increases in cancer tissues. Protein GPC3 consists of two subunits: a 40 kDa N-terminal fragment (GPC3-Nsub) and a C-terminal fragment of 30 kDa (GPC3-Csub). The N-terminal subunit can be cleaved and then modified to form soluble GPC3 (sGPC3) in the bloodstream, while the C-terminal unit remains in the cell membrane. This characterization makes GPC3-Csub potential in developing specific targeting liver cancer treatment strategies such as antibody-based drugs, chimeric antigen receptor-modified cells adoptive immunotherapy, and antibody-drug conjugate. There are many studies on developing therapeutic anti-GPC3 monoclonal antibodies such as GC33, YP7, and HN3. Interestingly, the C-terminal subunit is conserved among all isoforms and present in the soluble form of GPC3, moreover, its secondary structure exhibits a more immunogenicity potential compared to the N-terminal subunit [1] [2]. This is the reason we want to produce recombinant GPC3 C-terminal subunit and utilize it as a source for screening antibodies or peptides that have high affinity to HCC.

One big challenge in producing recombinant protein, especially in prokaryotic systems is proteins are expressed in the insoluble form known as the inclusion bodies. Currently, there are methods to recover protein from the inclusion bodies by using denaturants such as chaotropic or reducing agents [3] [4]. Here we found a combination method to dissolve our inclusion protein by using repeated freeze-thaw steps and urea in high pH conditions. The His-tagged GPC3-Csub protein after purification can be used as a material for investigating GPC3 protein functions and other potential therapies targeting HCC

2. Material and Method

2.1. HepG2 Cell Culturing, RNA Extraction, and cDNA Synthesis

HepG2 cell was cultured with Dulbecco's Modified Eagle Medium, 10% FBS, and penicillin/streptomycin until the confluent reached 90%. Then, 10^6 cells were harvested and isolated whole RNA using High Pure RNA Tissue Kit (Roche) and cDNAs were synthesized by reverse transcription reaction using RevertAid First Strand cDNA Synthesis Kit (Thermo) under the manufacturer's instructions.

2.2. Construction of His-Tag GPC3-Csub

GPC3-Csub coding sequence was amplified by PCR reaction carried out by Phusion High-Fidelity DNA Polymerase (Thermo) with the cDNAs of HepG2 cell line as templates and the primer pair Forward 5' (CGCGGATCC-AGATCTGC-TTATTATCCTGAAGAT) and Reverse 5' (CCGCTCGAGGTGC-ACCAGGA). We designed the reverse primer that omitted the stop codon so

our expressed protein will have 6xHis-tag fragment that can facilitate the purification steps. The PCR products were analyzed by gel electrophoresis and the gene of interest was isolated by gel extraction using GeneJET Gel Extraction Kit (Thermo). GPC3-Csub fragment was inserted into plasmid PET28a+ by restriction enzymes XhoI and BamHI and ligated by T4 ligase. The constructed plasmid has been sequenced and analyzed with the gene database by using the BLAST tool of NCBI (http://www.ncbi.nlm.nih.gov).

2.3. Monitoring Protein Expression by Different Culture Conditions

His-tag GPC3-Csub was expressed in *Escherichia coli* BL21(DE3) strain. Approximately 100 µg of pET28a(+)-GPC3-Csub plasmid was transformed to competent *E. coli* BL21 (DE3) by heat shock at 42˚C, 2 minutes, followed by incubation at 37˚C for one hour. The colony that appeared on LB/kanamycin plate was picked up and inoculated in 10 mL of LB medium with kanamycin (±3% Ethanol, Merck) as starter culture. Different culture conditions were investigated to maximize protein production (Table 1). In general, the starter culture was added to fresh LB medium (ratio 1:50) with or without an additional 3% Ethanol, inoculated at 37˚C, 180 rpm shaking. Until OD_{600nm} reached the target value, 1 mM of IPTG (Bioline) was added to the medium to induce protein production. The induced medium then was cultured at different time and temperature conditions, 180 rpm shaking as shown in Table 1.

2.4. Protein Extraction by Using Combination Methods Including Lysozyme, Free-Thaw, and Chemical Treatment

The bacteria biomass was harvested by centrifugation and resuspended in lysis buffer II (50 mM Tris pH 8.0, 10% glycerol (Merck), 0.1% Triton X-100 (Biobasic), 5 mM MgCl2 (Merck), 1 mM NaCl (Merck) with the ratio 2 mL lysis buffer II/ 10 mL culture, then added 1 mM PMSF solution (PMSF, isopropyl alcohol) and lysozyme (1 mg lysozyme/1 mL lysis buffer II). The mixture was then incubated on ice for 1 hour before freezing by liquid nitrogen and thawing in 37˚C. After that, adding DNase (3 U/1 mL lysis buffer II) (Roche) to the solution and incubate at 37˚C for 30 minutes to digest genomic DNA. The mixture was frozen and thawed three more times to facilitate the cell wall decomposition, and then sonicated on ice at ultrasonic level 6.0, 10 × 30 seconds, followed by centrifugation at 12,000 g, 20 minutes. The collected pellet was dissolved in a buffer containing Urea, SDS 10%, pH pH12.

2.5. GPC3-Csub Purification by FPLC System

Ni/NTA affinity purification was performed on an AKTA FPLC system using 1 mL HisPurTMNi-NTA Chromatography Cartridge column (Thermo Scientific). After equilibrating with binding buffer (300 mM NaCl, 20 mM NaH_2PO_4, 10 mM Imidazole, 8M Urea), protein sample was applied to Ni-NTA column. The

Table 1. Different culture conditions were applied to maximize GPC3-Csub protein production.

Culture conditions	LB				LB + 3% Ethanol		
Starter OD_{600nm}(*)	2.8(o/n)	0.5	2.8(o/n)	0.5	1.9(o/n)	1.9(o/n)	0.5
Expansion OD_{600nm}(**)	0.4	0.8	0.4	0.8	0.4	1.0	0.4
Temp (˚C)	37	37	26	26	37	26	26
Time (h)	4	4	24	24	4	24	24

(*): culture until this OD to do expansion culture; (**): culture until this OD to do induction with IPTG 1 mM; (o/n): culture overnight.

column was then washed with binding buffer and refolded gradually with refolding buffer (300 mM NaCl, 20 mM NaH_2PO_4, 10 mM Imidazole). The protein was then eluted with elution buffer (300 mM NaCl, 20 mM NaH_2PO_4, 500 mM imidazole). The total protein after elution was combined and exchanged with refolding buffer (to reduce Imidazole concentration) using ultrafiltration membrane with a cut-off 3000 MWCO (Da).

2.6. Coomassie Brilliant Blue (CBB) Staining

Protein was subjected to SDS-PAGE. The gel then was incubated with staining solution (0.6 mM Coomassie brilliant blue (CBB G-250, Merck) in 50% methanol (Merck), 10% acetic acid (Merck)) for 1 hour. After that, the gel was de-stained with a solution containing 25% methanol and 75% acetic acid.

2.7. Silver Staining

Protein was subjected to SDS-PAGE. The gel then was fixed with a fixer solution (50% Methanol (Merck), 12% acetic acid (Merck), 0.05% formalin (Merck)) for 2 hours, washed three times with 35% Ethanol (Merck) for 30 minutes. The gel was sensitized with 0.02% $Na_2S_2O_3$ (Biobasic) and washed with distilled water three times for 5 minutes. Then, the gel was stained with silver nitrate solution (0.2% $AgNO_3$ (Merck), 0.076% Formalin), washed two times with distilled water for 1 minute before developing in developing buffer (6% Na_2CO_3 (Merck), 0.05% Formalin, 0.0004% $Na_2S_2O_3$) until protein bands were visible and stop the reaction by 50% Methanol, 12% acetic acid for 5 minutes. ImageJ software was used to estimate the protein concentration before and after purification.

2.8. Western Blotting

Protein was subjected to SDS-PAGE and then electro-transferred to Nitrocellulose membrane (Thermo). Membrane was blocked with skim milk for 30 minutes; wash 3 times by TBST before adding primary antibody His-tag (Invitrogen, 1:5000). Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG (Invitrogen, 1:5000), and ImageQuant™ LAS 500 System (GE Healthcare) was used for the detection of antibody reaction.

3. Result and Discussion

3.1. GPC3-Csub Construction

Figure 1A shows the gel electrophoresis results of the PCR products that amplified the GPC3-Csub fragment from cDNA of HepG2 cells. We observed a clear band between 600 bp and 700 bp, which is expected to be the size 669 bp of the GPC3-Csub fragment. We then isolated this PCR product fragment and cloned it into the PET28a(+) vector by restriction enzyme digestion with BamHI and XhoI and ligation with T4 ligase. The PCR screening results of colonies show four clones that had positive results with the GPC3-Csub gene among six tested colonies (Figure 1B). We then picked randomly the clone at line 4 for growing to isolate the constructed plasmid. The size of the insert was confirmed by restriction enzymes BamHI and XhoI (Figure 1C), and then the plasmid was sequenced and aligned with the NCBI Genebank database by BLAST tool. Figure 2 shows that our insert gene is 100% identical to the human GPC3-C sequence (GenBank Accession No: AK222766). Thus, we have successfully constructed the expression plasmid PET28a(+)-GPC3-Csub.

3.2. GPC3-Csub Expression

After successfully constructing recombinant plasmid to produce the C-subunit of GPC3, this protein was induced by IPTG and the protein expression was confirmed by Western Blot (Figure 2B, Figure 2D). To maximize protein production, different culture conditions were applied. There are several factors that need to be considered to achieve the highest protein products such as cell density (OD_{600nm} of medium in the starter culture and expansion culture), temperature, culture time, shaking rate… In this experiment setting, we first investigated how cell density, temperature, and time affect the protein expression. The results showed that GPC3-Csub protein was in inclusion bodies and when induction by IPTG at 37˚C, 4 hours, this protein expression was much higher than at 26˚C, 24 hours (Figure 2A, Figure 2B). Especially, at 37˚C, 4 hours culture condition, even incubation overnight when starter OD_{600nm} reached to death phase, protein expression level was not affected as compared to the differences of expansion OD_{600nm}. When expansion OD_{600nm} reached 0.8 (final-log phase to death phase), the targeted protein band was significantly lower as compared to OD_{600nm} 0.4 (mid-log phase). In general, the GPC3-Csub protein can be achieved the most when cell density reaches OD_{600nm} 0.4 before induction with 1 mM IPTG for 37˚C, 4 hours.

Recently, with the development of biofuel products, there are some researchers have investigated and concluded that the using of ethanol can increase the bioproducts from *E. coli* [5]. The presence of ethanol can increase DNA synthesis and plasmid number, which facilitates the increase of protein expression with established investigation in laboratory by Chhetri and colleagues [6]. Therefore, we tested the effect of ethanol on protein expression with 3% ethanol added to LB culture medium from the beginning of the culture and induction process.

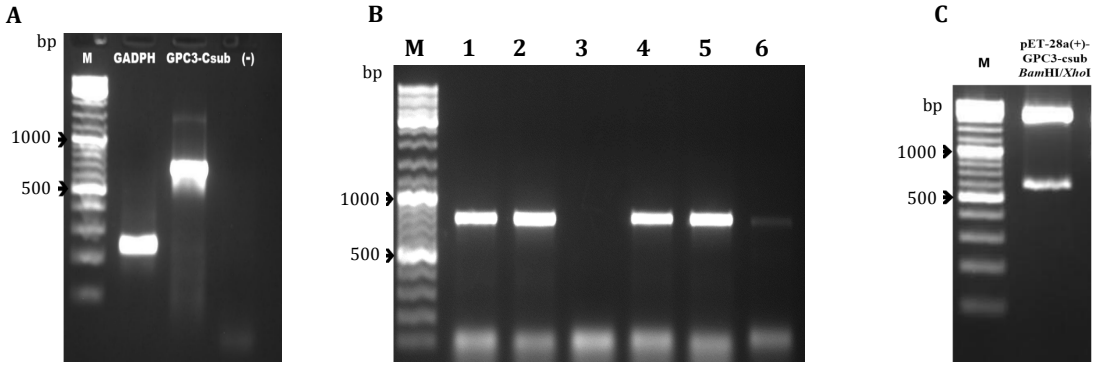

Figure 1. Construction of PET28a(+)-GPC3-Csub vector. (A) PCR amplification of GPC3-Csub from HepG2 cDNA, (B) PCR colonies amplification screening for positive colonies, (C) Digestion of constructed plasmid by restriction enzyme BamHI and XhoI.

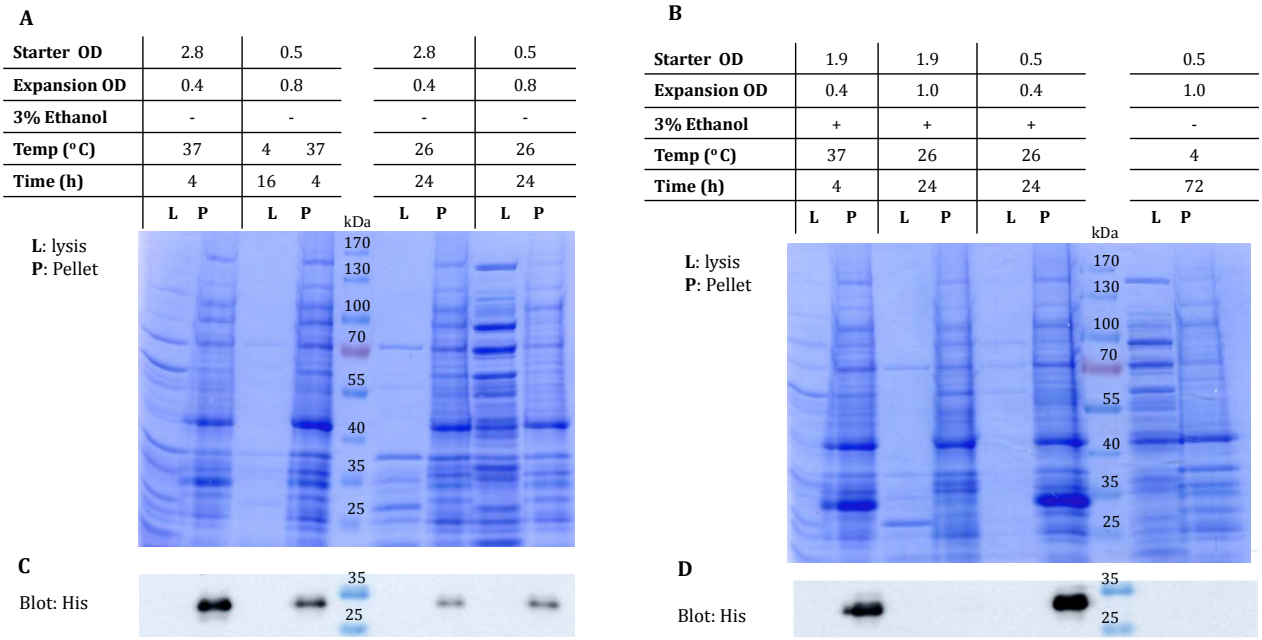

Figure 2. Protein expression at different culture conditions.

With the addition of ethanol, after induction with 1 mM IPTG, culturing medium at 26˚C, 24 hours can increase GPC3-Csub protein expression as compared to 37˚C, 4 hours culture condition (Figure 2C, Figure 2D). We still also observed that the high cell density after expansion culture (OD_{600nm} 1.0) significantly reduced GPC3-Csub protein expression. Especially, when considering other protein bands in the inclusion proteins, the expression of GPC3-Csub was strongest in the total protein expression as compared to other culture conditions. Therefore, we chose expansion OD_{600nm} 0.4, 26˚C, 24 hours as our final culture conditions for further experiments.

3.3. GPC3-Csub Solubilization

After confirming culture conditions which led to achieve the most amount of

GPC3-Csub inclusion body (IB), the solubilization of this IB was examined in different conditions. Firstly, as a well-known reagent for solubilization of inclusion protein, we used 8 M urea to dissolve protein after sonication. Urea 8 M is known as a traditional chaotropic agent to solubilize inclusion proteins by disrupting the hydrogen bonds in the networking of water molecules, reducing hydrophobic effect which maintains macromolecular structure as protein [7]. However, in our study, the inclusion bodies may be formed in more complex structures, which made the protein could not dissolve completely by only using 8 M urea. Therefore, GPC3-Csub together with other proteins were still in the pellet (**Figure 3**). Therefore, we tried using freeze-thawing method in combination with the addition of lysozyme to disrupt cell membrane proteins to expose internal proteins to lysis buffer. After the first free-thaw cycle, DNase was treated to degrade internal genome which also indicated that the cell membrane was broken down enough for releasing the composition inside their cytosol as compared to the old lysis buffer with sonication method (**Figure 3**). To check whether the sonication method after free-thawing process can help in separating GPC3-Csub to lysis buffer, we did sonication with the same level and time as previous method, and the protein after this treatment was even partially degraded without improving the solubilization of the IB.

To examine the role of denaturant in precipitate dissolution, urea was added with concentrations of 4 M, 6 M, and 8 M, respectively to the refolding buffer. The results in **Figure 4A** show that, under the condition of refolding buffer without the addition of urea, protein is almost present in pellet with a very small part in supernatant. As the concentration of urea gradually increased, the amount of protein dissolved into the soluble phase also increased. It can be seen through the density of the 30 kDa target protein band and the protein size between 40 - 50 kDa. This may be because the high concentration of urea makes it easier to denature proteins. However, the precipitated phase was still largely protein present, which was visible in the 30 kDa target protein, so it was necessary to examine whether increasing the pH value would lead to better conversion of the protein to the soluble phase. Besides, the results of **Figure 4B** showed that the protein was dissolved in urea-supplemented refolding buffer in pH 12 more than pH 7.4, but still had much amount of protein in pellet. It indicated that the high pH may help in increasing the negative charge of the inclusive proteins so that the urea 8 M can break the hydrogen bonds among these partially folded aggregates [8]; We observed that the concentration of urea supplemented was proportional to the amount of protein dissolved into the soluble phase (can be seen in the protein line between 40 and 50 kDa). Moreover, we demonstrated that the addition of SDS detergent at a concentration of 10% to the refolding urea buffer 8 M, pH 12 helped to dissolve the protein better than no addition. This can be seen clearly that the amount of protein GPC-Csub in the soluble phase when treated with SDS is much higher than that without SDS and similarly, the protein in the pellet phase is less when SDS is added.

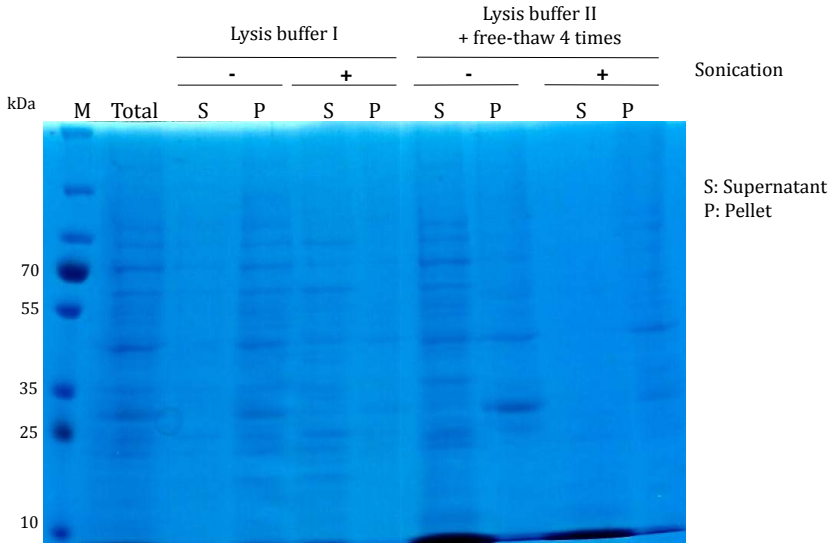

Figure 3. SDS-PAGE of four groups of protein extraction: lysis buffer I—treated with and without sonication groups, lysis buffer II in combination with freeze-thaw four times—treated with and without sonication groups. SDS-PAGE result was visualized by CBB staining.

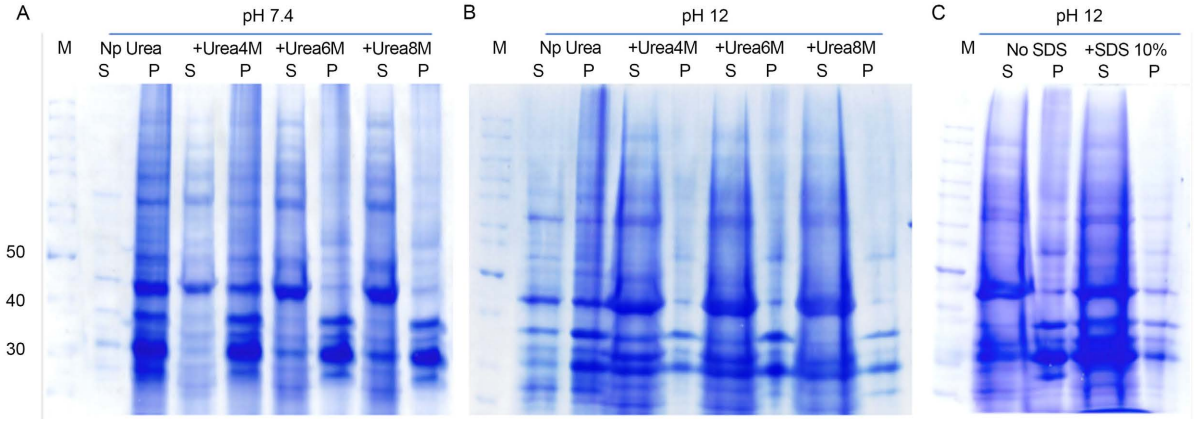

Figure 4. GPC3-Csub protein solubilization at different conditions. All samples were prepared after protein extraction process by lysis buffer II combined with freeze-thaw 4 times. SDS-PAGE result was visualized by CBB staining.

3.4. GPC3-Csub Purification

After identifying optimum conditions for expressing and solubilizing, protein GPC3-Csub was subsequently purified using affinity chromatography method. The pH of medium then was immediately neutralized as well as SDS concentration was reduced to 2% by diluting 5 times the supernatant with binding buffer (8 M urea in PBS with pH 7.4) to facilitate the binding of targeted protein on Ni-NTA column. GPC3-Csub protein was purified using 1 mL HisPurTM-Ni-NTA Chromatography Cartridge column managed by FPLC system (**Figure 5A**). The protein after purification was checked by Silver Staining (**Figure 5B**, **Figure 5C**) with expected GPC3-Csub protein was eluted starting from approximately 250 mM Imidazole until 500 mM Imidazole. The GPC3-Csub protein after buffer exchange was then confirmed by Western Blotting (**Figure 5D**).

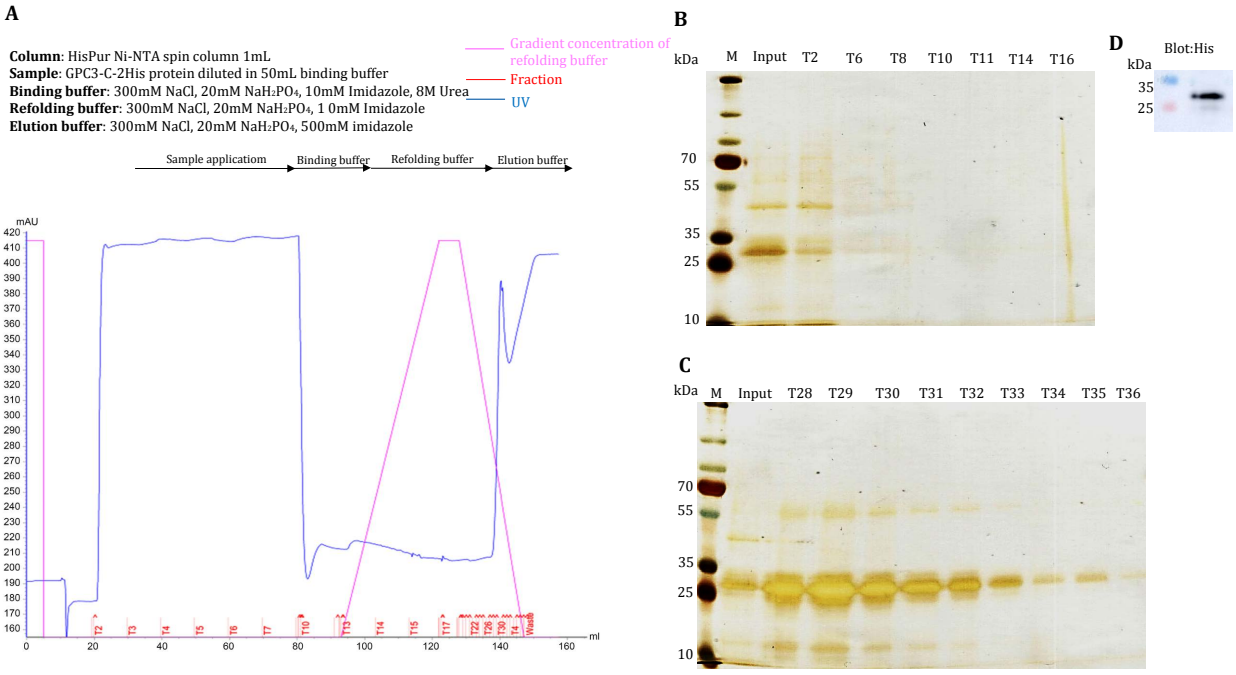

Figure 5. GPC3-Csub protein purification. (A) Histogram of GPC3-Csub purification by FPLC system; (B), (C) Silver staining. Ip: protein input; T2-T6: fraction in sample application step; T8: fraction in wash step with 10 mM Imidazole, 8M Urea; T10, T11, T14: fraction in refolding step with 10 mM Imidazole; T28-T36: fraction in elution step with 500 mM Imidazole; D: WB from the sample of a combination of elution fractions blotted with Histag-antibody.

4. Conclusion

In this study, we successfully established the protocol for GPC3-Csub protein expression and purification using affinity chromatography method. Recombinant protein GPC3-Csub was expressed in *E.coli* BL21 in the condition of adding 3% ethanol with IPTG induction. GPC3-Csub was extracted using repeated freeze-thaw cycles with lysozyme. High expression of GPC3-Csub was confirmed by Coomassie staining and western-blot, and inclusion bodies were almost solubilized by dissolving in refolding buffer supplemented with 8 M Urea, 10% SDS in pH 12. GPC3-Csub could be isolated with a Ni-NTA column and have a purity of about 90%.

The study succeeded in GPC3-Csub expression, however, optimized purify procedures need to be further examined to obtain a better purity GPC3-Csub. By using combination of 8 M Urea and 10% SDS and pH 12 to dissolve inclusion protein, the structure and function of protein were also affected. The process of refolding of denatured protein gradually on HisPurTM-Ni-NTA Chromatography Cartridge column can be solved by lowering the concentration of SDS and Urea in subsequent steps.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Phung, Y., Gao, W., Man, Y.G., Nagata, S. and Ho, M. (2012) High-Affinity Monoclonal Antibodies to Cell Surface Tumor Antigen Glypican-3 Generated through a Combination of Peptide Immunization and Flow Cytometry Screening. *Monoclonal Antibody*, **4**, 592-599. https://doi.org/10.4161/mabs.20933

[2] Filmus, J. and Capurro, M. (2013) Glypican-3: A Marker and a Therapeutic Target in Hepatocellular Carcinoma. *Federation of European Biochemical Societies Journal*, **280**, 2471-2476. https://doi.org/10.1111/febs.12126

[3] Marston, F.A.O. and Hartley, D.L. (1990) Solubilization of Protein Aggregates. *Methods in Enzymology*, **182**, 264-276. https://doi.org/10.1016/0076-6879(90)82022-T

[4] Singh, A., Upadhyay, V., Upadhyay, A.K., Singh, S.M. and Panda, A.K. (2015) Protein Recovery from Inclusion Bodies of *Escherichia coli* Using Mild Solubilization Process. *Microbial Cell Factories*, **14**, Article No. 41. https://doi.org/10.1186/s12934-015-0222-8

[5] Horinouchi, T., Tamaoka, K., Furusawa, C., Ono, N., Suzuki, S., Hirasawa, T., Yomo, T. and Shimizu, H. (2010) Transcriptome Analysis of Parallel-Evolved *Escherichia coli* Strains under Ethanol Stress. *BMC Genomics*, **11**, Article No. 579. https://doi.org/10.1186/1471-2164-11-579

[6] Chhetri, G., Kalita, P. and Tripathi, T. (2015) An Efficient Protocol to Enhance Recombinant Protein Expression Using Ethanol in *Escherichia coli*. *MethodsX*, **8**, 385-391. https://doi.org/10.1016/j.mex.2015.09.005

[7] Bennion, B.J. and Daggett, V. (2003) The Molecular Basis for the Chemical Denaturation of Proteins by Urea. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 5142-5147. https://doi.org/10.1073/pnas.0930122100

[8] Singh, S.M., Upadhyay, A.K. and Panda, A.K. (2008) Solubilization at High pH Results in Improved Recovery of Proteins from Inclusion Bodies of *E. coli*. *Journal of Chemical Technology and Biotechnology*, **83**, 1126-1134. https://doi.org/10.1002/jctb.1945