Efficient Protocol for Expression and Purification of DUSP5

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ABSTRACT

Dual Specificity Phosphatase 5 (DUSP5) is a human protein that targets specific kinases and dephosphorylates phosphoserine/threonine and phosphotyrosine residues. DUSP5 is found to be involved in cardiovascular diseases and many cancer pathways, including skin and breast cancer. For this reason, availability of an efficient protocol of expression and purification of DUSP proteins can play a crucial role in their studies towards better understanding of the disease process and development of better therapeutic approaches. For example, purification of DUSP5 could be used for the in vitro assays of the inhibitors of DUSP5 identified from the in-silico studies. This report provides the full procedure for protein purification thereby allowing the collection of desired amounts of DUSP5 using Glutathione S-transferase (GST) tag. The described method shows an efficient way to solubilize and purify DUSP5 for further protein studies.

KEYWORDS: DUSP5, expression, purification, and phosphatase.

INTRODUCTION

Protein expression is a process in which DNA is transcribed into mRNA and then translated into a protein (Overview of Protein Expression Systems | Thermo Fisher Scientific - US, 2019). This process occurs in both prokaryotic and eukaryotic cells and is a useful tool for studying individual proteins in living organisms. Protein expression through a bacterial system such as *Escherichia coli* (Min et al., 1994), takes advantage of the bacteria's short replication time of approximately 20 minutes and allows for a rapid expression of the protein of interest. The expressed protein can then be extracted from *E. coli* cells via the protein purification. The *E. coli* protein expression system is a common tool to express proteins from other organisms including human (Kucharsca et al., 2009).

Dual Specificity Phosphatase 5 (DUSP5) is a protein involved in many disease processes such as cancer (Rushworth et al., 2014; Liu et al., 2018) and cardiovascular diseases (Alleboina et al., 2019). Specifically, DUSP5 targets extracellular regulated kinases (ERKs) and dephosphorylates their phosphoserine/threonine and phosphotyrosine residues (NCBI, 1994). DUSP5 has two domains: an ERK-binding domain and a catalytic phosphatase domain (Kucharsca et al., 2009). Due to DUSP5's involvement in many disease processes, it is currently under investigation as a target for developing new drugs and repurposing exist-

ing FDA-approved drugs (Bongard et al., 2019; Bongard et al., 2017; Neumann et al., 2015). Development of the DUSP5 inhibitors is challenging due to a variety of issues including the necessity of optimization of the ADMET properties, efficient membrane transport, and, most importantly, selectivity issues related to the high similarity of the catalytic sites of phosphatases. Due to these challenges, a large scope of potential inhibitor candidates needs to be tested on DUSP5, which in turn necessitates a robust protocol for DUSP5 expression and purification in large quantities. Herein, we provide the full details of such protocol, which represents an optimized version of the protocol published by the Ramchandran group (Rushworth et al., 2014).

MATERIALS

- *Lysis buffer* – 50 mM tris-HCl buffer (pH=8.0), 1% triton X-100 detergent, 0.5 M NaCl, 10 mM EDTA, 10 mM EGTA, 10% (w/w) glycerol, 2 mM PMSF, 5 mM DTT, 0.4 ug/ml antipain, and 0.2 μg/ml leupeptin.
- *Tris Sucrose buffer* – 30 g of sucrose per liter of tris-HCl buffer (pH = 8.0).
- *Washing buffer* – 50 mM tris-HCl, 50 mM NaCl, and 5% (w/w) glycerol.
- *Elusion buffer* – 20 mM glutathione (oxidized), 1 mM DTT, and remainder washing buffer.
- *1X PBS* – 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 all at a pH of 7.3.
- *Lysozyme for tris-sucrose* – lysozyme concentration of 2.5 mg/ml.
- *Terrific broth (TB)* – yeast extract, tryptone, glycerol, and phosphate buffer (media was sterilized via autoclave).
- *Cracking buffer (CB)* – 250 mM tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, ddH_2O, and 0.1% Bromophenol Blue.
- *Antibiotics* – chloramphenicol and carbenicillin.
- *Precision Plus Protein standards* (purchased from Bio-Rad).

All reagents were purchased from Sigma-Aldrich, Inc. All buffers were filtered or sterilized prior to proceeding.

RESULTS & DISCUSSION

A cloned full length wild type Dual Specificity Phosphatase 5 (DUSP5) cDNA was used for transformation, as described by Ramchandran et al 2014 (Rushworth et al., 2014). DUSP5 was previously tagged with a glutathione S-transferase (GST) for simpler handling and purification. This was done through the PGEX-6P1 GST vector (Plasmid DNA Isolation, 2021), provided by the Ramchandran's lab. GST-DUSP5 was then transformed in Rosetta 2 Cells (Rosetta™ 2 Competent Cells, 2006) using a standard Novagen kit (Plasmid DNA Isolation, 2021) to prepare the cells. Once prepared, cells were plated and allowed to incubate over-

night at 37°C. The plates were prepared using a terrific broth (TB) solid media. After colonies formed, a single cell colony was used to incubate overnight in a mixture of liquid terrific broth (TB) and the appropriate antibiotics, that is carbenicillin (Crb) and chloramphenicol (Cam). A final concentration of 100 μg/ml Crb and 20 μg/ml Cam was added to 2 ml of TB. This mixture was incubated once again over night at 37°C and 200 revolutions per minute, to allow for cell growth. Once finished, 1 ml aliquots were frozen in liquid nitrogen with 80 μl of dimethyl sulfoxide (DMSO) to prevent crystallization and allow the cells to be stored for a longer period in -80°C freezer. These samples were used as the freezer stock for the rest of the experiment.

Solubility evaluation consisted of a three-day protocol. On day 1, 100 mg/ml Crb and 20 mg/ml Cam (final concentration) was added to 2 ml of TB and then inoculated over night with the freezer stock obtained from previous exercise. This was done at 37°C and 200 rpm, not to exceed a 16-hour period. On day 2, 25 ml of the TB broth were prepared, with appropriate antibiotics, adding 0.15 ml of overnight culture to the mix. This mixture was again agitated at 200 rpm and 37°C until the optical density (OD) 600 reached the value of OD_{600} = 0.5-1. A small portion of the cell culture (i.e., $0.3/OD_{600}$ ml) was saved for uninduced control to be used in SDS-PAGE gel electrophoresis. The remainder of the mixture was induced with a final concentration of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continued to shake at 37°C for three hours. A portion of the mixture ($0.3/OD_{600}$ ml) was saved for the induced control of overproduction via SDS-PAGE gel electrophoresis. The final mixture was then spun down and the pellet was saved for the third day's procedure (the supernatant was discarded). Both controls, induced and uninduced, were also spun down and the pellet reserved with the supernatant being discarded as well. At this point the cracking buffer (CB) was added to each of the controls to crack open the pelleted cells, which were then frozen to better stain the cells. On day 3, the frozen pellet was resuspended using 250 mM cold tris-sucrose buffer, 7.5 mM EDTA, 1mg/ml lysozyme, and 100 mM NaCl making sure the mixture was well dissolved. Next, the mixture was incubated at 4°C for 2 hours on a tilting table followed by 5 minutes in a 37°C-hot bath. Once well dissolved, it was centrifuged at 4°C and 13,000 rpm for 15 minutes followed by separating the supernatant from the pellet. About 75 ml of CB was added to the pellet and 10 ml of CB was added to 15 ml of supernatant. The samples were then heated to 95°C for 10 minutes along with the uninduced and induced control cell samples. About 5 ml of each sample was run through SDS-PAGE gel electrophoresis to check the solubility of DUSP 5. DUSP 5 protein is approximately 42 kDa in size and the GST-tag is approximately 30 kDa resulting in a 72 kDa total size when running the sample through an SDS-PAGE. The supernatant contains soluble protein whereas the pellet contains insoluble protein.

Using the product from a large-scale solubility test, the pellet was resuspended in a 20% by mass (of the pellet used) tris-sucrose buffer. Once the pellet had been resuspended, the lysis buffer with lysozyme was added to the mixture. Next, the solution was incubated for one hour at 4°C while being stirred to ensure that all solids were dissolved in mixture including the sides of the container. The solution was then sonicated using Branson Digital Sonifier 250. The sonication settings were set for *E. coli*: sonication was performed in four 12-second intervals at 60% amplitude with one-minute interval breaks in between so the solution is kept consistently cold. Once finished, the solution was run through the centrifuge at 8000 rpm for another hour at 4°C followed by separation of the supernatant from pellet. Another SDS-PAGE

gel was run to ensure consistency.

At this point the protein was fully resuspended in the supernatant and was ready to be purified using the gravity column chromatography. About 5 ml of Glutathione Sepharose 4B Beads (Glutathione Sepharose® 4B, 2021) were added to a hand gravity column for separation of product in the supernatant. Once added, the column was calibrated with the lysis buffer to ensure the beads didn't dry and destroy the protein. After the calibration, the supernatant containing DUSP5 protein was run through the column, followed by multiple washes of lysis buffer, and washing buffer described in the Materials section. Next, the column was eluted using two column volumes of the elution buffer. Used eluent was collected in 1 ml aliquots to check for protein presence/concentration in SDS-PAGE gel electrophoresis. Once all protein was eluted from the column, the beads were washed with 1X PBS buffer until all impurities were removed and the solution came out clear. The beads (GE Healthcare, 2008) were saved and stored at -20°C in 70% ethanol to prevent them from drying. The centric cones were then used to concentrate DUSP5 and minimize the impurities. To further concentrate the desired protein, filter centrifuge tubes with 10 kDA sized pores were used. This was done by filtering small aliquots of solution at ten minutes, followed by centrifugation at 13000 rpm. Once the entire solution was filtered, an SDS-PAGE gel was run to ensure DUSP5 was available in sufficiently high purity. The solution was then frozen in -80°C cooler for future use and study.

The SDS-PAGE gel electrophoresis of the GST-tagged DUSP5 protein (72 kDa, see Figure 1) (DUSP5, 1994) showed the protein band in the expected molecular weight range, see Figure 2. DUSP5 was predominantly found in the supernatant, which indicates that it is a soluble protein. The observed impurities (Figure 2) were removed using chromatography, as will be described below. The protein mixture was sent out for sequencing (DNA Sequencing Services | ACGT, 2015), which revealed a positive match for DUSP5 human protein as shown in Figure 5. The standard BLAST sequence comparison algorithm (Cloud-Based Informatics Platform for Life Sciences R&D | Benchling, 2021) showed perfect match of the obtained primary sequence of amino acids with the primary sequence of DUSP5 known from the literature (DUSP5, 1994), see Figure 3.

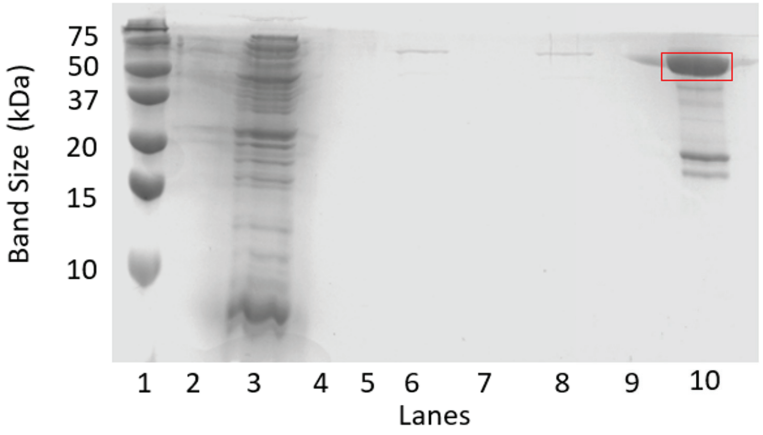

Figure 1. The SDS-gel electrophoresis of the concentrated DUSP5 solution. The lanes are marked as follows: **lane 1** marker, **lane 2** uninduced cells control, **lane 3** induced cells control, **lane 4** wash run-off, **lanes 5-7** three 30 kDa filter run-offs, **lanes 8-10** 10 kDa filter run-offs. Run-offs were collected in 1.5 ml aliquots to view placement of protein through electrophoresis.

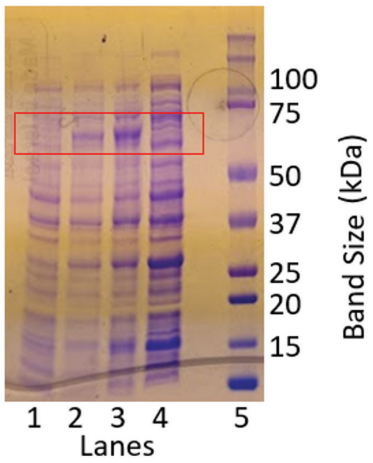

Figure 2. Initial SDS electrophoresis gel showing the solubility of DUSP5. The lanes are marked as follows: **lane 1** uninduced cells control, **lane 2** induced cells control, **lane 3** pellet, **lane 4** supernatant, and **lane 5** is the marker lane.

Figure 3. Comparison of the primary sequence of the expressed protein and DUSP5, showing the exact match. Only a part of the sequence comparison is shown for the better visibility.

Column chromatography showed positive results with the use of the Glutathione Sepharose 4B beads binding to the Glutathione S-transferase (GST) tagged DUSP5. After running gel electrophoresis on the elutions of DUSP5 protein obtained from chromatography, visuals showed the removal of impurities from the previous product. Figure 4 shows that the bands present were all in the allotted range of 50 to 75 kDa, with no other bands being present. With the use of a 10 kDa microcentrifuge filter, the protein was further concentrated to a usable concentration over 100 ng/uL. Subsequent gel electrophoresis showed, through band size alone, that the protein was significantly concentrated (Figure 1). The subsequent sample quantification using NanoDrop spectrophotometer showed that the concentration of DUSP5 in the final sample increased to 567.2 ng/ul. Further tests were performed using mass spectrometry (Mass Spectrometry Service - Applied Biomics, 2021), which gave a protein match score of 97 and total ion score of 75 (Figure 5). This was the highest match of any human

protein present in the data base leading to the conclusion that DUSP5 was successfully expressed and purified.

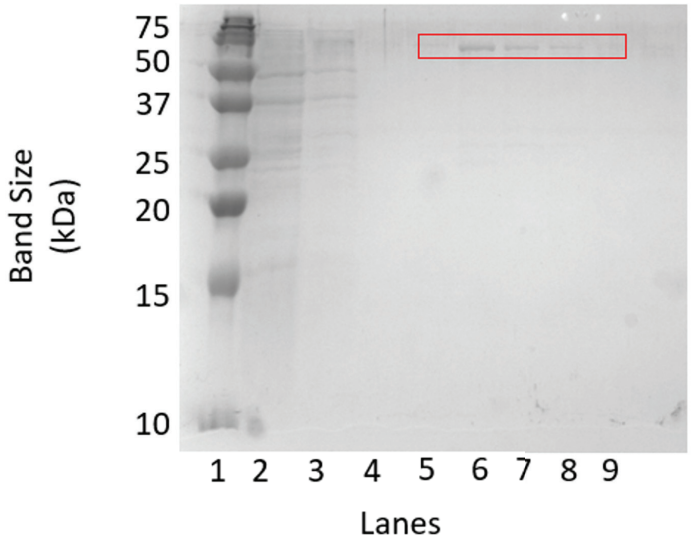

Figure 4. This gel shows the results of the column chromatography. **Lane 1** marker, **lane 2** un-induced, **lane 3** = induced, **lanes 4-9** first through sixth elutions. Elutions were collected in 1.5 mL aliquots to better view when protein was eluted, shown in the gel above through electrophoresis.

Spot number	MALDI well number	Match Quality	Top Ranked Protein Name [Species]	Accession No.	Protein MW	Protein PI	Pep.Count	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%
1	D1		Dual specificity protein phosphatase 5 OS=Homo sapiens OX=9606 GN=DUSP5 PE=1 SV=2	DUS5_HUMAN	42,020	8.7	7	97	100	75	100
2	D2		Histone deacetylase 7 OS=Homo sapiens OX=9606 GN=HDAC7 PE=1 SV=2	HDAC7_HUMAN	102,863	7.2	10	57	96	37	97
Control											
1 fmol	F6		Beta-galactosidase OS=Escherichia coli O9:H4 (strain HS) OX=331112 GN=lacZ PE=3 SV=1	BGAL_ECOHS	116,388	5.2	18	182	100	115	100
1 fmol	F7		Beta-galactosidase OS=Escherichia coli O9:H4 (strain HS) OX=331112 GN=lacZ PE=3 SV=1	BGAL_ECOHS	116,388	5.2	14	176	100	135	100
1 fmol	F8		Beta-galactosidase OS=Escherichia coli O9:H4 (strain HS) OX=331112 GN=lacZ PE=3 SV=1	BGAL_ECOHS	116,388	5.2	17	239	100	178	100
1 fmol	F9		Beta-galactosidase OS=Escherichia coli (strain K12) OX=83333 GN=lacZ PE=1 SV=2	BGAL_ECOLI	116,409	5.3	16	175	100	121	100
high confidence											
low confidence											
no confidence											

Figure 5. Mass Spectrometry results showing positive match for DUSP5 (Mass Spectrometry Service - Applied Biomics, 2021).

CONCLUSION

The dual specificity phosphatase 5 (DUSP5) protein attracts significant attention due to its connection to cancer and cardiovascular diseases. Therefore, availability of an efficient

protocol for expression and purification of this protein is imperative for the biochemical and drug development studies of this protein. In this research contribution, we present a detailed procedure of the GST-tagged DUSP5 expression/purification in large quantities necessary for the subsequent studies. The identity and purity of the expressed DUSP5 protein was validated by the primary sequence analysis, chromatography, and mass spectrometry.

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