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USP5$^{1-835}$ Expression and Purification & Development of a Mass Spectrometry USP5 Catalytic Activity Assay

Objective: expression and purification of full length USP5$^{1-835}$ for optimization of a mass spectrometry (MS) based assay to detect polyubiquitin cleavage (MS assay developed and optimized by Dr. Suzanne Ackloo)

Method & Results:

A. Growth

6 x 1 L TB media (Sigma Alrdrich) supplemented with 50 µM Kanamycin and 34 µM chloramphenicol used to grow clone SDC075-B03 (Table 1) in BL21 Codon Plus RIL cells at 37˚C, induced at OD_{600} ~0.75 with 0.5 mM IPTG. Cultures grown overnight at 15˚C.

Table 1. USP5$^{1-835}$ Clone Summary

SGC Construct ID	**Vector**	**Cloned AA Sequence**	**N-terminal Tag sequence**	**MW (+tag)**	**pI**
SDC075-B03	pET28-LIC	MAELSEEALLSVLPTIRVPKAGDRVHKDECAF SFDTPESEGGLYICMNTFLGFGKQYVERHFN KTGQRVYLHLRRTRRPKEEDPATGTGDPPRK KPTRLAIGVEGGFDLSEEKFELDEDVKIVILPD YLEIARDGLGGLPDIVRDRVTSAVEALLSADS ASRKQEVQAWDGEVRQVSKHAFSLKQLDN PARIPPCGWKCSKCDMRENLWLNLTDGSILC GRRYFDGSGGNNHAVEHYRETGYPLAVKLG TITPDGADVYSYDEDDMVLDPSLAEHLSHFG IDMLKMQKTDKTMTELEIDMNQRIGEWELI QESGVPLKPLFGPGYTGIRNLGNSCYLNSVV QVLFSIPDFQRKYVDKLEKIFQNAPTDPTQDF STQVAKLGHGLLSGEYSKPVPESGDGERVPE QKEVQDGIAPRMFKALIGKGHPEFSTNRQQ DAQEFFLHLINMVERNCRSSENPNEVFRFLV EEKIKCLATEKVKYTQRVDYIMQLPVPMDAA LNKEELLEYEEKKRQAEEEKMALPELVRAQVP FSSCLEAYGAPEQVDDFWSTALQAKSVAVKT TRFASFPDYLVIQIKKFTFGLDWVPKKLDVSIE MPEELDISQLRGTGLQPGEEELPDIAPPLVTP DEPKAPMLDESVIIQLVEMGFPMDACRKAV YYTGNSGAEAAMNWVMSHMDDPDFANPL ILPGSSGPGSTSAAADPPPEDCVTTIVSMGFS RDQALKALRATNNSLERAVDWIFSHIDDLDA EAAMDISEGRSAADSISESVPVGPKVRDGPG	MGSSHH HHHHSSG LVPRGS	95.3 kDa	5.1

		KYQLFAFISHMGTSTMCGHYVCHIKKEGRW VIYNDQKVCASEKPPKDLGYIYFYQRVAS			

B. Purification

Cells were resupsended in 400 mL resupension buffer (50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP, 5% glycerol (v/v) and supplemented with benzonase and 1xprotease inhibitor (PMSF) and lysed by sonication (5 sec intervals, 7 sec pause, power: 8.5). Clarified lysate (supernatant-SN) rocked with 5 mL equilibrated Talon resin for 1 hour at 4˚C before being poured through a open column (flow through-FT). Beads washed with 100 mL resuspension buffer (wash 1-W1) then 200 mL resuspension buffer + 5 mM imidazole (wash 2-W2) before elution with 30 mL resuspension buffer + 300 mM imidazole (Elution-E). Elution concentrated to 5 mL and loaded on gel filtration column Superdex 200 1660 (**Figure 1**).

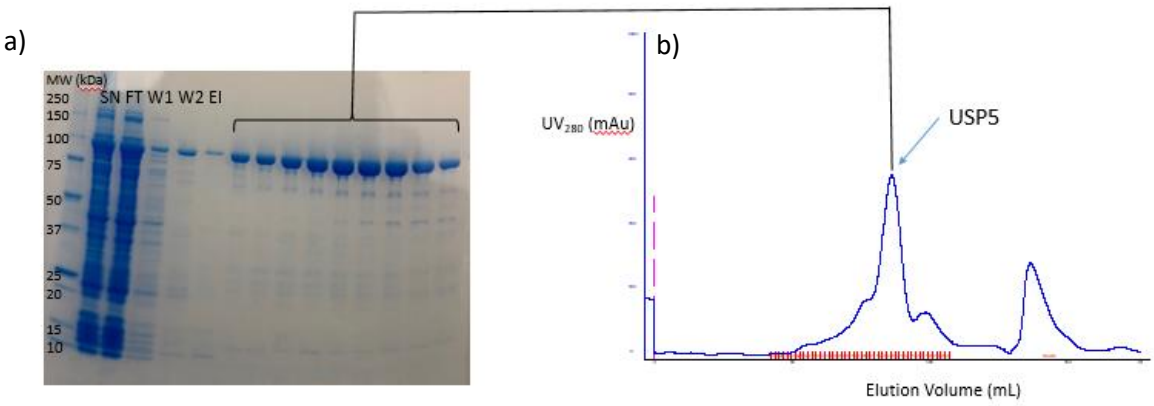

Figure 1. a) SDS-PAGE b) S200 1660 chromatogram

Column fractions were concentrated to 4.9 mg/mL and aliquoted (127x5 µL), frozen in liquid nitrogen and stored at -80˚C. The final yield was 3.1 mg.

C. Mass Spectrometry Catalytic Activity Assay Development

1. USP5 activity on Ub4K48 by adapting LCMS protocol in doi: 10.1021/bi200854q

1 µL of 600 nM USP5$^{1-835}$ added to 60 µL buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM DTT) and 1 µL of 1 µg/µL of substrate (Ub4K48) added to reaction mixture at room temperature. 10 µL aliquots were taken at 1, 5, 10, and 20 minutes, and 2 hours after the start of the reaction, mixed with 50 µL of 0.1% (v/v) formic acid to stop the reaction. The samples were then run on the C3 column LCMS (Agilent). The substrate was readily de-ubiquinated by USP5. Some representative time points are shown in **Figure 2**.

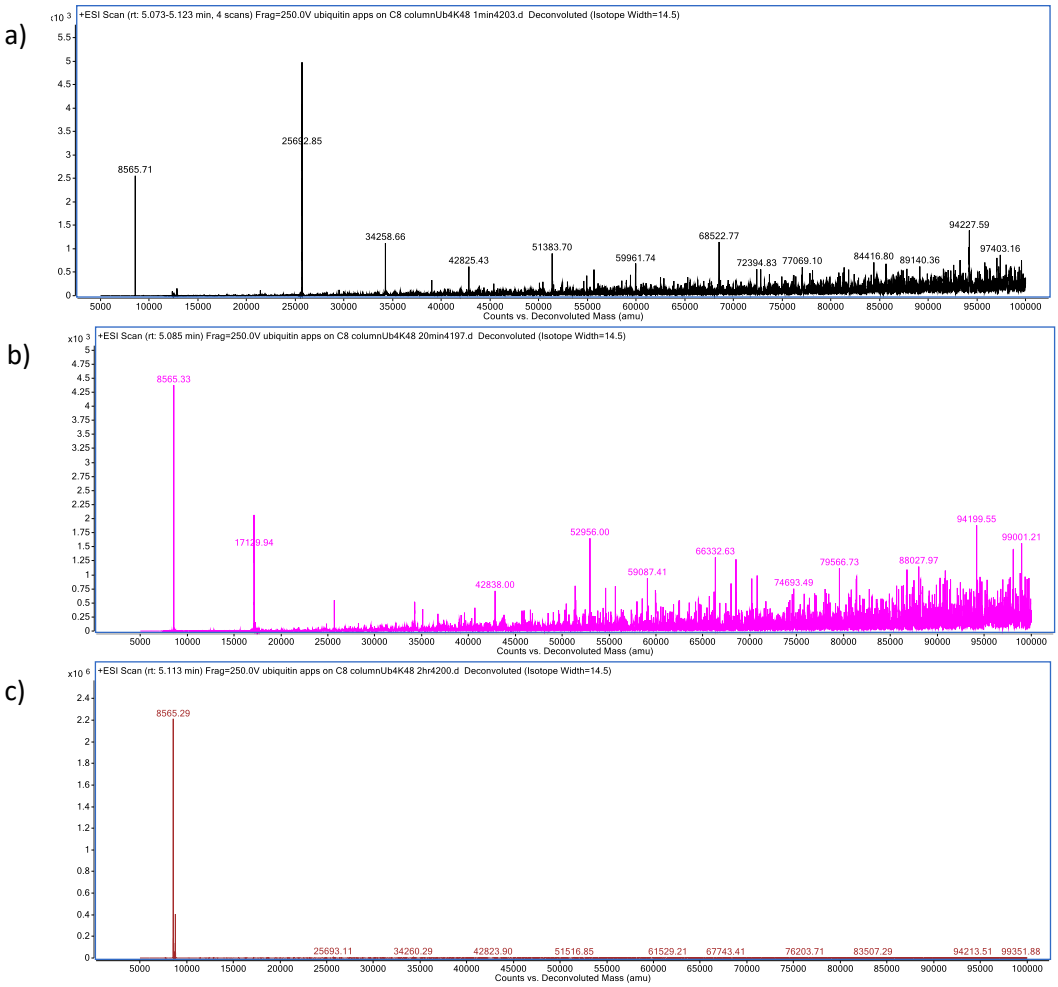

Figure 2. Spectra of USP5 and Ub4K48 reaction over time a) 5 min reaction time b) 20 min reaction time c) 2 hour reaction time

2. Optimizing USP5 concentration in MS assay

USP5$^{1\text{-}835}$ added to 60 µL buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM DTT) for final concentration of either 20 ng or 100 ng. 1 µL of 1 µg/µL of substrate (Ub4K63) then added to both reaction mixtures at room temperature. 10 µL aliquots were taken 1 hour after the start of the reaction, mixed with 50 µL of 0.1% (v/v) formic acid to stop the reaction. The samples were then run on the C3 column LCMS (Agilent). 20 ng of enzyme is sufficient for de-ubiquitination as mono, di- and tri-ubiquitin substrate peaks are seen (Figure 3).

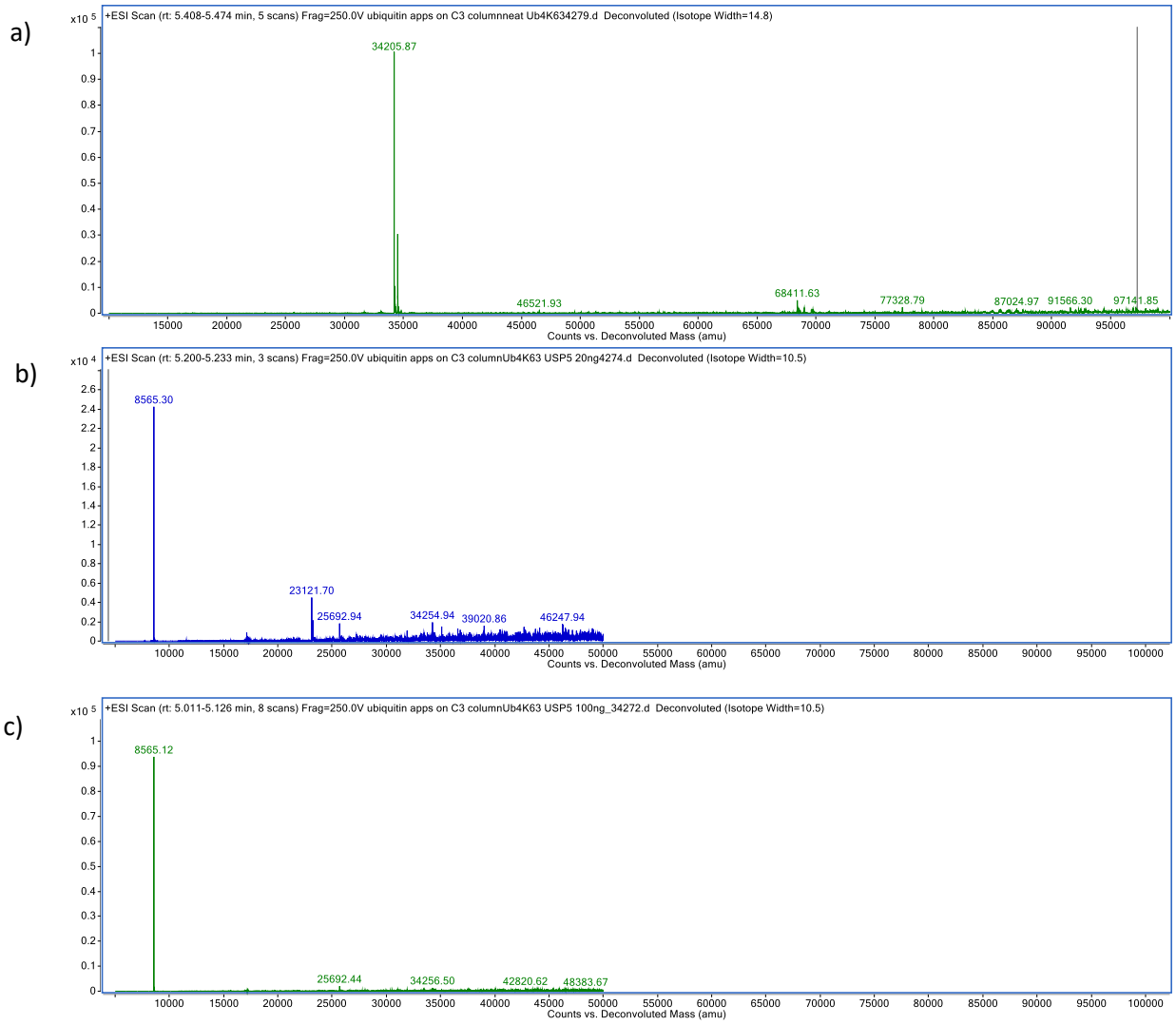

Figure 3. MS spectra a) neat substrate (Ub4K63) b) 20 ng USP5 reaction at 1 hour c) 100 ng USP5 reaction at 1 hour

3. Optimizing the intensity of USP5 in MS assay

2 columns were evaluated in the context of the USP5 ion. 20 pmol USP5 injected for C3 and PLRP column. USP5 was prepared as follows: equal volume of 10 uM USP5 and 0.5% formic acid (FA) were mixed and then 2 µL were injected in the LCMS (Agilent) to generate the spectra below (**Figure 4**). The intensity of the USP5 peak is 4 times higher for the PRLP column relative to the C3.

a)

b)

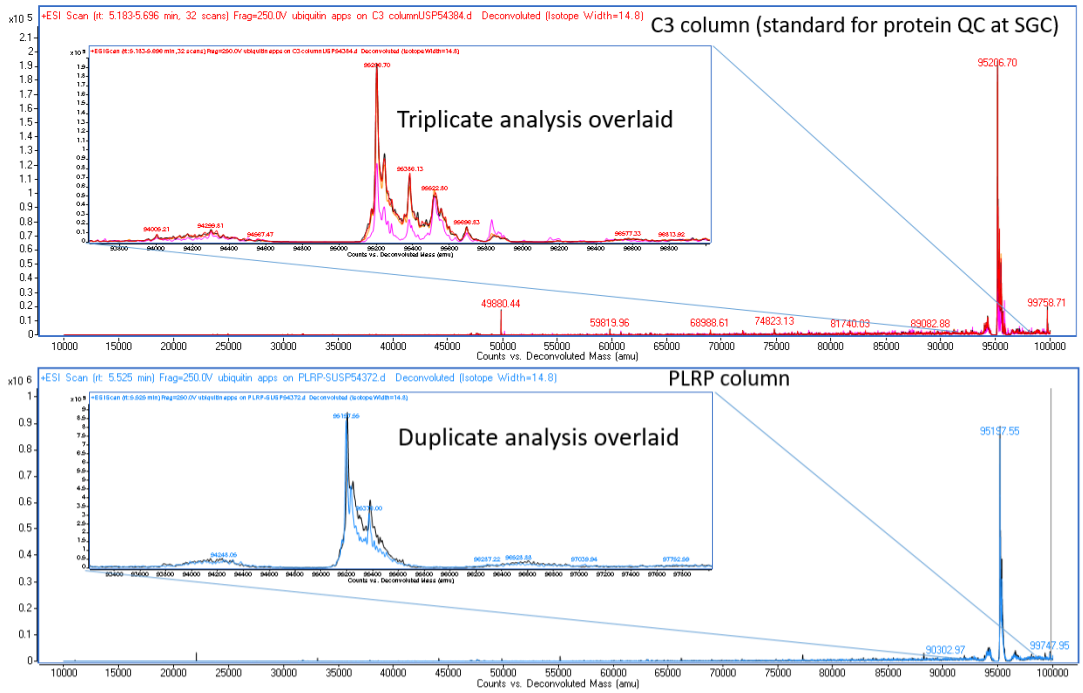

Figure 4. USP5 ion intensity in a) C3 column b) PLRP column

Conclusions & Future Directions:

I was able to express and purify full length USP5 in E.coli and Dr. Suzanne Ackloo developed and optimized a mass spectrometry assay to detect polyubiquitin cleavage. Next, I'll be testing some of the small molecule inhibitors of the zinc-finger ubiquitin binding domain (Zf-UBD) in the mass spectrometry assay to determine if the small molecules can antagonize the catalytic activity of USP5.