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Expression and Purification of Ubiquitin-Specific Protease (UBP1) of *Saccharomyces cerevisiae* in Recombinant *Escherichia coli*

Kang-In Na[1], Myoung-Dong Kim[1], Won-Ki Min[1], Jeong-Ah Kim[1], Woo-Jong Lee[2], Dae-Ok Kim[3], Kyung Moon Park[4], and Jin-Ho Seo[1]*

[1] Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea
[2] Korea Biotechnology Commercialization Center, Korea Institute of Industrial Technology, Incheon 406-130, Korea
[3] Department of Food Science and Technology, Kyung Hee University, Yongin, Kyounggi 449-701, Korea
[4] Department of Chemical System Engineering, Hongik University, Jochiwon, Choongnam 339-701, Korea

Abstract Truncated form of UBP1, an ubiquitin-specific protease of *Saccharomyces cerevisiae*, was overexpressed in *Escherichia coli*. The hexahistidine residue (His_6) was fused to the N-terminus of truncated UBP1 and the corresponding recombinant protein was purified with high yield by immobilized metal affinity chromatography. The truncated form of UBP1 protein was functional to cleave ubiquitinated human growth hormone as substrate. Effects of pH and temperature were investigated in order to optimize deubiquitinating reactions for the truncated UBP1. Optimum temperature and pH for the cleavage reaction were 40°C and pH 8.0, respectively.

Keywords: ubiquitin-specific protease, UBP1, *Saccharomyces cerevisiae*, *Escherichia coli*

Ubiquitin is a highly conserved 76-amino acid protein that functions as a marker for targeting proteins to proteasomes [1]. It has been used as a partner in the expression and purification of fusion proteins in plants, yeast and bacteria [2-5]. Ubiquitin-specific proteases such as UBP1 and UBP6 in *Saccharomyces cerevisiae* cleave off the ubiquitin moiety from ubiquitin fusions to produce unmodified proteins [6,7].

UBP1 of *S. cerevisiae*, an 809 amino-acid protein, cleaves at the carboxyl terminus of the ubiquitin moiety in natural and engineered fusions irrespective of their size or the presence of an amino-terminal ubiquitin extension [6]. This property distinguishes it from YUH1 (yeast ubiquitin hydrolase 1) that deubiquitinates relatively short ubiquitin fusions and hence, is practically inactive with longer fusions such as ubiquitin-β-galactosidase [8]. Such a property of YUH1 defines its application as deubiquitinating enzyme (DUB).

In this study, truncated form of UBP1 of *S. cerevisiae* was overexpressed in a recombinant *Escherichia coli* system and its enzymatic activity was confirmed by using human growth hormone fused with ubiquitin at N-terminus (ub-hGH) as substrate. Effects of temperature and pH were examined in an attempt to characterize enzymatic properties and to optimize deubiquitinating conditions.

E. coli DH5α was used for plasmid preparation and BL21-CodonPlus (DE3) RIL (F⁻, *ompT*, *hsd*SB(rB⁻ mB⁻), *dcm*⁺, Tet^r, gal λ(DE3), *endA*, Hte[*argU iley leuW* Cam^r]) purchased from Stratagene (La Jolla, CA, USA) was used as host for protein expression. To make plasmid pAP4UBPN6His, the truncated form of UBP1 (YDL122W) fused with the hexa-histidine residue at N-terminus was amplified by PCR using genomic DNA of *S. cerevisiae* as template and introduced into *Nde*I/*Bam*HI sites of pAP4, a kind gift from APTECH (Ansan, Korea). Two PCR primers, UBP1F (5'-AGGCCCATATGCACCACCACCACC ACCACAGAAGTAAGTTTACCCACTTA-3') and UBP1R (5'-AGGCGGATCCTCTATGGTCTTCTGTTCTCTCG-3'), were designed to truncate 91 amino acids from authentic UBP1 (92.8 kD) and hence to obtain 83.6 kD-size UBP1 (His_6-UBP1). Underlined characters in the primer sequences denote restriction enzyme sites. Plasmid pAP4, a pBR322 derivative, harbors a kanamycin-resistance gene and expression of the His_6-UBP1 gene in pAP4UBPN6His was under the control of the *tac* promoter.

LB medium (0.5% yeast extract, 1% tryptone and 1% NaCl) containing 25 mg/L kanamycin was used to cultivate recombinant *E. coli* strains. For large-scale expression, a bench-top bioreactor (Bioengineering, Wald, Switzerland) containing 1.5 L of fresh medium was used to cultivate recombinant *E. coli* at 37°C and pH 7.4. Medium acidity was controlled by adding either 1 N HCl or 1 N NaOH. Agitation speed of 500 rpm and aeration rate

*Corresponding author
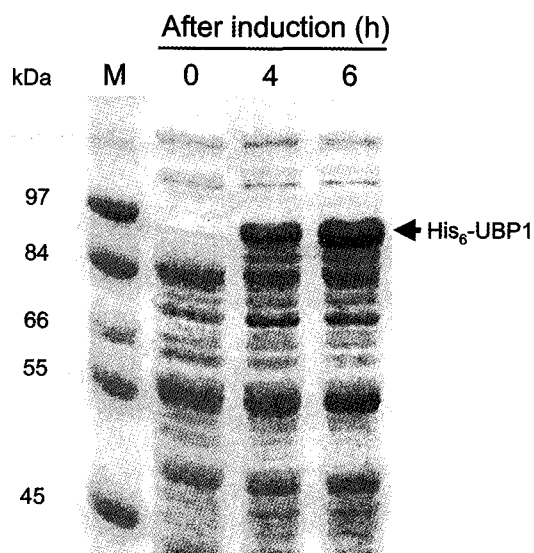Tel: +82-2-880-4855   Fax: +82-2-873-5095
e-mail: jhseo94@snu.ac.kr

Fig. 1. Expression of His_6-UBP1 in recombinant *E. coli* BL21 (DE3) RIL strain harboring pAP4UBPN6His plasmid. Cells were harvested at the indicated time after induction by 1 mM IPTG and disrupted by French Press. Soluble fraction of cell lysate (20 μg protein) was separated by SDS-PAGE (15% polyacrylamide). M denotes size marker.

Fig. 2. Mass spectrum of purified His_6-UBP1 from MALDI-TOF analysis. The arrow indicates the His_6-UBP1.

of 1 vvm were maintained throughout the cultivation. Expression of the His_6-UBP1 was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM at the late exponential phase of growth (OD_{600} ≈ 0.8) since these conditions were found to be optimum from preliminary experiments (data not shown). After induction, cultures were grown further for 6 h and harvested by centrifugation at 2,000 × g for 20 min. Harvested cells were resuspended in 50 mM sodium phosphate buffer (pH 7.4) and disrupted by French®Press (Thermo Spectronic, Rochester, NY, USA). Cell lysate was centrifuged at 15,000 × g for 20 min and the supernatant was filtered through a 0.45 μm membrane. The ÄKTA prime system (Amersham Biosciences, Piscataway, NJ, USA) that was installed with the HiTrap Chelating HP column (5 mL) was used for His_6-UBP1 purification and the bound protein was eluted by buffer A (50 mM Tris, pH 8.0, 150 mM NaCl) containing imidazole. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide) was used to analyze expression and purification of the fusion protein. Protein concentration was determined using the protein assay kit (Bio-Rad, Hercules, CA, USA). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was done to measure the molecular weight of the purified protein using Voyager-DE STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA).

Enzymatic activity of the truncated form of UBP1 was assayed using ub-hGH (M.W. ≈ 28.0 kDa) as substrate, which was donated from APTECH. Ubiquitinated human growth hormone protein was expressed in recombinant *E. coli* and purified with high yield using metal affinity
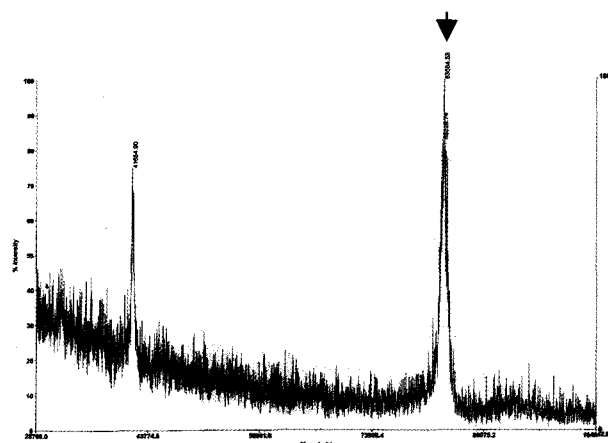
chromatography (APTECH, personal communication). Approximately 1 μg of His_6-UBP1 protein was preincubated in an appropriate condition for 5 min and ub-hGH was added to a final concentration of 240 mM to start the cleavage reaction. The reaction continued for 3 min. To evaluate the effects of temperature and pH, the reactions were performed by incubating reaction mixtures at various pHs and temperatures. Buffers to adjust pH were sodium acetate (pH 4.0~5.5), sodium phosphate (pH 6.0~8.0) and borate-KCl-NaOH (pH 8.5~10.0). After 3 min, an aliquot of the reaction product (15 μL) was withdrawn, stored at -80°C until analysis and then, analyzed by SDS-PAGE. Percent degradation of the ub-hGH was measured by densitometric analysis.

Plasmid pAP4UBP6His was introduced into the *E. coli* BL21 CodonPlus (DE3) RIL strain to overexpress truncated form of UBP1 protein. Our preliminary experiments showed that authentic UBP1 protein was very hard to overexpress in a recombinant *E. coli* system and the BL21 CodonPlus (DE3) strain was much better than usual BL21 (DE3) for the truncated UBP1 expression. Wojtowicz *et al.* [9] achieved an increased level of UBP1 expression in recombinant *E. coli* by removing the transmembrane region that encompasses amino acids 34-51. Most of the expressed His_6-UBP1 was found in soluble fraction and a maximum amount of the fusion protein was obtained by inducing at 37°C for 6 h (Fig. 1). The cell lysate of recombinant *E. coli* exibited enzymatic activity of UBP1 to cleave ub-hGH into ubiquitin and human growth hormone (data not shown) and hence, His_6-UBP1 was expressed in a large scale and purified by immobilized metal affinity chromatography (IMAC). A maximum purification yield was obtained with a final imidazole concentration of 200 mM in buffer A. Purity of the His_6-UBP1 protein was approximately 90% relative to the total protein content in the final eluant and 6.0 mg of the His_6-UBP1 protein was obtained from 1.5 L cultivation of recombinant *E. coli*. Molecular weight of the purified protein determined by MALDI-TOF analysis was approximately 83.6 kDa, as predicted theoretically (Fig. 2).
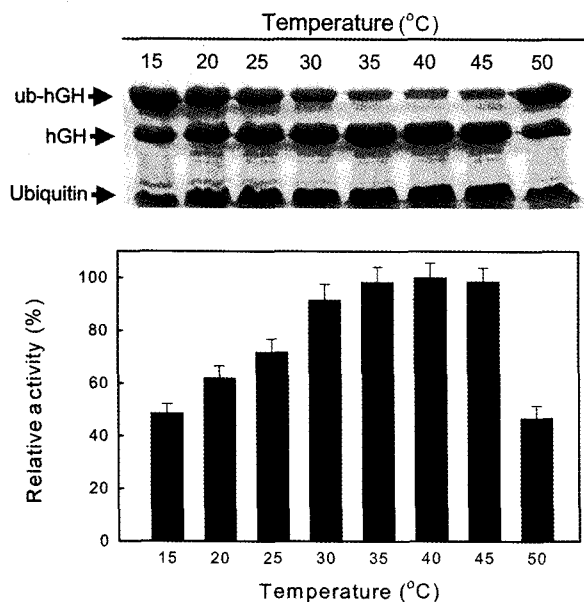
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Fig. 3. Determination of optimum reaction temperature for the purified His₆-UBP1. After 3 min of reaction in appropriate conditions, reaction mixtures were separated by SDS-PAGE (15% polyacrylamide). Percent degradation of substrate protein, ub-hGH, was measured by densitometric analysis.

An enzymatic reaction using ubiquitin-hGH as substrate was carried out in 100 mM buffers with various pHs. Maximum enzyme activity was obtained at pH 8.0 in 100 mM sodium phosphate buffer (data not shown).

To examine the effect of temperature on the activity of truncated UBP1 activity, the reaction mixture in 100 mM sodium phosphate buffer (pH 8.0) was incubated at various temperatures. The optimum reaction temperature appeared to be around 40°C. As shown in Fig. 3, UBP1 was active in the range of 35~45°C, indicating a wide application of truncated UBP1 protein to cleave the ubiquitin-fused protein.

Recent developments in recombinant protein manufacturing techniques allowed production of many value-added proteins in recombinant microbial systems [4,5,10]. However, a number of proteins are not still expressed well in commonly used host cells [5,11] and hence, the fusion of unstable and aggregation-prone proteins with a highly evolved structure has been widely used [4,11-13]. Fusion partners include glutathione S-transferase (GST), maltose-binding protein (MBP), FLAG, His₆ and c-Myc. However, problems might be encountered when these tags must be removed to study the protein's structure by X-ray crystallography or NMR. Proteases such as thrombin, Factor Xa and TEV (protease encoded by tobacco etch virus) have been used to cleave off these tags. However, all of these proteases recognize short degenerate peptide sequences and hence, the cleavage may occur within the protein of interest. Another problem is inaccessibility of the cleavage site within the fusion due to steric constraints. Accordingly, a 'moderate-size' fusion partner stimulating high-level expression is desirable for efficient purification and recovery of target proteins.

Ubiquitin is a small and highly conserved eukaryotic protein composed of 76 amino acids. Through covalent conjugation (ubiquitination), ubiquitin plays important roles in regulating the stability, activity and localization of many proteins in a cell [1]. Ubiquitin fusion has been used to improve both the quality and the quantity of foreign proteins expressed in *E. coli* and yeast. Ubiquitin as a fusion partner exhibited a chaperone-like property reflected on higher yields of active and properly folded proteins [4,14]. Moreover, expression of proteins as ubiquitin fusions allows the production of proteins with any desired N-terminal residue upon ubiquitin cleavage [14]. Generally, engineered ubiquitin fusion proteins are cleaved after the C-terminal glycine residue of ubiquitin by deubiquitinating enzymes. Although, several deubiquitinating enzymes have been reported in yeast and other eukaryotic cells [1], much information is not available about their enzymatic properties yet. Ubiquitin and DUBs do not exist in bacteria and hence, various ubiquitin fusion proteins have been expressed in bacteria and isolated after *in vitro* cleavage by DUBs [15]. Moreover, DUBs are unstable, difficult to produce and often have to be used at enzyme to substrate ratio of 1:1. Accordingly, it is highly demanded to overproduce DUBs using recombinant expression systems.

In this study, truncated form of *S. cerevisiae* UBP1 protein was overexpressed in a recombinant *E. coli* system and purified with high purity. The purified enzyme was functional to cleave ubiquitinated human growth hormone as substrate. Optimum reaction conditions were pH 8.0 and 40°C. More research is in progress to increase an expression level of the truncated UBP1 and to design an optimum enzyme reactor regime for cleavage of the ubiquitinated target proteins.

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