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ScienceDirect
Protein Expression and Purification 56 (2007) 20–26
Protein Expression & Purification
www.elsevier.com/locate/yprep

Characterization of ubiquitin C-terminal hydrolase 1 (YUH1) from *Saccharomyces cerevisiae* expressed in recombinant *Escherichia coli*

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Received 3 April 2007, and in revised form 11 July 2007
Available online 18 July 2007

Abstract

The *YUH1* gene coding for ubiquitin C-terminal hydrolase 1, a deubiquitinating enzyme, was cloned from the *Saccharomyces cerevisiae* genomic DNA and expressed in *Escherichia coli*. YUH1 was fused with the 6 histidine tag at the N-terminus (H6YUH1) or C-terminus (YUH1H6) and purified by an immobilized metal affinity chromatography with high purity. By using a fluorogenic substrate, Z-Arg-Leu-Arg-Gly-Gly-AMC, the deubiquitinating activities for H6YUH1 (1.72 U/mg) and YUH1H6 (1.61 U/mg) were about 18 times higher than 0.092 U/mg for H6UBP1, ubiquitin specific protease 1 of *S. cerevisiae* containing the 6 histidine residue at the N-terminus which is normally used in protein engineering. YUH1 had the optimal temperature of 27 °C and acidity of pH 8.5. Analysis of thermal deactivation kinetics of H6YUH1 estimated 3.2 and 1.4 h of half lives at 4 and 52 °C, respectively. Immobilization onto the Ni-NTA affinity resin and environmental modulation were carried out to improve the stability of YUH1. Incubation of the immobilized YUH1 in 50% glycerol solution at −20 °C resulted in 52% of decrease in specific activity for 7 days, corresponding to a 2.7-fold increase compared with that of the free YUH1 incubated in the same solution at 4 °C.

Keywords: YUH1; Ubiquitin C-terminal hydrolase; *Saccharomyces cerevisiae*; Recombinant *Escherichia coli*

Ubiquitin is widely used as a fusion partner in the production of foreign proteins and peptides by *Escherichia coli* and *Saccharomyces cerevisiae* [1–3]. Additionally, it plays an important role in protein degradation, cell-cycle control, stress response and DNA repair in eukaryotic cells. The compact and globular structure of ubiquitin protects the amino terminus of the fusion protein against proteolytic attack [4]. A peptide library containing 8∼70 amino acids and fused with ubiquitin was produced solubly in recombinant *E. coli* at 10–31% of specific yields based on biomass [3]. An increase in the production yield of yeast metallothionein by the fusion of ubiquitin seemed to be ascribed to stability improvement and/or more efficient translation of the fusion proteins [5]. Furthermore, deubiquitinating enzymes cleaved rapidly and precisely between the target fusion proteins and the carboxyl-terminal glycine residue of ubiquitin so that the target proteins with over 90% of purity can be obtained simply [3].

Deubiquitinating enzymes (DUBs)[1] categorized in cysteine proteases specifically cut ubiquitin conjugates at the

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[1] *Abbreviations used:* DUBs, deubiquitinating enzymes; UBP, ubiquitin specific protease; UCH, ubiquitin carboxyl-terminal hydrolase; PCR, polymerase chain reaction; DTT, dithiothreitol; YUH1, yeast ubiquitin C-terminal hydrolase 1.

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doi:10.1016/j.pep.2007.07.005

ubiquitin carboxyl terminus. DUBs are involved in the regulation of the ubiquitin dependent proteolytic pathway by direct or indirect association with the proteosome [2,6,7]. On the basis of sequence homology, DUBs can be grouped into ubiquitin specific protease (UBP) and ubiquitin carboxyl-terminal hydrolase (UCH). UBP includes over 90 proteases that have various cellular functions. UBPs are large size proteins of 60–300 kDa and show high divergency except for two short conserved sequences that surround the catalytic cysteine and histidine residues. They are mainly responsible for the hydrolysis of proteins in proteosome and generate free ubiquitin monomers from branched ubiquitin chains for recycling [2,6,8]. UBP1 derived from *S. cerevisiae* cuts the carboxyl terminus of the ubiquitin moiety in linear fusions, irrespective of their sizes or the presence of an amino-terminal ubiquitin extension [9]. UCHs are relatively small size proteins of 20–30 kDa with a few exceptions. UCHs can remove peptides and small molecules from the C-terminus of ubiquitin. However, most of them could not release ubiquitin from the ubiquitin–protein conjugates or disassemble poly ubiquitin chains. Thus, UCHs seem to play a role in the elimination of small adducts from ubiquitin and generation of free monomeric ubiquitin from its precursors. Yeast ubiquitin C-terminal hydrolase 1 (YUH1), the only UCH present in *S. cerevisiae,* is known to be specific for short ubiquitin fusion proteins with 50–80 amino acids [10]. A conformational change of YUH1 active site induced by substrate binding could explain the substrate specificity of YUH1 [10,11].

In this study, a gene coding for YUH1 of *S. cerevisiae* was cloned and overexpressed in recombinant *E. coli*. To characterize the deubiquitinating properties of YUH1, YUH1 fused with the 6 histidine tag was purified with an affinity chromatography, and optimal conditions of pH and temperature for the enzyme reaction were determined. Half lives of YUH1 were estimated using a thermal inactivation kinetic model. Protein immobilization and environmental modulation were performed to increase the stability of YUH1.

Materials and methods

Strain and plasmids

E. coli DH5α was used as a host strain for the manipulation and expression of the *YUH1* gene from *S. cerevisiae*. An expression vector, pTrc containing the *trc* promoter and ampicillin resistance gene was purchased from Invitrogen Co. (Carlsbad, CA, USA). *S. cerevisiae* UBP1 tagged with 6 histidines at the N-terminus with over 90% purity and 0.11 mg/ml of concentration (H6UBP1) was presented by AP Technology Co. (Suwon, Korea) [12].

Construction of YUH1 expression vector

Amplification of *YUH1* from the *S. cerevisiae* genomic DNA by the polymerase chain reaction (PCR) was carried out with the Accupower HL PCR PreMix (Bioneer, Daejeon, Korea) in a GeneAmp PCR System 2400 (Applied Biosystems, CA, USA). Two DNA oligomers of 5′-GAATTCCCATGGGCCATCATCATCATCATCATAGCGGAGAAAATCGTGCTGTG-3′ (H6YUH1-F) and 5′-AATCGCCTCGAGTTATTCCCAATTAGGGCCCAATCCTAGCAT-3′ (YUH1-R) were used to obtain the coding region of *YUH1* and to combine 6 histidine residues at its N-terminus. To fuse 6 histidines at the C-terminus of YUH1, two DNA primers including 5′-GAATTCCCATGGGCAGCGGAGAAAATCGTGCTGTGGTGCCG-3′ (YUH1-F) and 5′-AATCGCCTCGAGTTATTAATGATGATGATGATGTTCCCAATTAGGGCCCAATCC-3′ (YUH1H6-R) were added to the PCR reaction mixture. The recognition sites of restriction enzymes were underlined and incorporated into the PCR primer sequences such as *Nco*I for H6YUH1-F and YUH1-F, and *Xho*I for YUH1-R and YUH1H6-R. A temperature profile was programmed as follows: an initial denaturation step for 10 min at 95 °C followed by 30 cycles of 30 s at 94 °C; 60 s at 55 °C; and 150 s at 72 °C, and a final cycle of 10 min at 72 °C. After the digestion of the PCR products with two restriction enzymes of *Nco*I and *Xho*I, each DNA fragment was ligated with plasmid pTrc cut with the same enzymes. Two plasmids of pTrc-H6YUH1 and pTrc-YUH1H6 were constructed to express two types of YUH1 with the 6 histidine tag at the N-terminus (H6YUH1) and C-terminus (YUH1H6).

Culture conditions

Cells were grown at 37 °C in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) with 50 mg/L ampicillin for the selection and maintenance of *E. coli* transformants. Expression of YUH1 was induced by the addition of 1 mM IPTG at a range of optical density from 0.6 to 1.0 [13]. After 4 h of induction, culture broth was harvested by centrifugation at 6000 rpm and 4 °C for 10 min.

Purification

Purification of H6YUH1 and YUH1H6 followed the previous report with some modification [14]. The harvested cells were resuspended in buffer A (20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole (pH 7.4)) and disrupted by a ultrasonic processor (Cole-Parmer, IL, USA). After the centrifugation of the cell lysate at 12,000 rpm and 4 °C for 10 min and collection of the supernatant, the pellets representing insoluble components were stored at −80 °C for further analysis. The supernatant containing soluble proteins was filtered through 0.45 μm membrane. An ÄKTA FPLC SYSTEM (Amersham Biosciences, Uppsala, Sweden) installed with the HiTrap Chelating HP column was used for the purification of H6YUH1 and YUH1H6. Buffer B (20 mM sodium phosphate, 0.5 M NaCl and 0.5 M imidazole (pH 7.4)) was flowed constantly at 1.5 ml/min during equilibration and elution.

The absorbance of the eluted proteins was monitored by a UV detector (UPC-900, Amersham Biosciences, Sweden) at 280 nm of wavelength. The purified YUH1 proteins were desalted using the disposable PD-10 Desalting Column (Amersham Biosciences, Sweden) and their concentrations were determined by the protein assay kit (Bio-Rad, Hercules, CA, USA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 16% polyacrylamide) and protein visualization with Coomassie brilliant blue followed the previous report [15]. Western blotting with the INDIA His-HRP antibody (Pierce, Rockford, IL, USA) and protein detection with the Opti-4 CN substrate kit (Biorad, USA) were carried out as the standard method and the manufacturer's manual.

Immobilization onto Ni-NTA resin

Immobilization of H6YUH1 using a metal affinity resin was performed as the manufacturer's manual (Qiagen, Valencia, CA, USA) with slight modification. Briefly, the purified H6YUH1 was added into a solution containing the Ni-NTA resin (Qiagen, USA) pre-equilibrated with buffer A, which was gently shaked for 1 h at 4 °C. After centrifugation, solid materials were collected and washed with buffer A for the subsequent removal of unbound proteins. For the determination of protein content and deubiquitinating activity, an aliquot of H6YUH1-immobilized Ni-NTA resin was mixed with five to ten bead volumes of buffer B. After gentle mixing for 30 min, the solid materials were removed by centrifugation and the supernatant was subjected to the assay of YUH1.

Activity assay

Activities of the free and immobilized YUH1s, and UBP1 were determined using a fluorogenic substrate, Z-Arg-Leu-Arg-Gly-Gly-AMC which was purchased from Bachem (Bubendorf, Switzerland). The fluorescence of an AMC moiety released by YUH1 and UBP1 was monitored by a fluorescence spectrophotometer (RF5301, Shimadzu, Kyoto, Japan) at the wavelengths of 440 nm for emission and 380 nm for excitation. An enzyme reaction with some modification was carried out at 27 °C and pH 8.5 in 3 ml of a reaction mixture containing an assay buffer (20 mM Tris–Cl, 0.5 mM EDTA, 1 mg/ml ovalbumin and 1 mM dithiothreitol (DTT)), 50 μM of the substrate and 100 μl of the enzyme solution diluted appropriately [16]. The standard AMC was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). One unit of YUH1 and UBP1 was defined as the amount of an enzyme hydrolyzing 1 μmol of the substrate per minute under the reaction conditions.

Substrate specificity

As a substrate of deubiquitinating enzymes, the crude extract of human growth hormone tagged with ubiquitin at the N-terminus was provided by AP technology Co. (Korea). Ubiquitin fused human growth hormone was diluted appropriately with 20 mM Tris–HCl buffer (pH 8.5), and 20 μl of the purified UBP1 and YUH1 were added into 200 μl of the substrate solution, respectively. After 3 h of incubation at 37 °C, the reaction solution was analyzed by SDS–PAGE.

Determination of optimal environmental conditions, thermal deactivation kinetic parameters and enzyme stability

To determine an optimal temperature for YUH1, temperatures of the reaction mixture were varied from 16 to 55 °C. An optimal acidity was determined at 27 °C and over the pH ranges from 3.0 to 9.0 using a series of different buffers such as 20 mM acetate buffer (pH 3.0–5.0), 20 mM HEPES buffer (pH 5.0–8.0) and 20 mM Tris–HCl buffer (pH 7.8–9.0). All buffers contained 0.5 mM EDTA, 1 mg/ml ovalbumin and 1 mM DTT. The purified H6YUH1 in 20 mM Tris–HCl buffer (pH 8.5) was incubated at various temperatures and subjected to enzyme activity assay after 1, 2 and 4 h of incubation. For stability assay, the free and immobilized H6YUH1 were incubated at 4 and/or −20 °C in 50% glycerol solubilized in 20 mM Tris–HCl buffer (pH 8.5).

Results

Expression and purification of YUH1 fused with the 6 histidine tag

For the efficient purification of YUH1, two plasmid systems of pTrc-H6YUH1 and pTrc-YUH1H6 were constructed to contain the 6 histidine tag at the N- and C-termini of YUH1, respectively. IPTG induction triggered the accumulation of YUH1 in recombinant *E. coli* DH5α strains. Expression patterns of YUH1 are displayed in Fig. 1(a). SDS–PAGE analysis showed that the expression levels of H6YUH1 and YUH1H6 occupied 34% and 41% of the total protein content, respectively. H6YUH1 and YUH1H6 were mainly expressed as soluble form and only 8–11% of YUH1 proteins accumulated as inclusion body. YUH1 in the soluble fraction was purified using Ni-NTA affinity chromatography, and 0.15 mg/ml of H6YUH1 and 0.11 mg/ml of YUH1H6 solutions were obtained. The purified YUH1 proteins from the crude extract showed over 90% homogeneity as determined by SDS–PAGE analysis and immunoblotting with a monoclonal antibody against the 6 histidine tag confirmed the correct expression of YUH1 in recombinant *E. coli* (Fig. 1(b)). Activity assay of the purified H6YUH1 gave the highest specific activity of 1.72 ± 0.04 U/mg. YUH1H6 and H6UBP1 showed the specific activities of 1.61 ± 0.02 and 0.092 ± 0.01 U/mg, respectively. Eighteen times higher activity of YUH1 was achieved in comparison to UBP1, a usually used and major ubiquitin specific protease with the same function. Consequently, further enzymatic analy-

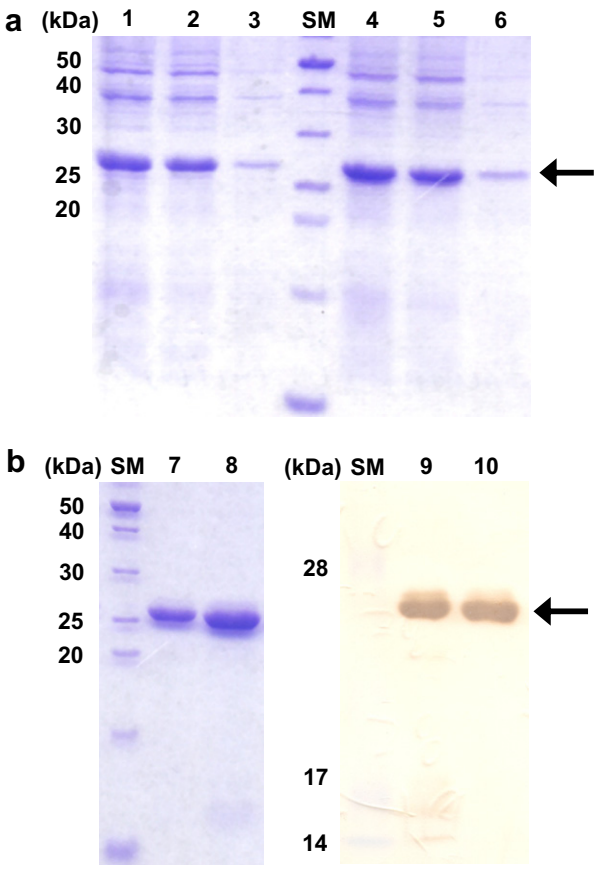

Fig. 1. SDS–PAGE and western blot analysis of the crude and purified proteins of H6YUH1 and YUH1H6 expressed in recombinant *E. coli* DH5α strains containing pTrc-H6YUH1 and pTrc-YUH1H6 vectors, respectively. After 4 h of IPTG induction, recombinant *E. coli* cells grown at 37 °C were collected by centrifugation. Crude protein samples (a) for H6YUH1 (lanes 1–3) and YUH1H6 (lanes 4–6) expression were fractionated into total cell lysate (lanes 1 and 4), soluble fraction (lanes 2 and 5) and insoluble fraction (lanes 3 and 6). Purified H6YUH1 and YUH1H6 (b) by RP-HPLC were shown in lane 7 and lane 8, respectively, of which immunoblotting with the INDIA His-HRP antibody (Pierce, USA) was presented in lanes 9 and 10. Lane SM represents protein size markers and the arrows indicate the positions of H6YUH1 and YUH1H6. Protein bands on SDS–PAGE and nitrocellulose membrane were visualized with Coomassie blue staining and the Opti-4-CN substrate kit (Bio-Rad, USA), respectively.

sis was carried out with YUH1 fused with the 6 histidine tag at the N-terminus (H6YUH1).

Substrate specificities of YUH1 and UBP1

The deubiquitinating activities of H6YUH1 and H6UBP1 were evaluated by their reactions on human growth hormone fused with ubiquitin at the N-terminus. As shown in Fig. 2, SDS–PAGE analysis of H6UBP1 reaction mixtures showed that about 32 kDa of ubiquitin tagged human growth hormone was precisely hydrolyzed, and two bands of human growth hormone and ubiquitin were observed with the estimated molecular sizes of 20 and 12 kDa (lanes 1 and 2). However, the bands of human

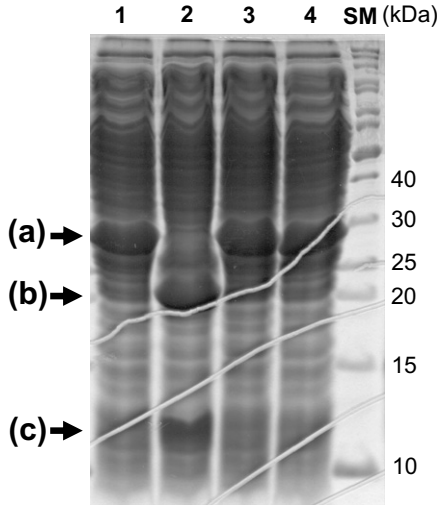

Fig. 2. SDS–PAGE analysis of H6UBP1 and H6YUH1 reaction mixtures using ubiquitin fused human growth hormone as a substrate. A crude protein solution with human growth hormone tagged with ubiquitin at the N-terminus was mixed with purified H6UBP1 (lane 2) and H6YUH1 (lane 4). Both lanes 1 and 3 show the crude substrate solution without H6UBP1 and H6YUH1 treatment. Arrows indicate ubiquitin fused human growth hormone (a), human growth hormone (b) and ubiquitin (c). SM presents a protein size marker.

growth hormone and ubiquitin were not observed in SDS–PAGE analysis of H6YUH1 reaction mixture (lanes 3 and 4), indicating that H6YUH1 was unable to hydrolyze the linkage of ubiquitin with human growth hormone.

Determination of optimal reaction conditions

Effects of temperature and acidity on the deubiquitinating activity were investigated with the purified H6YUH1 (Fig. 3). When reaction temperatures were changed from 16 to 55 °C, the highest activity was obtained at 27 °C. Activity assay in various reaction buffers with different acidities showed that the highest activity was achieved in 20 mM Tris–HCl buffer at pH 8.5. Interestingly, no deubiquitinating activity was detected under pH 6.5. As a result, the optimal conditions of temperature and acidity for the deubiquitinating reaction of *S. cerevisiae* YUH1 were determined as 27 °C and pH 8.5.

Thermal deactivation kinetics of H6YUH1

As a temperature change affected YUH1 activity considerably as shown in Fig. 3(a), thermal deactivation kinetics was used to analyze its deactivation behavior [17]. To determine kinetic parameters, the purified H6YUH1 was incubated in 20 mM Tris–HCl buffer (pH 8.5) at various temperatures from 4 to 52 °C. The half life of H6YUH1 according to the incubation temperature was estimated as shown in Fig. 4. The estimated half lives were similar to the experimental values by 5% standard deviation. A half of deubiquitinating activity remained after 1.4 h of incuba-

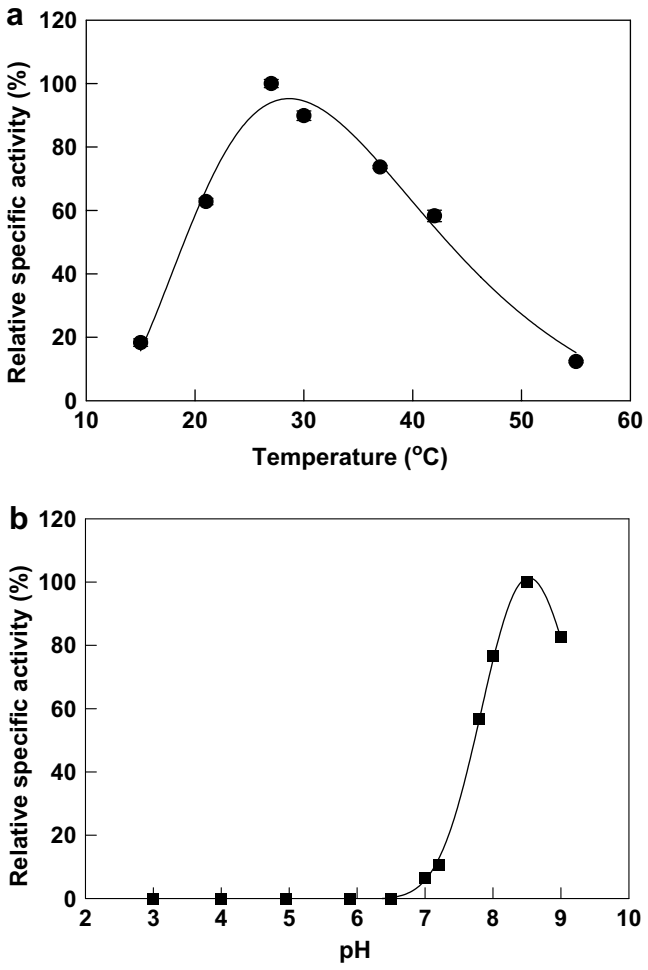

Fig. 3. Determination of optimal reaction conditions of temperature (a) and pH (b). Temperature was changed from 16 to 55 °C. Acidity was varied from pH 3.0 to pH 9.0 using a series of different buffers, all of which contained 0.5 mM EDTA, 1 mg/ml ovalbumin and 1 mM DTT. Specific activity of H6YUH1 was assayed in duplicate.

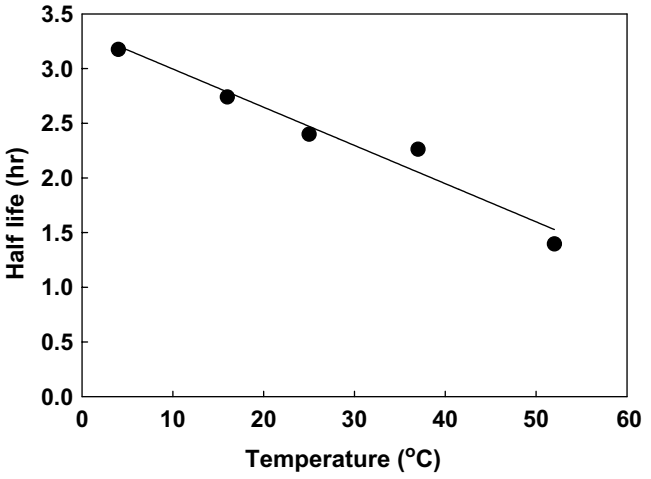

Fig. 4. A profile of the estimated half lives of H6YUH1 according to the reaction temperatures. Values were calculated based on the deactivation constants.

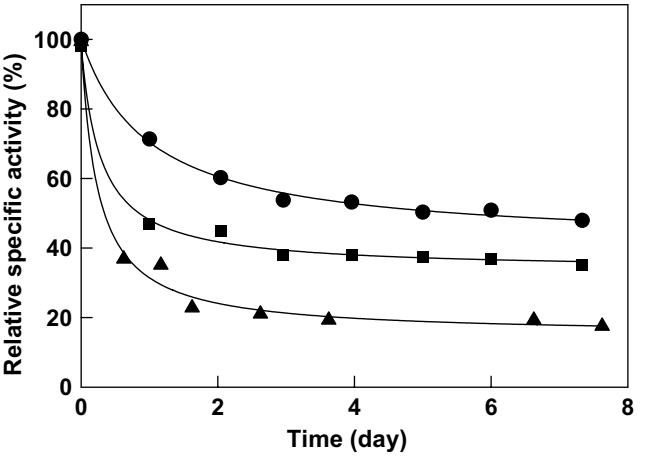

Fig. 5. Time-course profiles of the relative specific activities of the free and immobilized H6YUH1 enzymes. The free enzyme was incubated at 4 °C (▲) and the immobilized enzyme was placed at 4 °C (■) and −20 °C (●). All solutions contained 50% glycerol in 20 mM Tris–HCl (pH 8.5).

tion at 52 °C. Moreover, only 3.2 h was taken for YUH1 to lose it's half activity at 4 °C.

Stability of the free and immobilized YUH1

To overcome the unstable behavior of YUH1, its immobilization was carried out with a metal affinity resin and the specific activities of the free and immobilized YUH1 were monitored for about 8 days (Fig. 5). Adding a stabilizer such as 50% glycerol and storing at low temperatures (4 and −20 °C) enhanced the stability of YUH1. After 2 days of incubation without 50% glycerol at 4 °C, white aggregates were observed at the bottom of a container, which seemed to be a denatured form of YUH1 (personal communication). By incubation in 50% glycerol solution at 4 °C and pH 8.5, 35.1% of the specific activity of the immobilized YUH1 remained after 7.5 day of incubation, which was two times higher than that of the free YUH1. Change of incubation temperature from 4 to −20 °C in the same solution resulted that 48.0% of the immobilized YUH1 remained actively.

Discussion

To characterize the ubiquitin C-terminal hydrolase 1 (YUH1) from *S. cerevisiae*, its coding region was successfully introduced and expressed in *E. coli*. Soluble and active YUH1 was produced and the histidine tag facilitated its efficient separation and purification with high purity. In comparison to the soluble expression of YUH1 under the control of the *trc* promoter in this study, Mildner et al. [18] reported that *S. cerevisiae* YUH1 tagged with 6 histidines at the N-terminus was expressed under the *T5* promoter in recombinant *E. coli*, of which 75% accumulated as inactive inclusion body. H6YUH1

showed 18 times higher specific activity on the short and artificial fluorogenic substrate than H6UBP1. Ubiquitin carboxyl-terminal hydrolase including YUH1 is usually deficient in binding domains for ubiquitin-leaving groups and more efficiently utilizes free energy released from its remote interaction with ubiquitin-containing substrates, which seemed to be due to a higher activity of H6YUH1 [19]. In comparison to high activity on the artificial substrate with a short length, H6YUH1 was unable to remove ubiquitin from ubiquitin fused human growth hormone which was composed of 191 amino acid residues. Usually UCHs including *S. cerevisiae* YUH1 are specific for small polypeptides with 50–80 amino acids because of their specific structural feature of cross over loop [11]. UCH-L3 found in human thymus released 8–33 residue peptides more quickly than 70 amino acid extension from their ubiquitin fusion peptides [3]. It was recently reported that YUH1 exactly cleaved off ubiquitin fused with amyloid-β peptide 42 showing 6 kDa of molecular weight [20]. Moreover, YUH1 tagged with 6 histidines at the N-terminus precisely cut off a fusion protein of 6 histidine–ubiquitin-connective tissue activating peptide with 85 amino acids [18]. On the other hand, YUH1 expressed in recombinant *E. coli* and purified by an ubiquitin affinity chromatography partially hydrolyzed the *Arabidopsis* 52-amino acid ubiquitin extension protein [21]. To determine the optimal environmental conditions for the YUH1 reaction, activity assay was performed at various temperatures and pHs and the highest activity of YUH1 was achieved at 27 °C and pH 8.5. Interestingly, any deubiquitinating activity of YUH1 was not detected under pH 6.5, which coincided with the pH sensitive and alkaline UBP6 of *S. cerevisiae*. It was reported that the purified UBP6 of *S. cerevisiae* was maximally active at a range of pH 8.5–9.0, but no or little activity was detected below pH 7 and above pH 9 [22]. The highest activity of the truncated *S. cerevisiae* UBP1 expressed in *E. coli* was achieved at 40 °C and pH 8.0 in 100 mM sodium phosphate buffer [12]. Thermal deactivation kinetics showed that YUH1 had a short half life like other DUBs. A UCH from bovine erythrocytes lost 70% activity toward a substrate of histone H2A in isopeptide linkage with ubiquitin after preincubation for 1 h at 37 °C [23]. However, the enzyme treated under the same conditions kept the same activity toward the human ubiquitin carboxyl extension protein. The reason for the heat unstable and pH sensitive behavior of YUH1 was unclear and it was necessary to develop a method for the stable storage of YUH1 such as protein immobilization and stabilizer addition. The immobilization of YUH1 on a metal affinity resin and its incubation in 50% glycerol solution at −20 °C increased its stability by 2.7-fold in comparison with the free YUH1 incubated at 4 °C in the same solution. More research is in progress to increase the expression level of YUH1 in recombinant *E. coli* and to design an enzyme reactor for the deubiquitination of therapeutic proteins with high productivity.

Acknowledgments

This research was supported by the Korea Science and Engineering Foundation Grant (R01-2004-000-10221-0) and the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund, KRF-2006-005-J04702). Hyun-Ah Yu, Sung-Gun Kim and Eun-Jeong Kim were supported by Ministry of Education and Human Resources Development through the BK21 Program.

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