

Immobilization of Ulp1 protease on NHS-activated Sepharose: a useful tool for cleavage of the SUMO tag of recombinant proteins

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Abstract

Objective To fabricate an active and stable enzyme through covalent immobilization, a Ubl-specific protease (Ulp1) was used to cleave small ubiquitin-like modifier (SUMO) fusion proteins.

Results We immobilized Ulp1 on *N*-hydroxysuccinimide (NHS)-activated Sepharose with a coupling efficiency of 1.7 mg/ml. The immobilized Ulp1 maintains 95% substrate-cleavage ability and significantly enhances pH and thermal stability, especially can withstand pH of 10.5. Besides resistance against some small molecules, the immobilized Ulp1 can tolerate 15% (v/v) DMSO and 20% (v/v) ethanol. It can be reused for more than 15 batch reactions with 90% activity retention. This provides a fast purification system to quickly obtain cleaved recombinant proteins with 95% purity from cell lysates with the application of immobilized Ulp1.

Conclusions Ulp1 used in immobilization form is a potentially useful tool for cleavage of SUMO-tagged proteins and may reduce time and cost of protein purification.

Keywords Enzymatic cleavage · Extreme pHs tolerance · Immobilization · Thermal stability · Ubl-specific protease (Ulp1)

Introduction

The small ubiquitin-like modifier (SUMO) tag is often used in the fusion expression of insoluble and easy misfolding proteins, such as (SARS-CoV) 3CL protease, nucleocapsid and membrane proteins, just like GST, MBP and TRX fusion tags (Marblestone et al. 2006). However, no matter if the fusion tags are large or small, they will possibly interfere with the structure or normal functions of the fused proteins (Horchani et al. 2009). Therefore, protein tags sometimes need to be removed by chemical agents, proteases or possibly self-splicing intein. For example, ribosome-nascent chain complexes (RNCs) have been expressed with SUMO fusion technology and purified with Ni-NTA but the SUMO-tag needs to be cleaved for further NMR studies (Rutkowska et al. 2009).

The most commonly used proteases include enterokinase, tobacco etch virus (TEV) protease, thrombin, PreScission and Ulp1. Ulp1 was the first described SUMO protease in *S. cerevisiae* (Li and

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Hochstrasser 1999). It can cleave the epsilon-linked peptide bond between the C-terminal glycine of the mature SUMO and the lysine epsilon-amino group of the target protein (Malakhov et al. 2004). Ulp1 is expressed in soluble form, it is easy to use and is widely used for protein purification. However, there are some problems for the application of Ulp1: the activity of Ulp1 is gradually lost during preservation especially after long time storage, even at $-80\text{ }^{\circ}\text{C}$. Free Ulp1 cannot be reused, and further purification process is needed to remove Ulp1 and SUMO from cleaved proteins.

Covalent immobilization is often used to improve properties of enzymes, such as activity, stability and specificity, due to the formation of the irreversible covalent bond and the generation of a more rigid structure for multi-point covalent binding (Rodrigues et al. 2012). The N-terminal, epsilon-amino group of lysine residues, sulfhydryl group of cysteine residues, beta- or gamma- carboxyl group of aspartic acid and glutamic acid residues, and C-terminal are commonly used for immobilization of enzymes (Homaei et al. 2015). TEV protease can be immobilized through covalent immobilization on a solid matrix to promote its stability without loss of activity, which makes separation accomplished simply by centrifugation or filtration (Miladi et al. 2012).

We have now immobilized Ulp1 on N-hydroxy-succinimide (NHS)-activated Sepharose without decreasing its activity. The immobilization of Ulp1 gives enhanced thermal and pH stabilities, improves resistance to organic reagents and operational reusability, and it can be applied to fast purification of proteins without a tag.

Materials and methods

Materials

Sepharose 6B-CL and Hitrap Q FF were from GE Healthcare. All other chemicals and solvents are analytical or HPLC grade.

Protein purification

Ulp1^(403–621) was cloned into pET28a and expressed in *E. coli* BL21 (DE3). The Ulp1 protein was purified with Ni-NTA, followed by iron exchange with Hitrap

Q FF column and dialyzed against sodium phosphate buffer, pH 8.0. Substrates of Ulp1 have been simply purified by Ni-NTA.

Immobilization matrix synthesis and Ulp1 immobilization

N-Hydroxysuccinimide (NHS)-activated Sepharose, CHO-Sepharose, and CNBr-activated Sepharose were prepared as described (Besselink et al. 1993; Shaniff 1980; Hughes et al. 2001). Ulp1 was immobilized as indicated in Supplementary Fig. 1 and as given by McCarney et al. (2007).

Enzymatic activity determination

SUMO-GR (1 mg/ml) was cleaved by free (10^{-4} – 10^{-5} mg/ml) and immobilized Ulp1 in Ulp1 buffer (50 mM Tris/HCl, pH 8.0) at $4\text{ }^{\circ}\text{C}$ for 24 h or at $25\text{ }^{\circ}\text{C}$ for 12 h with gentle agitation. The cleavage efficiency was analyzed by 15% gel SDS/PAGE. For the time course study, free Ulp1/substrate ratio used was 1:1000, immobilized Ulp1/substrate ratio used was 3:1000. To determine the optimal reaction pH, free and immobilized Ulp1 cleavage assays were accomplished in Britton-Robinson buffer within pH 4.5–11. Scion Image was chose for quantitative analysis in this study.

Reusability of immobilized Ulp1

20 μl immobilized Ulp1 was incubated with 250 μg SUMO-GR lysate in 250 μl reaction buffer (50 mM sodium phosphate buffer, pH 8.0, 1 mM EDTA) at $25\text{ }^{\circ}\text{C}$ for 24 h. Beads were filtered and 40 μl taken from the supernatant was mixed with 10 μl $5\times$ loading buffer and then 5 μl was subjected to SDS/PAGE analysis. The filtered beads were washed with sodium phosphate buffer (pH 8) for three times, another 250 μg SUMO-GR lysate was added for next cleavage.

Fast purification strategy

SUMO-GR (150 μg) lysate was incubated with Ni-NTA beads in a micro-column for 15 min. After washing with sodium phosphate buffer, pH 8, 1% (v/v) NP-40 and equilibrating with 50 mM Tris/HCl (pH 8.0), the immobilized Ulp1 was added to the micro-

column and made the mixture fully reacted on a horizontal rotator for 15 min at 25 °C. Beads were filtered via centrifugation (12,000×g for 30 s) and the no-tag target protein was collected in the filtrate.

Results and discussion

Ulp1 immobilization

The molecular weight of Ulp1^(403–621) is 25.5 kDa. Considering H514 and C580 but not any lysine residues are important activity sites for Ulp1, immobilization strategies for protein coupling through ε-amino group of lysines were employed (Li and Hochstrasser 1999). After immobilization on NHS-activated Sepharose, CHO-Sepharose and CNBr-activated Sepharose (Supplementary Fig. 1), proteins recovered in the supernatant and offered to the support were subjected to SDS/PAGE (Fig. 1a). NHS-activated Sepharose was shown as the most efficient matrix for immobilization and the binding capacity was estimated to be 1.68 mg/ml of the matrix.

Assessment of free and immobilized Ulp1 activity

The activity of immobilized Ulp1 was tested; more than 95% of substrate was cleaved after overnight incubation at 4 °C compared to soluble Ulp1 (Fig. 1b). Due to the insufficient mixing of substrates, a 3-fold excess of immobilized enzyme as the soluble one was used. Figure 1c shows that the immobilized Ulp1 can give similar reaction velocities to free Ulp1. Both free and immobilized Ulp1 have a broad pH adaptation: they work well within pH 4.5–11 (Fig. 1d). More than 90% of the substrate could be cleaved, although a little activity decreasing for the immobilized Ulp1 at pH 11.

Effects of chemical reagents addition on enzyme activity

Since some organic reagents are used in the cell assays, we tested the effect of increasing concentration of DMSO up to 15% (v/v) and observed no significant effects on enzymatic activity of the immobilized Ulp1

(Fig. 2a). As for the free Ulp1, its activity was sharply decreased with DMSO up to 5% (v/v). We also examined the influence of ethanol (Fig. 2b) and found both free and immobilized Ulp1 work well when ethanol was up to 20% (v/v). Stability in these solvents indicates that DMSO or ethanol can be added to minimize bacterial growth and be useful for some catalyzing and modification reactions (Yang et al. 2014). Interestingly, SUMO was degraded when no DMSO was added while no perspective degradation existed after 10% DMSO was added (Supplementary Fig. 2). Therefore, presumably the existence of DMSO prevented the degradation of cleaved SUMO. Both free and immobilized Ulp1 are resistant to some small molecules such as NaCl, imidazole, DTT and urea (Supplementary Fig. 3a–d). These properties will be helpful for purifying some specific proteins (Rutkowska et al. 2009).

Storage stability of Ulp1

Activities of free and immobilized Ulp1 stored in sodium phosphate buffer pH 8.0 were checked. Figure 3a shows that the free Ulp1 lost about 84% of activity after 24 h at 37 °C. However, the immobilized Ulp1 retained 96% of activity even after 36 h. With storage at 25 °C free Ulp1 maintained about 50% of its initial activity over 7 days (data not shown) but lost 77% of its activity after 14 days, whereas only 7% decreasing of activity for the immobilized Ulp1 within the same period (Fig. 3b). Immobilized Ulp1 also showed good stability at 4 and 30 °C (Fig. 3b, Supplementary Fig. 4). Unexpectedly, cleaved SUMO (Fig. 3a, Supplementary Fig. 4) was degraded which is consistent to the speculation that DMSO will prevent degradation of cleaved SUMO. The cleavage efficiency of Ulp1 usually increases with the increase of temperature, the optimum temperature is generally 25–37 °C, and increasing hydrolysis time can reduce the cost of Ulp1. Ulp1 used in its immobilized form has greatly enhanced thermal stability and can be utilized in rapid cleavage and industrial applications (Kokufuta 1992).

The stability of free and immobilized enzymes stored for 24 h at various pH buffers were also examined (Fig. 4). The free Ulp1 retained full activity

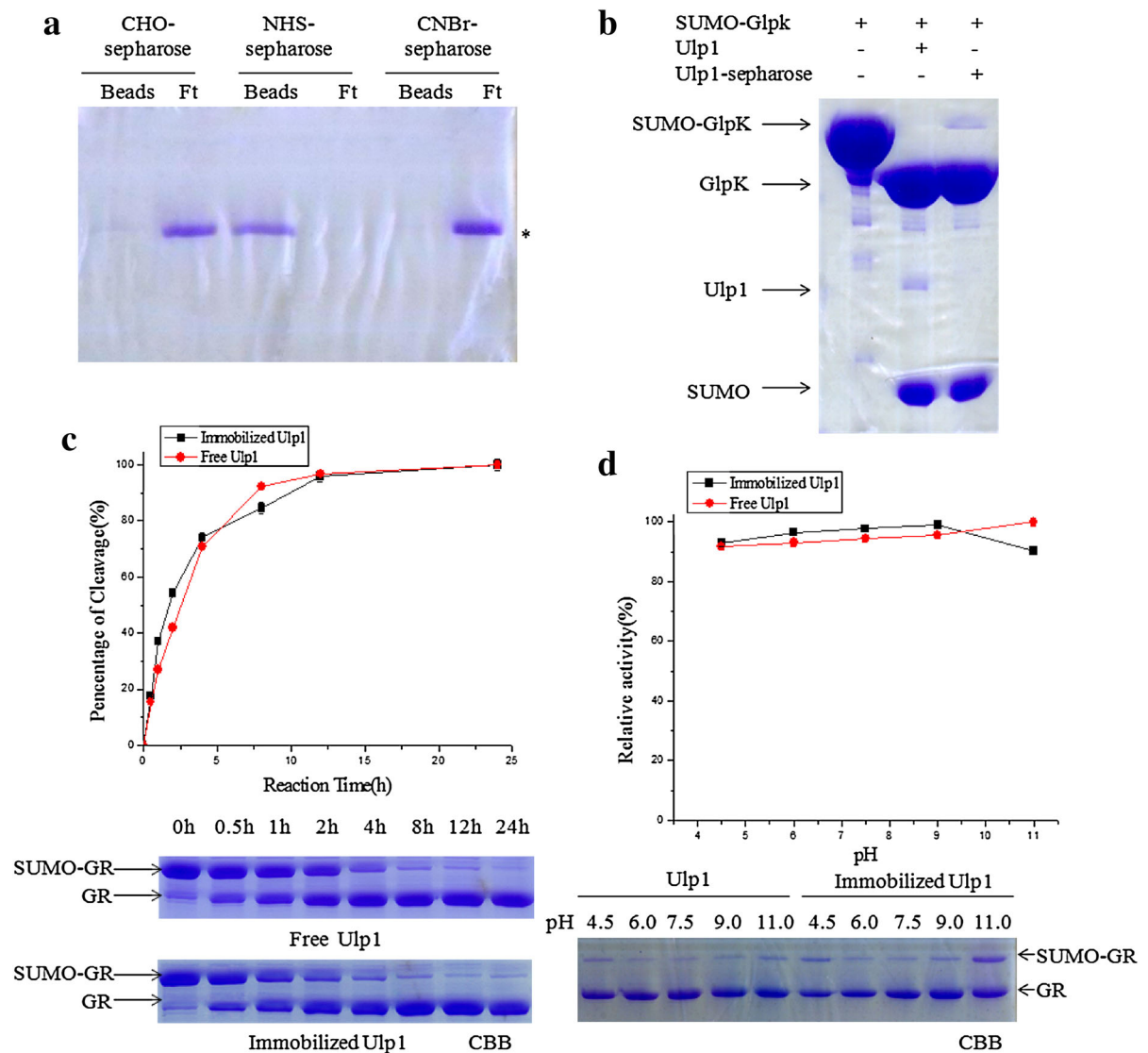


Fig. 1 The immobilization of Ulp1 and activity test. **a** SDS/PAGE analysis of the activity of Ulp1 immobilized on NHS-activated Sepharose. SUMO-Glpk was used as substrate and protein concentration was quantified by BCA Protein Assay Kit and Bradford method, using bovine serum albumin as standard. *Ft* flow-through fraction. *Asterisk* (*) marks Ulp1. **b** SDS/PAGE (15% gel) of Ulp1-substrate digested by free and immobilized

proteases. **c** Kinetic profile of free (*filled circle*) and immobilized (*filled square*) Ulp1 at 4 °C with the 3 ratio of immobilized Ulp1. **d** The effect of pH on the activity of free and immobilized Ulp1 were checked at different pHs ranging from pH 4.5 to 11.0 using B-R buffer at 4 °C. Gels were stained with Coomassie Brilliant Blue R-250

at pH 4.5–6.5, decreasing its activity slightly at pH 8.5 and lost most of its activity at pH 10.5. The immobilized Ulp1 retained full activity at pH 4.5–8.5, only a slightly activity decreasing at pH 10.5. It seems that

Ulp1 is an acid-tolerant protease and immobilization enhances its pH stability. Carrier-bound active group (CAG) used here was NHS. NHS can react with amino group of lysines to form stable amido bond and may

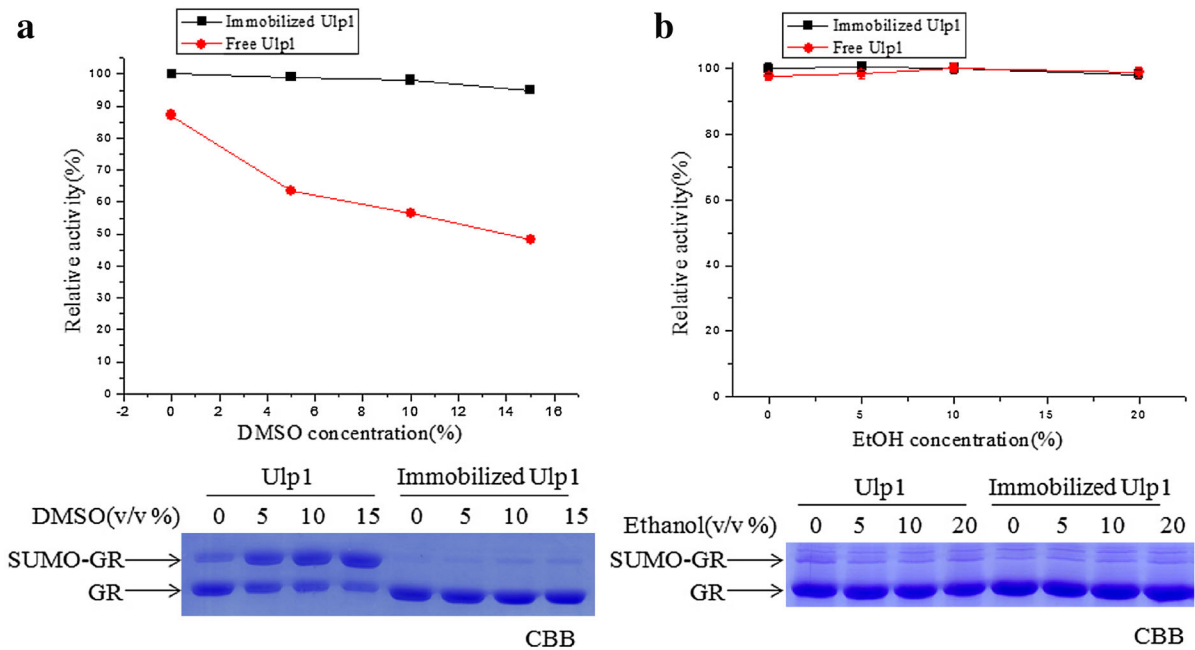


Fig. 2 Effects of organic reagents on enzyme activity. **a** The immobilized Ulp1 successfully endure 15% DMSO while free Ulp1 can't bear the DMSO. **b** Both free and immobilized Ulp1

show good performance in the exist of 20% ethanol. Gels were stained with Coomassie Brilliant Blue R-250

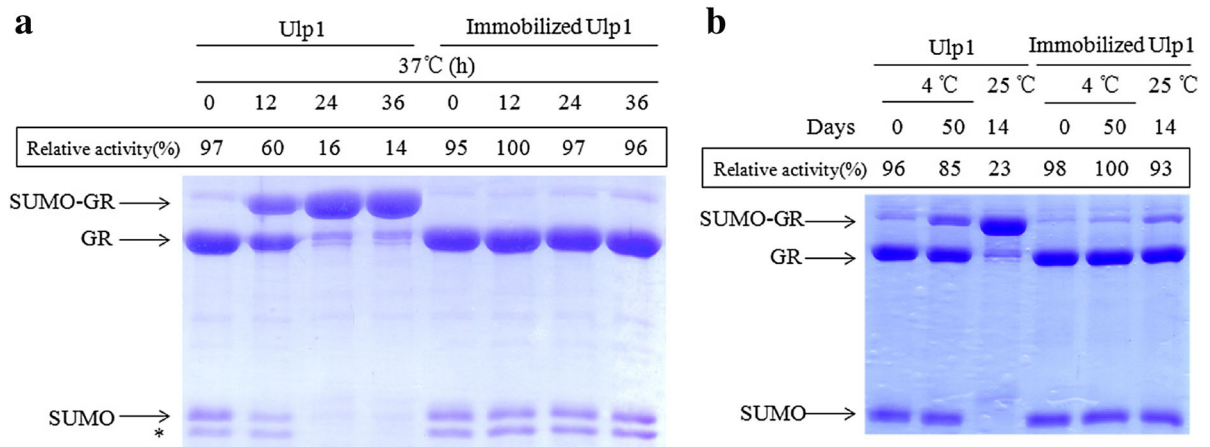


Fig. 3 Ulp1 stability at different temperatures. **a** The activity of free and immobilized Ulp1 were incubated at 37 °C during 36 h, the remaining activity was determined by cleavage assays. Asterisk (*) marks the degraded SUMO. **b** Free and immobilized

Ulp1 were incubated respectively at 4 °C for 50 days, and 25 °C for 14 days, the remaining activity was determined as described previously

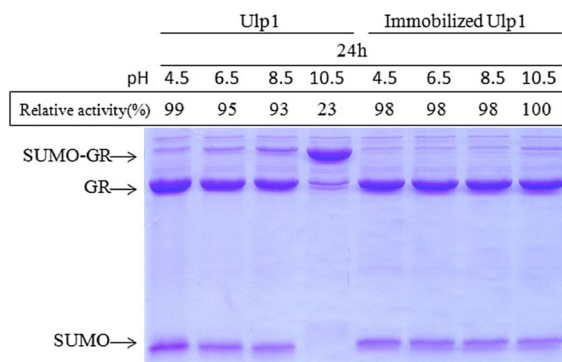


Fig. 4 Ulp1 pH tolerance. Stability at pH 4.5–10.5 [pH 4.5 (Britten-Robinson buffer), pH 6.5 (phosphate buffer), pH 8.5 (HEPHES/NaOH), pH 10.5 (glycine/NaOH)] for free and immobilized Ulp1 after 24 h incubation

improve structural stability of immobilized Ulp1 (Ferreira et al. 2003).

Reuse of immobilized Ulp1 and fast purification strategy

Reusability of immobilized Ulp1 was tested at 25 °C using SUMO-GR lysate as substrate. The immobilized Ulp1 retained over 90% of its starting

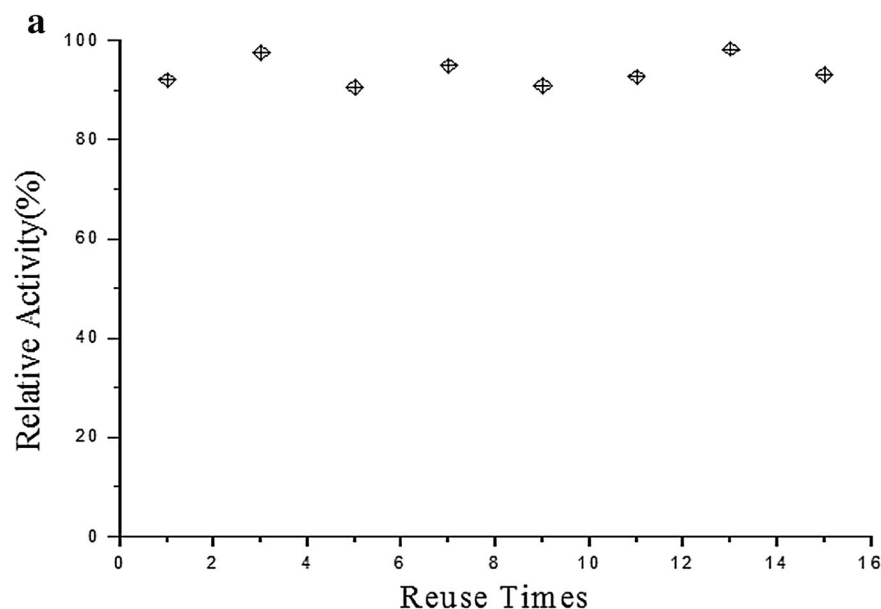


Fig. 5 Reuse of immobilized Ulp1 and GR quickly purification. **a** The immobilized Ulp1 showed reusability for more than 15 batch reactions. **b** Quickly purification of no-SUMO tag GR from lysate by immobilized Ulp1

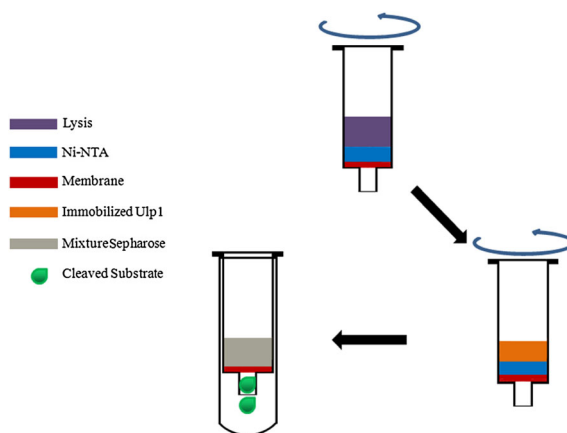


Fig. 6 Sketch map of fast purification column. Step 1, Ni-NTA beads was incubated with lysis and washed with sodium phosphate buffer, pH 8.0, 1% NP-40. Step 2, the immobilized Ulp1 was added and incubated for 15 min at 25 °C. Step 3, simple centrifugation to get purified no tag protein

activity after 15 reuses within 15 days (Fig. 5a). High operational stability of the immobilized Ulp1 was considerably enhanced by the long linker of NHS attached to Sepharose to form a micro-environment favorable for retention of Ulp1 activity (Ferreira et al. 2003).

Fast purification strategy applied in no-tag GR purification (Fig. 6) gives pure products within 1 h (Fig. 5b). This way may be a feasible approach to replace the streamlined method in industrial uses to produce various no-tag proteins at the same time. The repeated use of immobilized Ulp1 makes it possible to reduce the cost of purification, so it is of value for industrial productions (Vandamme 1989).

Conclusions

Immobilization of Ulp1 on NHS-activated Sepharose improves stability and tolerance to high temperature. These changes enable immobilized Ulp1 to be used for more than 15 times. Immobilized Ulp1 retained more than 90% activity after 50 days storage at 4 °C and more than 14 days storage at 25 °C. In addition, immobilized Ulp1 stable within extreme pHs and tolerant to organic reagents. Purification of SUMO tagged proteins is achieved within 1 h using the immobilized Ulp1 and Ni-NTA Sepharose.

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Supporting information Supplementary Fig. 1—Different immobilization strategies for Ulp1.

Supplementary Fig. 2—DMSO inhibits the degradation of SUMO.

Supplementary Fig. 3—The influence of small molecules on the activity of Ulp1.

Supplementary Fig. 4—Storage stability of free and immobilized Ulp1 at 30 °C.

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