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REGULAR PAPER

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Expression of adenylate kinase fused mouse ubiquitin active enzyme in *Escherichia coli* and its application in ubiquitination

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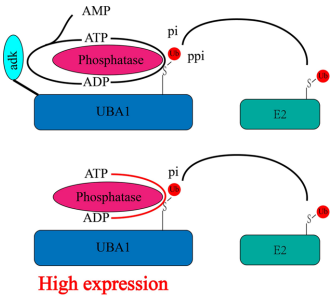
ABSTRACT

Ubiquitination is involved in the regulation of numerous cellular functions. Research works in the ubiquitin realm rely heavily on ubiquitination assays *in vitro* and require large amounts of ubiquitin-activating enzyme (UBA1) and keep ATP supplies. However, UBA1 is hard to be obtained with large quantities using reported methods. We fused *Escherichia coli* adenylate kinase (adk) and mouse UBA1 and obtained fusion protein adk-mUBA1. The expression level of adk-mUBA1 increased about 8-fold compared with mUBA1 in an *E. coli* expression system, and adk-mUBA1 was easily purified to 90% purity via 2 purification steps. The purified adk-mUBA1 protein was functional for ubiquitination and could use ATP in addition to ADP as energy supply and had a higher catalytic activity than mUBA1 in cell lysis. adk-mUBA1 can be applied to preparing ubiquitin-modified substrates and kinds of ubiquitin chains in a chemical synthesis process and is a preferable application than mUBA1 *in vitro* ubiquitination.

Graphical Abstract



High expression of adk-mUBA1 and its application in ubiquitination.

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Keywords: adk, mUBA1, ubiquitination, ATP, purification

Protein modification by ubiquitin (Ub), a process known as ubiquitination, is involved in the regulation of numerous cellular functions, including protein stability, cell-cycle progression, receptor transport, gene transcription, immune responses, and viral infection (Bennett and Harper 2008; Komander and Rape 2012; Moudry *et al.* 2012; Dobbelstein and Moll 2014; Groen and Gillingwater 2015; Kumbhar *et al.* 2018). Protein ubiquitination is a post-translational modification (PTM) in which the 76-amino acid protein Ub is covalently attached via its carboxyl terminus to usually Lys residues in target proteins (Michelle *et al.* 2009). Ub, as a single entity, can be attached to proteins on 1 or multiple sites, yielding mono- or multi-monoubiquitinated proteins, respectively. Polyubiquitination is the formation of a ubiquitin chain on target proteins. In a polyubiquitin chain, ubiquitin molecules can be linked through 1 of the 7 ubiquitin Lys residues (which are Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) or through the ubiquitin amino-terminal Met1 residue (Ikeda and Dikic 2008; Komander and Rape 2012).

Ubiquitination is brought about by ubiquitin-activating enzymes (UBAs), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligase enzymes (E3s). These families of proteins are part of a pyramid-like organization of enzymes, with 2 UBAs containing UBA1 and UBA6 at the top, about 40 E2s in the middle, and more than 600 E3s at the bottom (Michelle *et al.* 2009). In the first step of ubiquitination, the ATP-dependent activation reaction, ubiquitin is covalently attached to a cysteine residue of the UBA component through a thioester bond. In the second step, the activated ubiquitin molecule is transferred to an E2 through a transthiolation reaction. In the third and final step, ubiquitin is transferred from the E2 to a protein substrate, a reaction catalyzed by E3s (Ikeda and Dikic 2008).

Research works in the ubiquitin realm rely heavily on ubiquitination assays *in vitro* (Beaudenon and Huibregtse 2005; Carvalho *et al.* 2011). A key component in these assays is, of course, the UBA1 component, a 100-120-kDa protein. Two main strategies to obtain purified mammalian UBA1 are currently

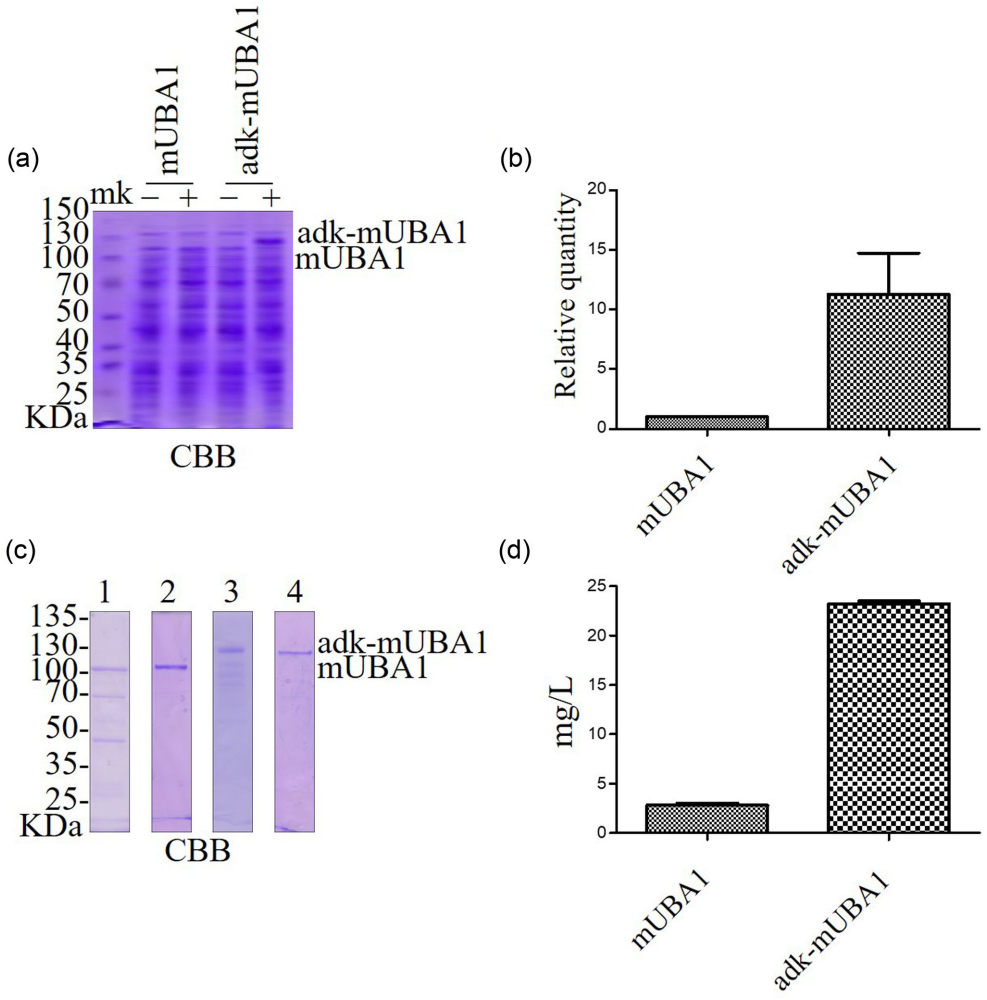

Figure 1. The expression and purification of mUBA1 and adk-mUBA1. (a) 10.5% SDS-PAGE analysis of mUBA1 and adk-mUBA1 expression. pET28a mUBA1 and pET28a adk-mUBA1 plasmids transformed into BL21 (DE3) codon plus cells were expressed with or without IPTG induction as indicated. (b) Relative quantity assay and the expression level of mUBA1 and adk-mUBA1. (c) The purification of mUBA1 and adk-mUBA1 used Ni TED 6FF (lines 1 and 3) and cascaded Q FF column (lines 2 and 4). (d) Purified protein quantity of mUBA1 and adk-mUBA1 per liter *E. coli*. Error bars in (b) and (d) represent the SD of at least 3 independent experiments.

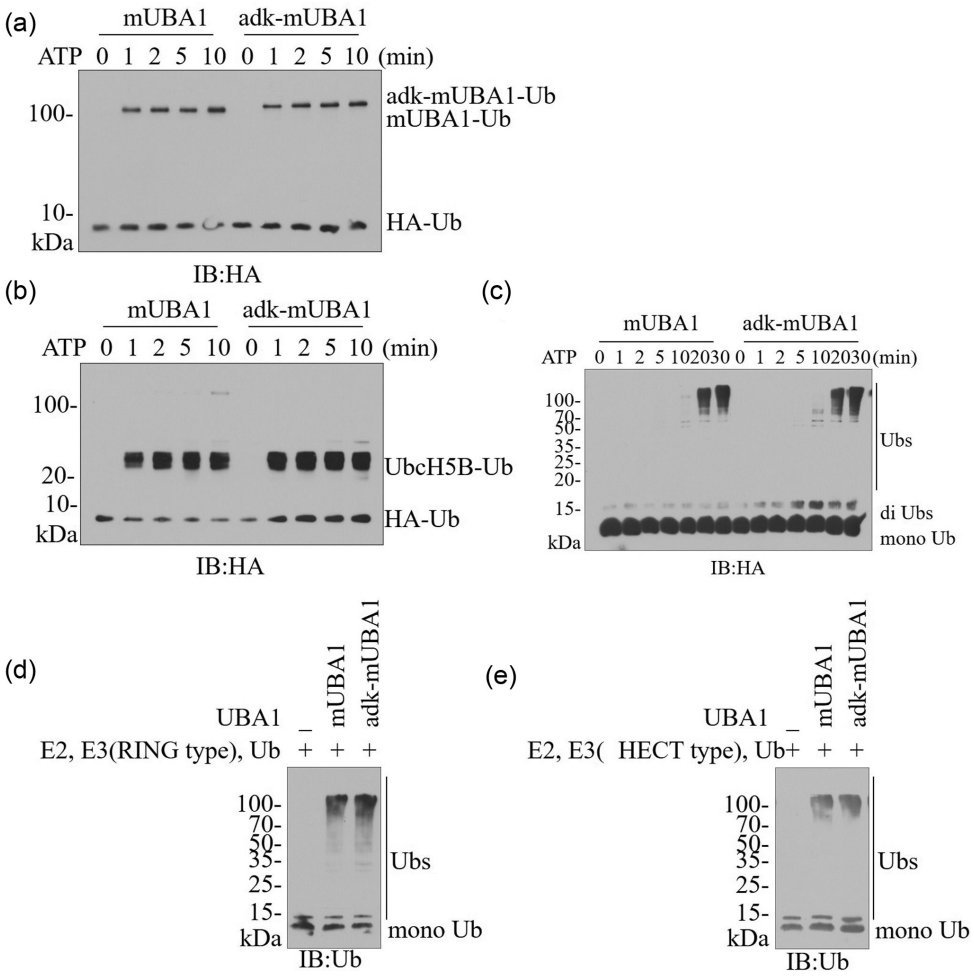

Figure 2. Ubiquitination assay of mUBA1 and adk-mUBA1. (a) Compared mUBA1 and adk-mUBA1 charge ubiquitin at different times. The reactions were stopped by SDS loading buffer (without DTT). (b, c) Compared mUBA1 and adk-mUBA1 transfer ubiquitin to UbcH5 and UBE2S: stopped by SDS loading buffer—without DTT (b) and with DTT (c). (d, e) Compared mUBA1 and adk-mUBA1 transfer ubiquitin to different type E3 ubiquitin ligases (RING-type and HECT-type) and synthesized polyubiquitin chains. Both reactions were stopped at 10 min by SDS loading buffer (with DTT). All of these reactions were analyzed with 13.5% SDS-PAGE.

used. The first strategy explores the efficiency of covalent affinity chromatography and uses human erythrocytes, rabbit reticulocytes, or other UBA1-rich biological materials as sources of the enzyme. Approximately 1 mg of UBA1 per liter of outdated blood can be obtained using this procedure. The second strategy consists of expressing tagged versions of mammalian UBA1 in an appropriate host organism followed by an affinity chromatography purification step. Both *Escherichia coli* and baculovirus/insect cell–based protein expression systems have been used to obtain recombinant UBA1, but the latter, despite being much more laborious and expensive, is by far the most frequently employed. Approximately 5 µg of UBA1 per 15 cm^2 plate of subconfluent cells is obtained using the baculovirus/insect cell–based expression system. Expression of rabbit UBA1 (rUBA1) in *E. coli* yielded only 0.05-0.1 mg of enzyme per liter of culture (Beaudenon and Huibregtse 2005).

ATP supply, *in vitro* ubiquitination, is also an important issue. ATP is hydrolyzed to AMP and PPi by UBA1 to adenylate ubiquitin, is unstable at room or reaction temperature, and is quickly hydrolyzed to ADP by ATP enzymes contained in reaction components (Tokgoz, Bohnsack and Haas 2006). All of these reasons make ATP hydrolyzed in a short time and the ubiquitination *in vitro* difficult.

Adenylate kinase (adk) is an essential phosphotransferase in species from bacteria to mammals. It catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP, coordinating macromolecular synthesis and the production of adenosine triphosphate (Kupriyanov, Ferretti and Balaban 1986). In foregoing research, we found that the active adk from *E. coli* could be expressed in a soluble form at a high level with little toxicity to *E. coli* cells (Yang and Li 2017).

Here, we show that adk fused mouse UBA1 (adk-mUBA1) not only greatly improved the expression of UBA1 in *E. coli*, providing a direction for the industrial production of UBA1 and other hardly expressed proteins, but also enabled UBA to be used in more complex reactions.

Materials and methods

Plasmids, antibodies, and reagents

The adk gene amplified from *E. coli* BL21(DE3) and was cloned into pET28a vector between Nde1 and BamH1 colon sites, and contributed a new vector named pET28a adk. mUBA1 cDNA was amplified from a mouse cell L5178Y ± tk cDNA library and separately cloned into pET28a and pET28a adk. pSumo

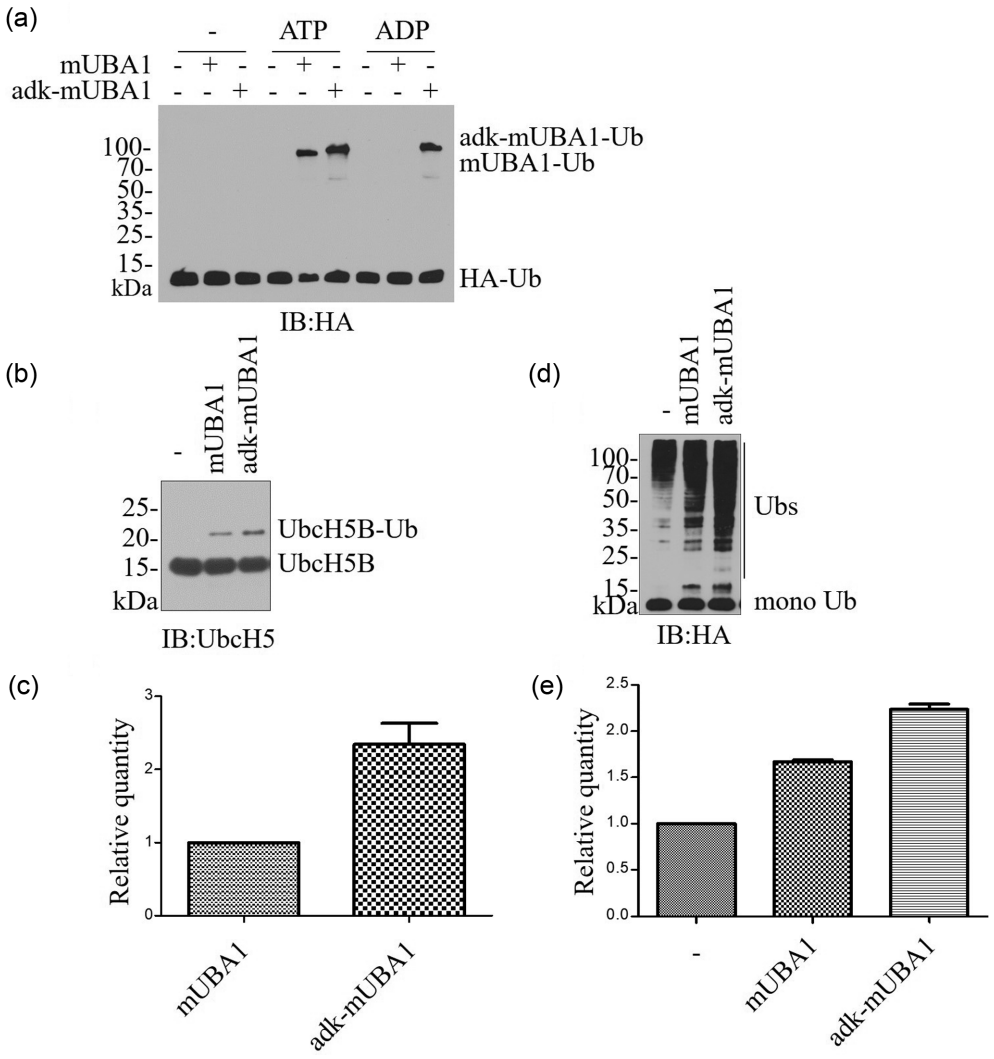

Figure 3. Ubiquitination assay of mUBA1 and adk-mUBA1 under different conditions. (a) Without or with mUBA1 or adk-mUBA1 charged ubiquitin in ATP, ADP, or no energy source conditions. Reactions were stopped at 10 min by SDS loading buffer (without DTT) and analyzed with 13.5% SDS-PAGE. (b and c) UbcH5B C85S charged ubiquitin in reaction buffer plus HA-ubiquitin and HeLa cell lysis (final concentration 5 mg/mL) and were transferred ubiquitin by mUBA1 or adk-mUBA1. Reactions were stopped at 10 min by SDS loading buffer (continue DTT). UbcH5B-ubiquitin was relatively quantitated and analyzed as (c). Level of UbcH5B-ubiquitin was transferred by mUBA1 and set to 1. (d, e) Reaction in HeLa cell lysis. 100 nM mUBA1 and adk-mUBA1 respectively added to reaction buffer plus 2 μM HA-ubiquitin and HeLa cell lysis (final concentration 5 mg/mL). Reactions were stopped at 10 min by SDS loading buffer (with DTT) and analyzed with 13.5% SDS-PAGE. Polyubiquitin chains were relatively quantitated and analyzed as (e). Level of polyubiquitin chains was synthesized without adding mUBA1 and set to 1. Error bars in (c) and (e) represent the SD of at least 3 independent experiments.

HA-Ub (HA-epitope tagged ubiquitin), pET28a UbcH5B (ubiquitin-conjugating enzyme E2 D2, also called UBE2D2), pET28a Ube2s (ubiquitin-conjugating enzyme E2 S) were stored in laboratory stocks. All plasmids were verified by DNA sequence. Purified His-Ube2s, His-UbcH5B, His-UbcH5B C85S, and HA-Ub were stored in laboratory stocks. Antibodies for Ub and Ube2D were obtained from Santa Cruz Biotechnology. The HA Epitope antibody was obtained from Biolegend. Ni-TED was from HUIYAN Bio and the anion exchange columns were from GE Healthcare. All of the other chemicals were Sigma-Aldrich products unless noted.

Protein expression and purification

The *E. coli* BL21 codon plus (DE3) strain was used as the host for the expression of recombinant adk-mUBA1 or mUBA1. Protein expressions were induced for 16 h at 16 °C with 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) after OD600 reached 0.6. Cell pellets were harvested by centrifugation at 9000 *g* for 20 min and resuspended in 1/25 vol of buffer A (50 mM PBS8.0, 1 mM DTT, 5 mM EDTA). The cells were lysed by sonication and cell debris was removed by centrifugation at 16 000 *g* for 30 min at 4 °C. Protein extracts were passed through a Ni-TED column (HUIYAN). After washing with buffer B (50 mM PBS8.0, 1 mM EDTA, 5 mM DTT, and 20 mM imidazole) to remove unbound proteins, proteins were eluted with buffer C (50 mM Tris-HCl, 200 mM imidazole, and pH 8.0) and subjected to SDS-PAGE analysis with Coomassie Blue staining.

To obtain pure proteins, samples eluted from Ni-TED column were loaded on a 5 mL QFF column (GE Healthcare), which was connected to the AKTA-Prime purification system (GE Healthcare). Unbound proteins were washed with buffer D (50 mM Tris-HCl, 15 mM NaCl, 1 mM EDTA, 1 mM DTT, and pH

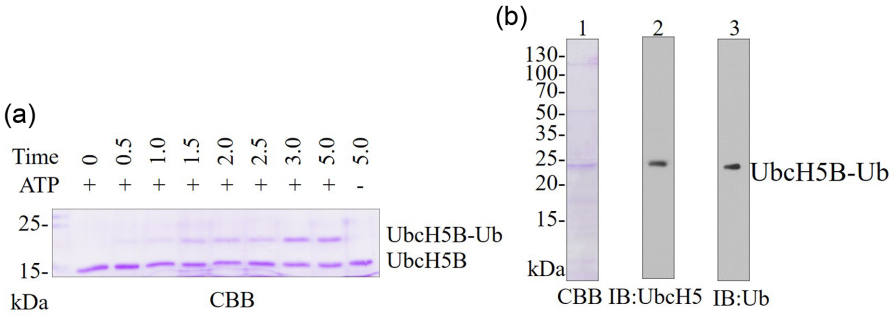

Figure 4. Production of UbcH5B-ubiquitin *in vitro*. (a) adk-mUBA1 transferred ubiquitin to UbcH5B C85S. The reaction was stopped at different times up to 5 h by SDS loading buffer (contain DTT) and analyzed with 16.5% SDS-PAGE. (b) adk-mUBA1 transferred ubiquitin to UbcH5B C85S and analyzed UbcH5B-ubiquitin. Products were tandem affinity purified by Ni-NTA and HA beads.

8.0) until OD280 detection declined to near baseline. Finally, the protein was eluted with buffer E (50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and pH 8). For each step of the purification process, the flow rate was at 2 mL/min. All purified proteins were stored with 20% Glycerol at −80 °C prior to use in the assays.

In vitro ubiquitination assay

Ubiquitination assays were carried out in a reaction buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT 1 mM ATP, and 1.5 mg HA-epitope tagged ubiquitin). For ADP as energy source assay, Ubiquitination assays were carried out in a reaction buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT 1 mM ADP, and 1.5 mg HA-epitope tagged ubiquitin). For UBA1 charge Ub assay, 10 nM adk-mUBA1 or mUBA1 added to the reaction buffer, and stopped the reaction at a different time with SDS-PAGE sample loading buffer (DTT free) and analyzed by immunoblotting using an anti-HA antibody. For UbcH5B charge Ub assay, 2 nM adk-mUBA1 or mUBA1 and 10 nM UbcH5B added to the reaction buffer, and stopped the reaction at a different time with SDS-PAGE sample loading buffer (DTT free) and analyzed by immunoblotting using an anti-HA antibody. For Ube2S synthesize polyubiquitin chains assay, 2 nM adk-mUBA1 or mUBA1 and 10 nM Ube2s added to the reaction buffer, and stopped the reaction at a different time with SDS-PAGE sample loading buffer (0.1 M DTT) and analyzed by immunoblotting using an anti-HA antibody. For E3 synthesize polyubiquitin chains assay, 2 nM adk-mUBA1 or mUBA1 and 10 nM UbcH5B, 20 nM LRSAM1 (RING-type) or SlrP (HECT-type) added to the reaction buffer, and stopped the reaction at 15 min with SDS-PAGE sample loading buffer (0.1 M DTT) and analyzed by immunoblotting using an anti-HA antibody. For synthesizing Ub chains in cell lysis assay, 5 nM adk-mUBA1 or mUBA1 and cell lysis (final concentration: 5 mg/mL) added to the reaction buffer, and stopped the reaction at 60 min with SDS-PAGE sample loading buffer (0.1 M DTT) and analyzed by immunoblotting using an anti-HA antibody. For synthesizing UbcH5B-Ub in cell lysis assay, 5 nM adk-mUBA1 or mUBA1 added to the reaction buffer, 10 mM UbcH5B, cell lysis (final concentration: 5 mg/mL) and stopped the reaction at 60 min with SDS-PAGE sample loading buffer (0.1 M DTT) and analyzed by immunoblotting using an anti-Ube2D antibody.2.4 synthesized and purified UbcH5B-Ub.

For synthesizing UbcH5B-Ub, 10 nM adk-mUBA1 or mUBA1 and 20 nM His-UbcH5 added to the reaction buffer, and stopped the reaction at a different time with SDS-PAGE sample loading buffer (0.1 M DTT) and analyzed by Coomassie Brilliant Blue (CBB) staining. For purifying UbcH5B-Ub, the reaction was stopped at 5 h with 20 mM EDTA, and purified with Ni-TED plus HA beads. Finally, the protein was analyzed by CBB staining or immunoblotting using an anti-UbcH5 and anti-Ub antibody.

Results

Cloning, expression, and purification of adk-mUBA1 in *E. coli*

As shown previously, a pET28b plasmid containing the coding region of mUBA1 can be used to produce the active enzyme in *E. coli*. However, the expression levels attained with this plasmid are quite low (Beaudenon and Huibregtse 2005). For this purpose, we cloned adk cDNA from *E. coli* to pET28a and constructed a vector pET28a adk. Then, mUBA1 cDNA was cloned into pET28a and pET28a adk, and resulting plasmids, pET28a mUBA1 or pET28a adk-mUBA1, were transformed into BL21 codon plus (DE3) *E. coli* cells for protein expression. As our prediction, induction of protein expression using standard conditions (ie 0.5 mM IPTG, 16 °C, 16 h) resulted that the expression level of adk-mUBA1 is about 10 times higher than that of mUBA1. adk-mUBA1 accounts for 10% of the total proteins and was easily find with Coomassie blue stain (Figure 1a and b). We used the Ni-TED column to affine purification adk-mUBA1 or mUBA1, and giving proteins with 60% and 40% purity, respectively (Figure 1c, lines 1 and 3). To obtain purer proteins, an anion-exchange column was used. Finally, we harvested adk-mUBA1 fusion protein at about 24 mg per liter *E. coli* with purity 90% and mUBA1 at about 3 mg per liter *E. coli* with 90% purity (Figure 1c, lines 2 and 4, and Figure 1d).

Ubiquitination activity of adk-mUBA1

Protein fusion may change protein structure and influence protein functions(Sabourin *et al.* 2007). adk fused mUBA1 can enhance its expression level, but does it influence the activity of mUBA1? The functions of UBA1 are activating ubiquitin and sending Ub to E2s *in vitro* ubiquitination assay. We found that adk-mUBA1 and mUBA1 can activate the same amount of Ub in the same amount of time, and that means they have the same Ub activating enzyme activity (Figure 2a). We also found that adk-mUBA1 and mUBA1 can transfer activity Ub at the same rate to E2(UbcH5B) or though E2(Ube2S) to synthesize polyubiquitin chains, and that signify fused adk does not influence the Ub transfer rate of mUBA1 (Figure 2b and c). Of course, fused adk does not influence the Ub transfer rate though E2 (UbcH5B) to HECT-type or RING-type of E3(ubiquitin transferase) (Figure 2d

and e). All of these tests illustrated that fused adk does not influence the activity of mUBA1 *in vitro* ubiquitination (Figure 2).

adk-mUBA1 applications

In vitro ubiquitination has been used for many purposes, such as to finding substrates of a known E3 or E3s of substrate proteins that are modified with Ub. In these reactions, cell lysis has been used to supply substrates or E3s. Because cell lysis is very complex and contains a lot of ATP enzymes, these can hydrolyze ATP to ADP and pi in a short time, and UBA1 does not have enough ATP as an energy source to active Ub. adk can catalyze 2 mol ADPs to produce 1 mol ATP and AMP. adk-mUBA1 not only uses ATP as an energy source but can also utilize ADP as an energy source to active Ub (Figure 3a). In order to verify the function of adk-mUBA1 in cell lysis, we mutated cysteine 85 of UbcH5B to serine (UbcH5B C85S) as a stable receptor of active Ub. As we excepted, compared with mUBA1, adk-mUBA1 can transfer more than 2 times ubiquitin to UbcH5B C85S (Figure 3b and c). When we added adk-mUBA1 or mUBA1 in cell lysis to detect the poly ubiquitin chains by cell lysis E2s and E3s, we found that polyubiquitin chains synthesized by adk-mUBA1 were more than 2 times than mUBA1 (Figure 3d and e).

E2–Ub complex has been used in many labs but its preparation was not easy. Here, we modified His-UbcH5B C85S with HA-tagged Ub (HA-Ub) by adk-mUBA1. In 5 h at 37 °C, HA-Ub-modified his-UbcH5B C85S (UbcH5B-Ub) rises to the highest rate. Stopping the reaction with 10 mM EDTA and purifying the reactants with the Ni-TED column and HA-Beads Tandem affinity, we obtained UbcH5B-Ub with >90% purity (Figure 4).

Discussion

Here, we show that about 24 mg of active recombinant adk-mUBA1 can be produced per liter *E. coli*. The high-yield expression observed in this study is probably related to the expression vector used to produce the enzyme. Because both pET28a mUBA1 and pET28a adk-mUBA1 have same carrier skeleton and promoter, adk-mUBA1 having a higher expression level may be due to differences in translation rates. The addition of adk may be more conducive to the translation of UBA1 in *E. coli*. adk-mUBA1 having a higher expression level may also be due to the differences of solubleness or stability because adk was reported as a solubility tag and has little toxicity to host cells (Liu *et al.* 2015). It was also found that adk fused proteins such as human mitogen-activated protein kinase kinase 1 and T4 DNA ligase can promote the expression level (Liu *et al.* 2015; Yang and Li 2017).

Because of the instability of ATP, many ubiquitination reactions use the creatine/creatine kinase system to provide ATP. However, in the presence of a large number of ATP hydrolases, the capacity provided by the creatine/creatine kinase system is not sufficient to meet the needs of the ubiquitination reaction for ATP. With the fusion of adk and UBA1, the ATP catalyzed by adk can be directly utilized by UBA1, thus completing the supply of ATP more efficiently and facilitating the ubiquitination.

It has been reported that the expression of UBA1 in insect cells can be improved through fusing glutathione S-transferase (GST) tag (Beaudenon and Huibregtse 2005). We have previously attempted to express the fused protein GST-mUBA1 in *E. coli* and found that GST tag also promoted the mUBA1 expression level (data not shown). Like GST, other usual tag proteins may enhance the expression of mUBA1. However, the adk-mUBA1 not only has a higher expression level but also has higher ubiquitination activity. So, adk is an excellent soluble and promoting expression tag to purify mUBA1.

In a word, the fusion of adk and mUBA1 not only greatly improved the expression of mUBA1 in *E. coli*, providing a direction for the industrial production of mUBA1, but also enabled mUBA1 to be used in more complex reactions, providing a good tool for the study of *in vitro* ubiquitination.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Author contribution

H.L. and X.L. conceived and designed experiments; L.H. and X.L. performed experiments, analyzed data, prepared figures, and helped with writing of the manuscript; and Y.Z. performed experiments.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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