Synthesis of K48-linked diubiquitin using dual native chemical ligation at lysine†

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The dual native chemical ligation at lysine strategy was revised by replacing the acid-labile Cbz protecting group with photolabile NVOC at the 4-mercaptolysine side chain. The optimized strategy was subsequently applied to the synthesis of K48-linked diubiquitin.

Ubiquitination is one of the most important protein post-translational modifications.[1] It refers to the linking of the C-terminal glycine of the ubiquitin protein (76 amino acids) to the lysine side chain of the modified proteins. Proteins can undergo monoubiquitination or polyubiquitination.[1] As a well-known example of monoubiquitination, the modification at the C-terminal region of histone H2A is associated with gene silencing.[2] The monoubiquitination at the C-terminus of H2B was shown to be associated with transcription elongation.[3] In the case of polyubiquitination, a chain consisting of multiple ubiquitins with defined linkage is attached to the lysine side chain of a modified protein target.[4] Ubiquitin contains seven lysine residues (K6, 11, 27, 29, 33, 48, 63) and each of these lysine residues can be employed for ubiquitin chain formation. The biological roles of polyubiquitin may be related to its linkage specificity.[4] K48-linked polyubiquitin is the best understood polyubiquitination and is shown to serve as a signal for targeting the protein to proteasomal degradation.[5] In contrast, K63-linked polyubiquitination is involved in a signaling pathway and associated with kinase activation.[6] To elucidate the biological role of ubiquitination, it is important to isolate a sufficient amount of ubiquitinated proteins for *in vitro* study. In eukaryotes, ubiquitination is catalyzed by three classes of enzymes, ubiquitin-activating enzymes E1, ubiquitin-conjugating enzymes E2 and ubiquitin ligase E3.[1] *In vitro* enzymatic synthesis of ubiquitinated proteins is limited by the requirement for identifying the ligases and the availability of the ligase. In recent years, chemical ubiquitination was succeeded by either Nα-auxiliary-mediated[7] or mercaptolysine-mediated[8] peptide ubiquitination. These methods generate the native Gly76–Lys(ε) isopeptide bond which is typical for ubiquitination. Cysteine mediated ligation/desulfurization enables the synthesis of Gly76Ala monoubiquitinated proteins.[9] Essentially, all these methods are based on the native chemical ligation (NCL) approach.[10] Recently, chemical ubiquitination employing a disulfide linkage was also reported.[11] These two forms of non-native monoubiquitination were shown to exert a similar effect to the native form.

Previously, we reported the synthesis of a monoubiquitinated peptide through dual native chemical ligation at lysine.[8a,12] A lysine derivative with a thiol group at C-4 of the lysine side chain was used to mediate the chemical ligation at both the α- and ε-amino group of lysine. While the ligation at the α-amino group enables the chemical synthesis of the target protein itself, the side chain ligation can be used for the site-specific installation of the ubiquitin tag. In the previous study, we employed benzyloxycarbonyl (Cbz) as the orthogonal protecting group for the ε-amino group of 4-mercaptolysine to make sure that the ligation first selectively occurred at the α-amine. Then the Cbz group was removed with a strong acid, such as trifluoromethanesulfonic acid (TFMSA), to free the ε-amine for the side chain ligation. We realize that the harsh conditions for Cbz removal may limit the application scope of our methodology for large protein ubiquitination. In this communication, we revise our orthogonal protection strategy by replacing Cbz with a photolabile protection group, *o*-nitroveratryloxycarbonyl (NVOC) (Fig. 1). We then apply our revised approach for the synthesis of K48-linked diubiquitin.

The *N*ε-NVOC protected 4-mercaptolysine derivative **1** was synthesized in a similar way to that previously reported[8a] by using NVOC-Cl instead of Cbz-Cl as the side chain protecting reagent (For the details, see Electronic Supplementary Information).

The overall strategy of synthesizing the diubiquitin is shown in Scheme 1. To synthesize K48-linked diubiquitin (**8**) using the dual NCL at lysine method, the crucial step is the installation of 4-mercaptolysine at position 48 of the monoubiquitin. The synthesis of ubiquitin and its mutants through stepwise synthesis or sequential chemical ligation/desulfurization has been reported.[13] Here, we synthesized the K48(4-SH)-containing ubiquitin (**6**) using C-to-N sequential ligation with Ala28Cys and K48(4-SH) as the ligation junctions. After we got **6**, the NVOC group was removed by 365 nm UV irradiation. Monoubiquitin **7** with free ε-NH_2 on K48 was then reacted with ubiquitin thioester Ub(1–76)-MES

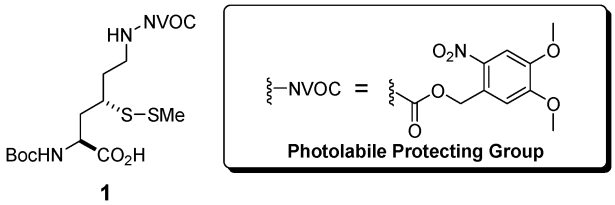

Fig. 1 *N*ε-NVOC protected 4-mercaptolysine derivative.

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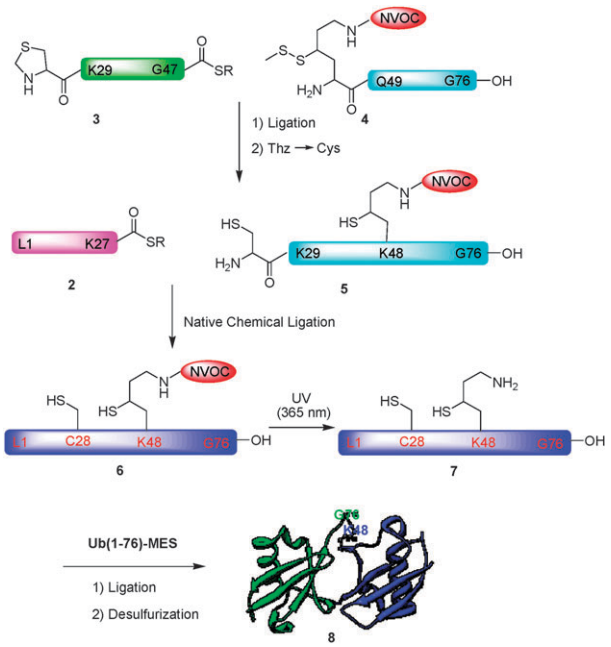

Scheme 1 The strategy used for the synthesis of K48-linked diubiquitin. Note: the Met1 of the synthesized ubiquitin was changed to Leu to avoid the oxidation.

which was generated by thiolysis of ubiquitin–intein fusion protein with sodium mercaptoethanesulfonate (MESNa). To generate the native K48-linked diubiquitin, free radical mediated desulfurization[14] was performed for the ligation product to convert Cys28 to Ala and K48(4-SH) to Lys.

HPLC data for the synthesis of **6** are shown in Fig. 2. The middle segment of ubiquitin, peptide $^{\alpha}$thioester **3**, was reacted with C-terminal segment **4** through 4-mercaptolysine mediated ligation. 8 mg of **3** and 10 mg of **4** were dissolved in ligation buffer (6 M Gdn·HCl, 0.2 M phosphate, 20 mM TCEP, pH 7.5). In the presence of TCEP, the disulfide in **4** was immediately reduced and gave the free thiol. Upon addition of the thiol additive MESNa (0.2 M), **3** was converted to its MES thioester. The ligation proceeded efficiently. After 12 h, the reaction was completed. The conversion of Thz (1,3-thiazolidine-4-carboxo group) to Cys was directly performed in the ligation mixture. 0.4 M $MeONH_2$·HCl (final concentration) was added into the mixture and the pH was adjusted to 4.0. After 4.5 h at room temperature, the deprotection was completed (Fig. 2C). Product **5** was purified by C18 semi-preparative HPLC. About 10 mg of ligation product was obtained after lyophilization. For the next ligation step, 7.3 mg of peptide thioester **2** and 10 mg of **5** were dissolved in the ligation buffer (6 M Gdn·HCl, 0.2 M phosphate, 0.2 M MESNa, 20 mM TCEP, pH 7.5). The reaction was completed overnight. The major side reaction was the self-cyclization of **2** involving possibly the C-terminal lysine residue. The full-length ubiquitin **6** was purified by C18 semi-preparative HPLC. About 5.1 mg of **6** was obtained after lyophilization.

To remove the photolabile protection group, 1.7 mg of **6** was dissolved in 60% acetonitrile aqueous solution (containing 0.045% trifluoroacetic acid) at a concentration of 1 mg mL^{-1}. The solution was irradiated with 365 nm UV light. The deprotection process was monitored with electrospray

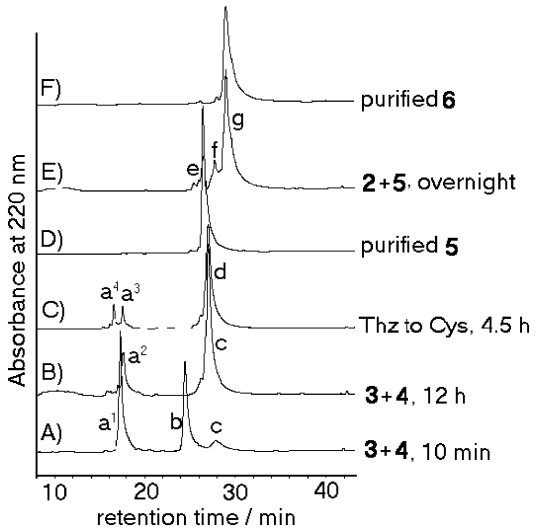

Fig. 2 C8 analytic HPLC data for the synthesis of **6**. (A) and (B): Ligation between **3** and **4** at 10 min and 12 h, respectively. Peak a^1, the 1 : 10 mixture of **3** and its MES thioester; peak b, reduced **4**; peak c, ligation product **Thz28-K48(4-SH, Nε-NVOC)-G76**-OH; peak a^2, about 1 : 1 mixture of **3** and its MES thioester. (C): The *in situ* conversion of Thz to Cys for 4.5 h. peak a^3, peptide **3**; peak a^4, Cys28-G47-$NHOCH_3$; peak d, product **5**. (D): Purified **5**. (E): Ligation between **2** and **5** overnight. Peak e, the MES thioester of **2**; peak f, cyclization product of **2**; peak g, ligation product **6**. (F): Purified **6**.

ionization mass spectrometry (ESI-MS). After 2 h, ESI-MS confirmed that the NVOC group was completely removed to afford product **7** (Fig. 3). The solution was diluted and subjected to C18 semi-preparative HPLC purification. 1.3 mg of **7** was obtained after lyophilization.

To synthesize K48-linked diubiquitin, ubiquitin **7** was reacted with Ub(1–76)-MES (Fig. 4). About 1.3 mg of **7** and 1.5 mg of Ub(1–76)-MES were dissolved in 150 μL ligation buffer (6 M Gdn·HCl, 0.1 M phosphate, 40 mM TCEP, 1% v/v benzyl mercaptan, pH 8.0). After 6 h, the ligation product was formed with a yield of about 65% based on analytic HPLC. After another 4 h, the yield of ligation product increased slightly. 0.8 mg of ligation product was isolated after purification by HPLC.

To get the native K48-linked diubiquitin, free radical mediated desulfurization was performed to convert cysteine 28 and 4-mercaptolysine 48 at the ligation junctions to alanine and lysine, respectively. After 9 h of treatment with the free radical initiator VA-044, both sulfur atoms on the two residues were removed, as confirmed by ESI-MS (Fig. 5C). Deconvoluted ESI-MS showed the correct molecular weight of the final

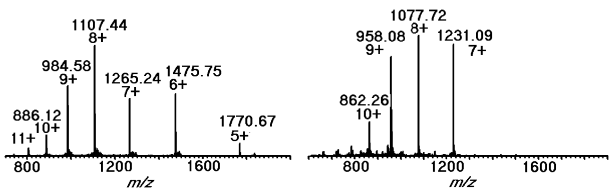

Fig. 3 ESI-MS monitored the removal of the NVOC group. Left panel: before UV irradiation, calculated M = 8850.0 Da, deconvoluted M = 8850.8 Da; right panel: UV irradiation for 2 h, calculated M = 8610.8 Da, deconvoluted M = 8612.6 Da.

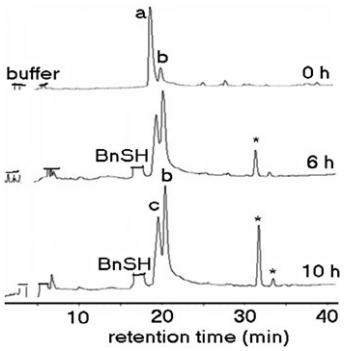

Fig. 4 C4 analytic HPLC monitored ligation between **7** and Ub(1–76)-MES at 0 h, 6 h and 10 h, respectively. Peak a: mixture of **7** and ubi(1–76)-MES. Peak b: ligation product. Peak c: mixture of ubi(1–76)-OH and small amount of remaining **7** and ubi(1–76)-SBn. Peak *: nonproteinous product. Note: 0 h HPLC was run before adding benzyl mercaptan. Bn = benzyl.

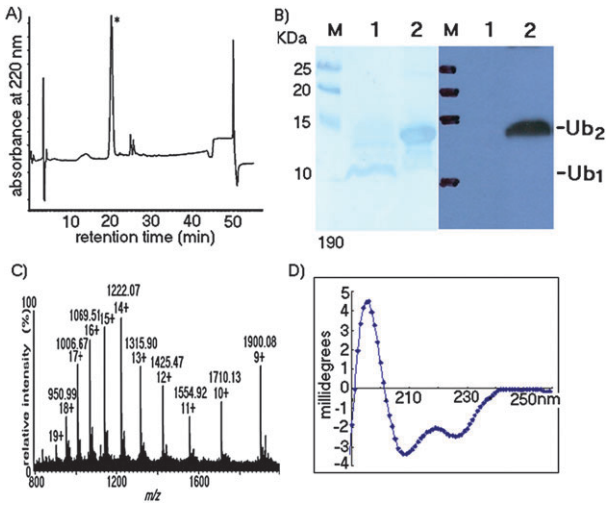

Fig. 5 Characterization of the synthesized K48-linked diubiquitin. (A) C4 analytic HPLC. (B) 18% SDS-PAGE analysis stained with Coomassie blue (left) and Western blot (right). M, protein marker; Lane 1, monoubiquitin; Lane 2, K48-linked diubiquitin **8**. (C) ESI-MS profile. MW calculated 17093.4 Da, found 17094.8 Da. (D) CD spectrum of folded **8**.

native K48-linked diubiquitin. For the characterization of the final product, the diubiquitin was checked with 18% SDS-PAGE. Coomassie blue staining showed a single band. Western blot with antibody FK2H, which is an HRP-conjugated antibody against mono- and polyubiquitinated conjugates but not free ubiquitin, detected the same band as the one in Coomassie blue staining (Fig. 5B). To test whether the synthesized diubiquitin can be folded to its native form, circular dichroism (CD) was measured with dialyzed diubiquitin **8**. The CD spectrum indicated that the diubiquitin was well folded after dialysis (Fig. 5D). Next we performed the cleavage assay using ubiquitin C-terminal hydrolase. It was found that the diubiquitin can be hydrolyzed by the hydrolase, UCH-L3 (Fig. S11, Electronic Supplementary Information).

In summary, herein we have optimized the dual native chemical ligation approach by replacing the strong acid-labile Cbz with the photolabile NVOC protecting group for the side-chain amine of 4-mercaptolysine. The synthesis of K48-linked diubiquitin using the improved protocol demonstrates the practical utility of this ligation strategy in synthetic protein chemistry.

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