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Ubiquitin Chains

Chemical Synthesis of Ubiquitinated Peptides with Varying Lengths and Types of Ubiquitin Chains to Explore the Activity of Deubiquitinases**

Sudhir N. Bavikar, Liat Spasser, Mahmood Haj-Yahya,
*Subramanian Vedhanarayanan Karthikeyan, Tal Moyal, K. S. Ajish Kumar, and Ashraf Brik**

The remarkable diversity of the ubiquitin (Ub) signal is achieved by attaching Ub as a monomer or as chains of varying lengths and types as well as at varying anchoring sites to a protein target.[1] These chains are attached to a protein target with the assistance of the E1–E3 enzymes, and the chain elongations occur through the attachment of Ub through one of its seven lysines or through the attachment of its N terminus to the C terminus of the subsequent Ub.[1,2] Notably, emerging data support different roles of these chains in Ub signaling.[3] For example, whereas chains linked through lysine (Lys = K) residue K48 signal for proteasomal degradation, the K63-linked chains are involved in intracellular processes such as kinase activation and endocytosis. Moreover, changes in the length of a specific Ub chain attached to a protein target could impose different behaviors and dynamics of the components involved in the signaling pathway. For example, it has been generally accepted that the minimal signal necessary for proteasomal degradation is a chain of four Ub molecules linked through residue K48.[4] Yet, some relatively small proteins are degraded efficiently in their mono-ubiquitinated form.[5] For chains linked through the other lysine residues, the preferred length to promote specific signals is still unclear. Also unclear is how the variations of the chain lengths, in the free or the conjugated form, affect the specificity and function of deubiquitinases (DUBs) as well as the binding specificities of ubiquitin-binding domains.

The preparation of these chains in their free or anchored form is a crucial step for understanding the effect of chain lengths and types on biological function. However, the preparation of ubiquitin chains using enzymatic approaches has been a very challenging task,[6,7] which prevented several studies aiming to shed light on these aspects. Recent advances in chemical and semisynthetic approaches bring us closer than ever to understanding the above-described aspects of the Ub signal.[8] In this regard, several research groups, including ours, reported the synthesis of di-Ub chains with natural and unnatural linkages.[9,10] More recently, we also reported the first total chemical synthesis of a K48-linked tetra-Ub unanchored chain, composed of 304 residues, which represents the largest chemically made protein to date.[11] Thus, by using these approaches the synthesis of unanchored chains with various lengths is possible.[11] However, these synthetic advances have not been applied to date to examine how the variations of the length of a specific chain affect their structure and function. Moreover, none of these approaches have been applied for the preparation of a Ub chain anchored to a specific substrate, regardless of the type or length of the chain.[12,13] Herein we report the first efficient synthesis of a tripeptide substrate linked to mono-, di-, tri-, and tetra-Ub chains, the monomers of which are connected through residue K48 or K63. These targets were prepared in good quantities and their activities as substrates for two different DUBs (UCH-L3 and IsoT) were studied.

To examine the syntheses of the ubiquitinated peptides with varying ubiquitin chain lengths (mono-, di-, tri-, and tetra-Ub) we chose the tripeptide Phe-Lys-Thr (FKT, Phe = phenylalanine, Thr = threonine) from the P53 protein as our model peptide to be attached through an isopeptide bond to K48- or K63-linked Ub chains. We recently reported the synthesis of Ub from two fragments (Ub1-45 and Ub46-76) by applying native chemical ligation (NCL)[14,15] coupled with desulfurization to convert the Cys46 residue, which was needed to promote NCL between these fragments, to the native Ala46 residue (Cys = cysteine, Ala = alanine).[9a,11,12e] This strategy has assisted in the efficient synthesis of Ub thioester and all the Lys-linked di-Ub chains without particular obstacles. However, in the case of the tetra-Ub species, the presence of the four Cys46 residues in addition to three thiol groups near the isopeptide bonds imposed difficulties in the final desulfurization step.[11] To avoid the presence of seven thiol groups in the final step, it was necessary to desulfurize the Cys46 residues in each of the four Ub building blocks so that the final ligation product (i.e. tetra-Ub) would only have three thiol groups. This requirement also forced us to adopt the linear strategy, which is time-consuming and less efficient than the convergent approach using two di-Ub building blocks. Although the approach using the linear strategy was eventually successful and the final tetra-Ub product was obtained and fully characterized, we reasoned that an alternative synthetic strategy with fewer synthetic

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[*] Dr. S. N. Bavikar, L. Spasser, M. Haj-Yahya, Dr. S. V. Karthikeyan, T. Moyal, Dr. K. S. Ajish Kumar,[+] Prof. A. Brik
Department of Chemistry and the National Institute for Biotechnology in the Negev
Ben-Gurion University of the Negev
Beer Sheva 84105 (Israel)
E-mail: abrik@bgu.ac.il
Homepage: http://www.bgu.ac.il/~abrik

[+] Current address: Bioorganic division, Modular lab
Bhabha Atomic Research Centre (BARC)
Trombay, Mumbai-400085 (India)

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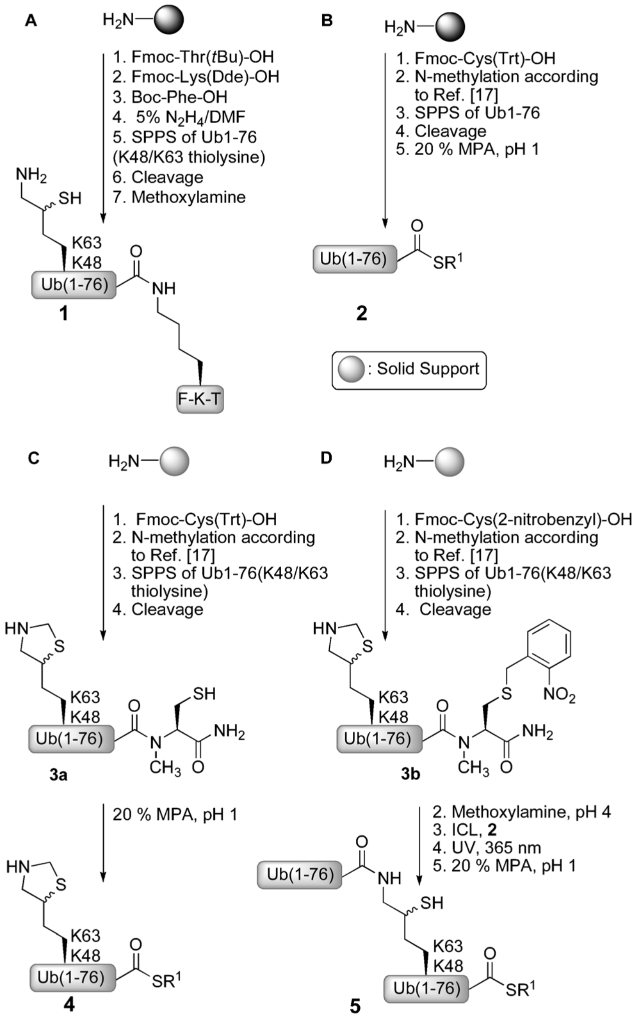
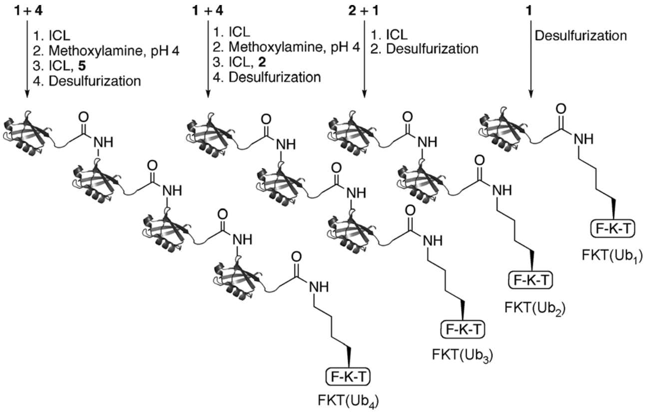
steps must be employed to allow highly efficient and scalable synthesis of any of the longer chains linked to a peptide and ultimately to a protein substrate.

To avoid the use of Cys residues in the preparation of Ub, we endeavored to directly synthesize Ub and its analogues (Scheme 1). Recently, the Ovaa group[9c] exploited the direct SPPS approach in an effort to optimize the synthesis that was reported by Ramage et al.[16] In this synthesis, four pseudoproline dipeptide building blocks and two dimethoxybenzyl dipeptides were incorporated, and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and diisopropylethylamine (DIEA) were used for all the coupling steps. To further optimize this synthesis, we have examined several conditions for the Fmoc-SPPS of Ub-COOH and obtained a highly efficient synthesis of Ub by applying only two dipeptide analogues (pseudoproline dipeptide Leu56-Ser57 (Leu = leucine, Ser = serine) and dimethoxybenzyl-Asp52-Gly53 (Asp = aspartic acid, Gly = glycine)) when using the coupling reagents 2-(6-chloro-1H-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU)/DIEA for all coupling steps (see the Supporting Information).



Scheme 1. Synthesis of the different Ub building blocks (**1**–**5**) applying direct solid-phase peptide synthesis (SPPS). $R^1 = CH_2CH_2COOH$, Boc = *tert*-butyloxycarbonyl, Dde = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl, Fmoc = 9-fluorenylmethyloxycarbonyl, ICL = isopeptide chemical ligation, MPA = 3-mercaptopropionic acid, Trt = triphenylmethyl.

For the challenging synthesis of our final seven synthetic targets, including FKT(Ub_1) and the K48- or K63-linked FKT(Ub_2), FKT(Ub_3), and FKT(Ub_4) shown in Scheme 2, various Ub building blocks (**1**–**5**) were needed (Scheme 1).



Scheme 2. The synthesis of ubiquitinated peptides linked to varying lengths and types (K48 or K63) of Ub chains.

We used our optimized SPPS protocol for the synthesis of all these Ub monomers in combination with our recent synthetic advances to introduce the desired modifications at various regions of Ub (see the Supporting Information). Hence, the ubiquitinated peptide building block **1**, bearing δ-mercaptolysine[12f] at position 48 or 63, was prepared according to the sequence of reactions described in Scheme 1 A. This sequence included our recent method of using orthogonal protection to form the isopeptide bond on the solid phase and subsequent direct synthesis of full-length ubiquitin.[12e] Building blocks **2** and **3** were prepared using our recent method of chemically synthesizing Ub thioester[17] and by exploiting direct SPPS wherein the C terminus of Ub was equipped with *N*-methylcysteine as an N–S acyl transfer device for latent thioester functionality (Scheme 1 B,C). After this synthesis, building block **3a**, in the crude form, was converted to **4** (Scheme 1 C) by treatment with 20 % (v/v) 3-mercaptopropionic acid (MPA). Building block **3b** was prepared in a similar manner to **3a**, however the use of the photolabile nitrobenzyl protecting group on the Cys residue was required, because the nucleophilic methoxylamine, used to unmask the mercaptolysine, was found to attack the partially formed N–S acyl transfer intermediate (25–30 % as monitored by HPLC–MS). When we used the nitrobenzyl-protected *N*-methylcysteine, this side reaction was completely diminished. Building block **5** was prepared from **3b** by also first unmasking the mercaptolysine with methoxylamine to allow ligation with **2** (Scheme 1 D). Subsequently, the C terminus of the proximal Ub was converted to the thioester functionality by first irradiating with UV light to unmask the *N*-methylcysteine and then applying treatment with 20 % MPA at 37 °C.

The syntheses of these Ub building blocks allowed a significant improvement in the preparation of the final synthetic targets. These building blocks were synthesized in parallel on a 0.1 mmol scale using an automated peptide synthesizer and afforded, after purification steps, 60–70 mg of a highly pure material for each Ub variant. This result has permitted the assembly of the two sets of ubiquitinated peptides as shown in Scheme 2. Thus, **1** was desulfurized using the free-radical-based approach or H_2/Raney Nickel[18] to give FKT(Ub_1), which was isolated in 60–70 % yield (Figure 1 A) or linked with **2** by ICL and subsequent desulfurization to give FKT(Ub_2) (Figure 1 B), which could be isolated in 30–35 % yield (for two steps). Building block **1** was also ligated with **4** and subjected to methoxylamine treatment, which after ligation with **2** and subsequent desulfurization gave FKT(Ub_3) in 24 % overall yield of isolated product (Figure 1 C). Alternatively, this target was also achieved by ligation of **1** and **5** and subsequent desulfurization. Finally, FKT(Ub_4) was prepared in a convergent manner starting from **1** and **4**, which were ligated and subsequently treated with methoxylamine to give the di-Ub fragment. Then, this product was ligated with di-Ub **5** and, after a desulfurization step, gave the desired product in 17 % yield of isolated FKT(Ub_4) for the two steps (Figure 1 D). This convergent synthetic approach to the tetra-Ub was faster and afforded the final product in a better yield than the linear approach that we had previously adopted.[11]

The successful syntheses of these sets of peptides having K48- and K63-linked Ub chains gave us a unique opportunity to start examining the effects of the different linkages and chain lenghts on the activity and specificity of different DUBs. For this study we focused on the enzymes IsoT and UCH-L3 from the ubiquitin-specific processing proteases (USP) and the ubiquitin C-terminal hydrolase (UCH) families, respectively (Scheme 3).[19] UCH-L3 catalyzes the removal of small adducts such as small molecules and short peptides from the C terminus of Ub (Scheme 3 A).[20] Biochemical and structural analyses revealed that this enzyme and other members of this family use a disordered active-site crossover loop of 20 residues, which imposes substrate filtering and restricts access of larger substrates.[21] In regards to substrates that consist of Ub linked to peptides, it has been suggested that UCH-L3 cleaves peptide extensions composed of up to 20 amino acids with high efficiency and low sequence preference.[12e,21] The natural substrate of UCH-L3 remains unidentified to date. Although we currently have a better understanding of the influence of the length of the peptide linked to the C terminus of mono-Ub on the UCH-L3 activity, such knowledge regarding the types and lengths of an ubiquitin chain linked to a peptide extension is still lacking.

To shed more light on these parameters, we examined the FKT(Ub_1) and K48- or K63-linked FKT(Ub_2), FKT(Ub_3), and FKT(Ub_4) as substrates for UCH-L3. Thus, each of the seven substrates was treated with hydrolase UCH-L3 (1:200 enzyme/substrate). The percentage of cleavage was determined by analyzing each enzymatic reaction with analytical HPLC, and the entire peak of the cleavage mixture was analyzed by using mass spectrometry. As shown in Figure 2, the mass of the hydrolyzed product was exactly 393.5 Da lower than that of the starting material, which corresponds to

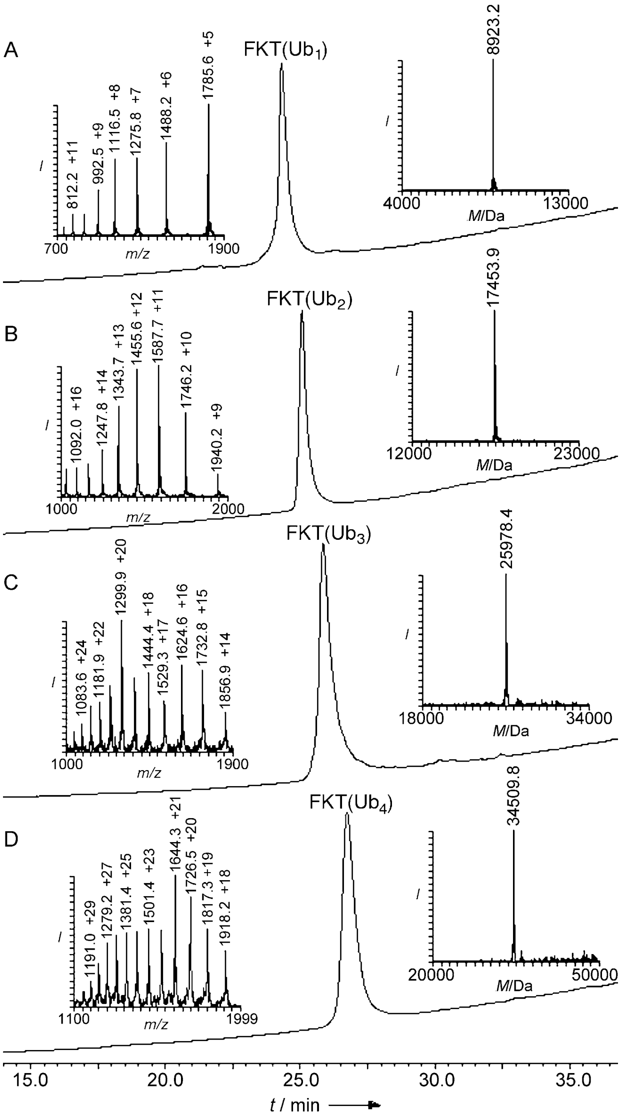
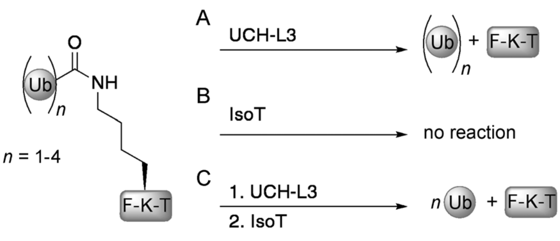


Figure 1. Analytical HPLC traces of the purified ligation products and ESI-MS spectra (left insets) and corresponding deconvoluted mass spectra (right insets) of the FKT tripeptide with the mono-, di-, tri-, and tetra-Ub chains linked through residue K48. A) FKT(Ub_1) with the observed mass of 8923.2 Da (calcd 8922.3 Da), B) FKT(Ub_2) with the observed mass of 17 453.9 Da (calcd 17 451.1 Da), C) FKT(Ub_3) with the observed mass of 25 978.4 Da (calcd 25 979.9 Da), D) FKT(Ub_4) with the observed mass of 34 509.8 Da (calcd 34 508.7 Da). For more details on the preparation process of these targets and the K63-linked substrates, see the Supporting Information.



Scheme 3. Schematic representation of the reactions catalyzed by UCH-L3 and IsoT of the ubiquitinated peptides anchored to Ub chains with varying lengths and connectivities.

the mass of the tripeptide Phe-Lys-Thr. Although ESI-MS is not strictly quantitative, it is very reasonable to assume that the ionization efficiency for both proteins (with or without the tripeptide) is nearly identical and that the relative intensities of the MS signals reflect relative molar ratios. More than 90 % of FKT(Ub_1) was cleaved (Figure 2 A). However, when the remaining substrates having varying chain lengths and types were analyzed, two clear observations were made. 1) In both types of chains (K48 and K63) a decrease in the cleavage efficiency was observed when the chain length was increased from FKT(Ub_1) to FKT(Ub_4). 2) The decrease in the cleavage efficiency in the case of K63-linked chains was more pronounced compared to the K48-linked chain.

The molecular bases for these differences are not clear to date; however, we speculate that both the chain size and conformation could play an important role in the observed outcome. Previous structural data of the UCH-L3-Ub have clearly shown that the crossover loop forms multiple interactions with Ub residues, which stabilize the C terminus of Ub for the cleavage step.[21] These interactions could be disturbed when the length of the Ub chains is increased. Moreover, the remarkable differences between the open conformations of the K63-linked chain[22] and the closed ones of the K48-linked chain[23] could also play an important role in the recognition of these substrates and the subsequent hydrolysis of the tail peptide from these chains.

Next, we subjected the two sets of the ubiquitinated peptides to the enzyme IsoT, which is responsible for the disassembly of the majority of unanchored poly-Ub chains in a nonprocessive manner, that is, it releases one Ub at a time from the proximal end of the chain.[24] When any of the chains bearing the tail peptide was treated with enzyme IsoT, regardless of conditions, no cleavage products could be detected by LC–MS (Figure 3 A; for FKT(Ub_2)). Indeed, biochemical and structural analyses revealed that this enzyme is specific for an unmodified C terminus of the proximal Ub unit of the polyubiquitin chain.[25] Hence, our results are consistent with these findings on the importance of an unmodified C terminus, because this part of the chain is occupied with the tripeptide. Thus, we reasoned that releasing this tail from the proximal end of the chain to generate the free acid would allow cleavage by IsoT. Indeed, upon addition of UCH-L3 to these reaction mixtures, IsoT showed normal activity and cleaved the chains with an efficiency that depended on the ability of UCH-L3 to cleave the tail peptide

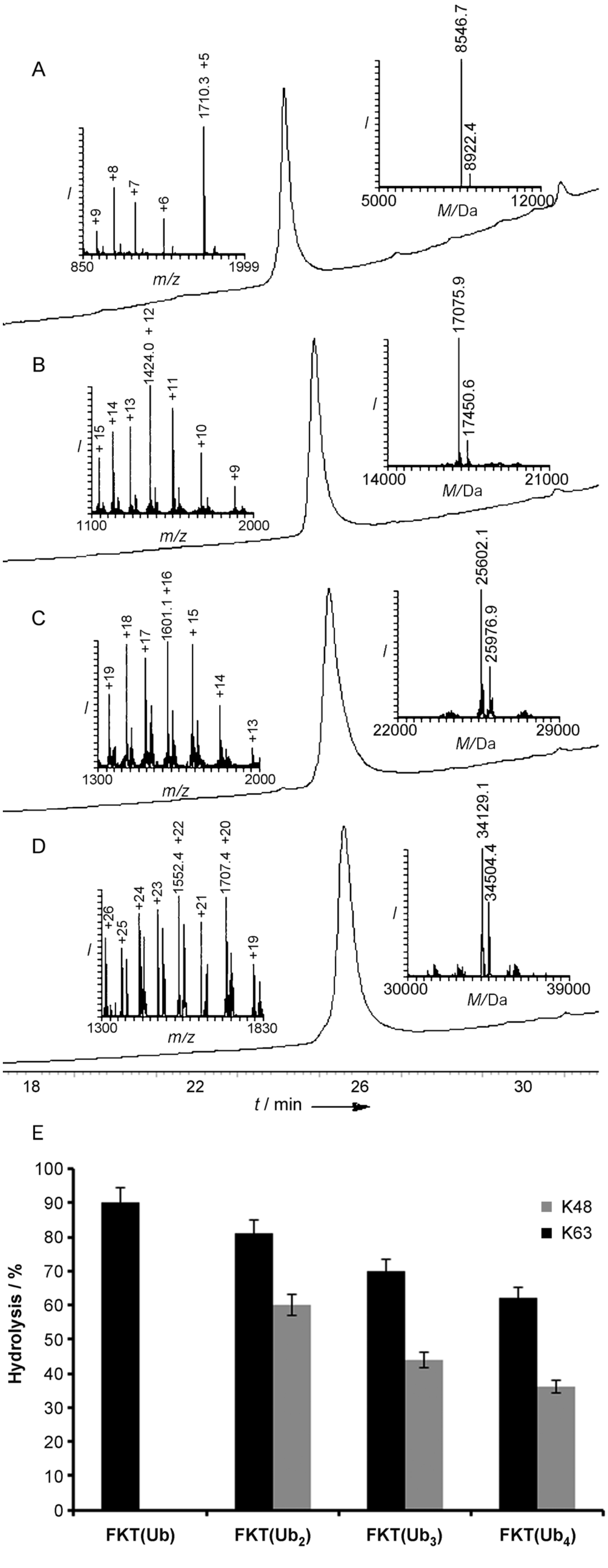


Figure 2. A)–D) Analytical HPLC traces of the UCH-L3 cleavage reactions of the ubiquitinated peptides (K48) and mass spectrometry analysis (ESI-MS) of the entire HPLC peak, which corresponds to the starting material and the cleavage product. Left insets show the ESI-MS spectra and right insets show the corresponding deconvoluted masses of the hydrolysis product (lower mass) and the starting material. A) FKT(Ub_1); observed mass of hydrolysis product 8546.7 Da (calcd 8547.8 Da). B) FKT(Ub_2); observed mass of hydrolysis product 17075.9 Da (calcd 17076.6 Da). C) FKT(Ub_3); observed mass of hydrolysis product 25602.1 Da (calcd 25603.4 Da). D) FKT(Ub_4); observed mass of hydrolysis product 34129.1 Da (calcd 34133.2 Da). E) The cleavage percentage of the FKT(Ub_1)–FKT(Ub_4) substrates (K48- and K63-linked Ub chains) as determined by comparing the intensities of the MS signals of the deconvoluted spectra.

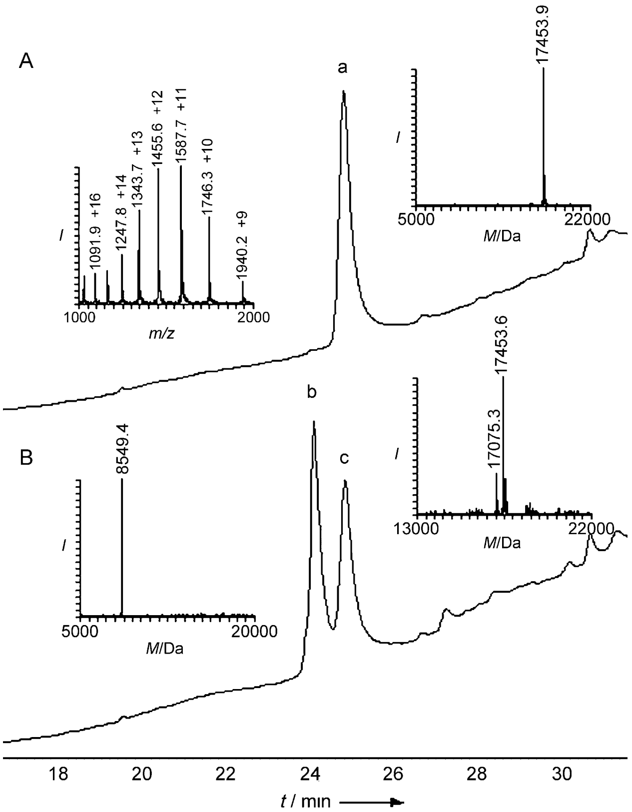


Figure 3. The hydrolysis reaction of K48-linked di-Ub catalyzed by UCH-L3/IsoT. A) Analytical HPLC trace of FKT(Ub_2) with IsoT: peak a shows the unhydrolyzed product; the left inset is the ESI-MS spectrum of peak a and the corresponding deconvoluted spectrum is shown in the right inset with the observed mass of 17453.9 Da (calcd 17451.1 Da). B) Analytical HPLC trace for FKT(Ub_2) initially treated with UCH-L3 for five minutes and then with IsoT for five minutes. Peak b corresponds to the hydrolysis product Ub with the observed deconvoluted mass of 8549.4 Da (calcd 8547.8 Da, see left inset); peak c, of which the deconvoluted mass spectrum is shown in the right inset, corresponds to the unhydrolyzed product, with the observed mass of 17453.6 Da (calcd. 17451.1 Da), along with remaining hydrolyzed product di-Ub of UCH-L3 with the observed mass of 17075.3 Da (calcd 17076.6 Da).

from the specific chain (Scheme 3). For example, when K48-linked FKT(Ub_2) was initially treated with UCH-L3 for five minutes to ensure release of over 50 % of the tail peptide from these chains (Figure 2E), and subsequently IsoT was added (1:50, enzyme/substrate), over 75 % of the UCH-L3 hydrolysis product (i.e., K48-linked (Ub_2)) was disassembled to the mono-Ub product within the first five minutes (Figure 3). Similar findings were observed in the cases of K48-linked (Ub_3) and K63-linked (Ub_2) and (Ub_3) chains (see the Supporting Information). These results further support the importance of an unmodified C terminus of the Ub chain for the IsoT activity regardless of its type and length.

In summary, the preparation of different sets of ubiquitinated peptides anchored to Ub chains with varying lengths and types was achieved. Our advances in the synthesis of the Ub building blocks allowed the efficient synthesis of Ub thioester with or without thiolysine at position K48 or K63 as well as tripeptide Ub monomer (proximal) having δ-mercaptolysine at any of these positions. Combining these monomers by ICL and desulfurization steps furnished two different sets of peptides having mono-, di-, tri-, and tetra-Ub linked homogeneously through K48 or K63. For the first time, we were able to assess the activity of two DUBs (UCH-L3 and IsoT) with these sets of ubiquitinated peptides. Our results further support the requirement of an unmodified C terminus of the proximal Ub in a specific chain for the IsoT activity regardless of its type and length. On the other hand, UCH-L3 clearly prefered the shorter chains as well as the K48-linked chains over the K63-linked chains. Our work paves the road to examine the effect of varying the lengths and types of Ub chains on other DUBs. Moreover, the synthetic advances made in this study bring us a step closer to achieving the synthesis of a protein target linked to a specific ubiquitin chain of a desired length and type.

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