Polymerization Behavior of a Bifunctional Ubiquitin Monomer as a Function of the Nucleophile Site and Folding Conditions

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Synthesis of Ub^{48} and Ub^{63} monomers

The synthesis was carried out according to the following scheme:

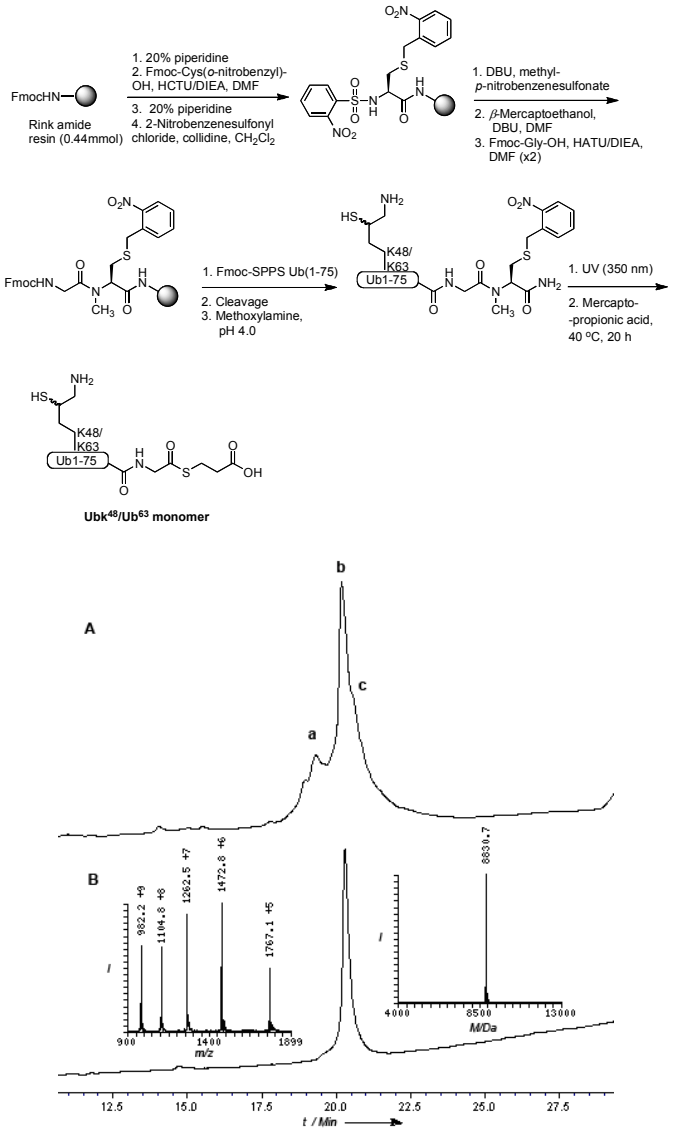

Figure 1: Analytical HPLC / ESI-MS of the synthesis of **Ub^{48}**. A) Crude peptide before methoxylamine treatment; peak a corresponds to unidentified peptide; peak b corresponds to the desired product with the observed mass 8842.4 Da (calcd 8843.8 Da); peak c is piperidine adduct (+67 Da) with observed mass 9033.3 Da; B) purified peptide after methoxylamine treatment with the observed mass 8830.7 Da (calcd 8830.2 Da).

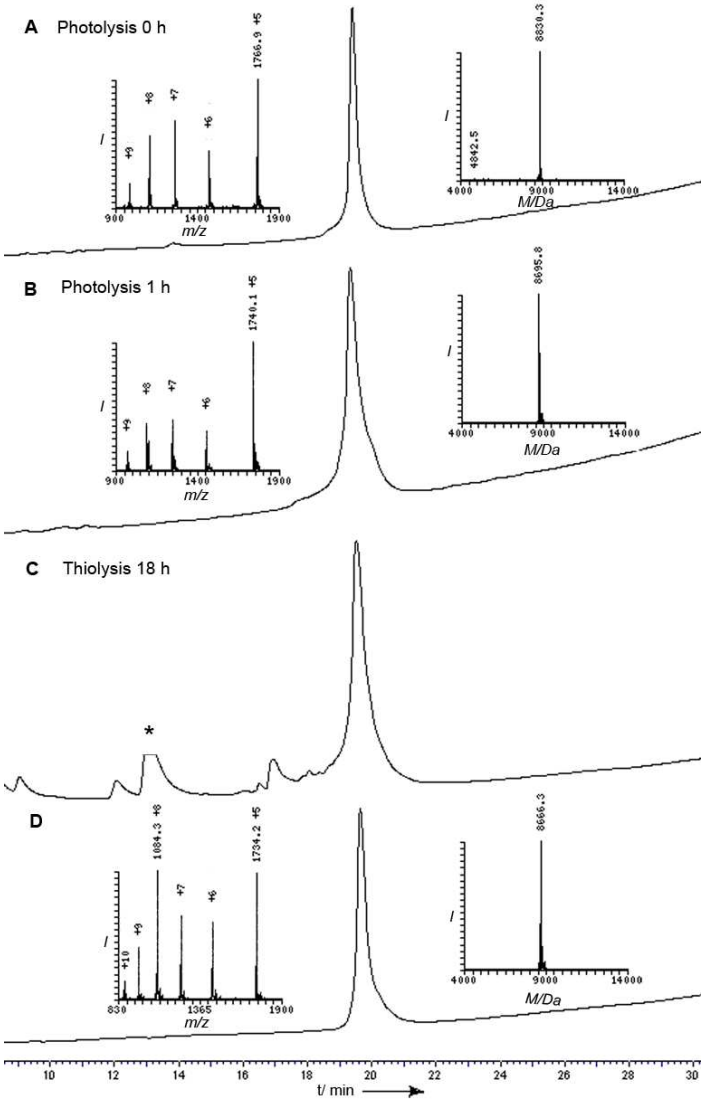

Figure 2: Synthesis of **Ub**^{48} monomer. A) Analytical HPLC traces / ESI-MS of **Ub**^{48} after Thz removal with the observed mass 8830.3 Da (calcd 8830.2 Da); B) photolysis after 1 h; the HPLC peak corresponds to the desired product with the observed mass 8695.8 Da (calcd 8694.9 Da); C) thiolysis after 18 h; with the observed mass 8666.3 Da (calcd 8667.8 Da). Peak * corresponds to thiol additives; D) purified **Ub**^{48} monomer.

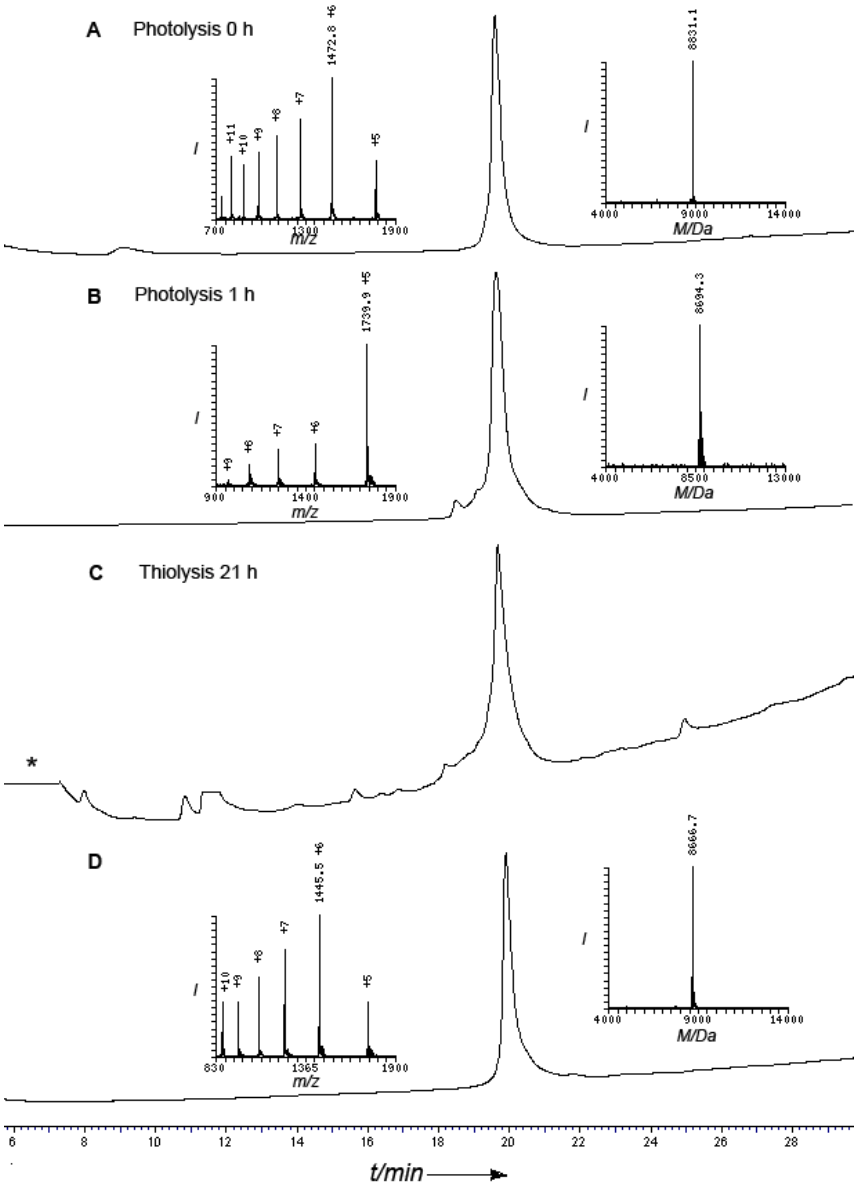

Figure 3: Synthesis of **Ub**^{63} monomer. A) Analytical HPLC traces/ESI-MS of **Ub**^{63} after Thz removal with the observed mass 8831.1 Da (calcd 8830.2 Da; B) photolysis after 1 h afforded the desired product with the observed mass 8694.3 Da (calcd 8694.9 Da); C) thiolysis after 21 h afforded the desired product **Ub**^{63} with the observed mass 8667.7 Da (calcd 8667.8 Da); peak * corresponds to thiol additives; D) Purified **Ub**^{63} monomer.

Analytical HPLC and mass spectrometry data for Ub[1]

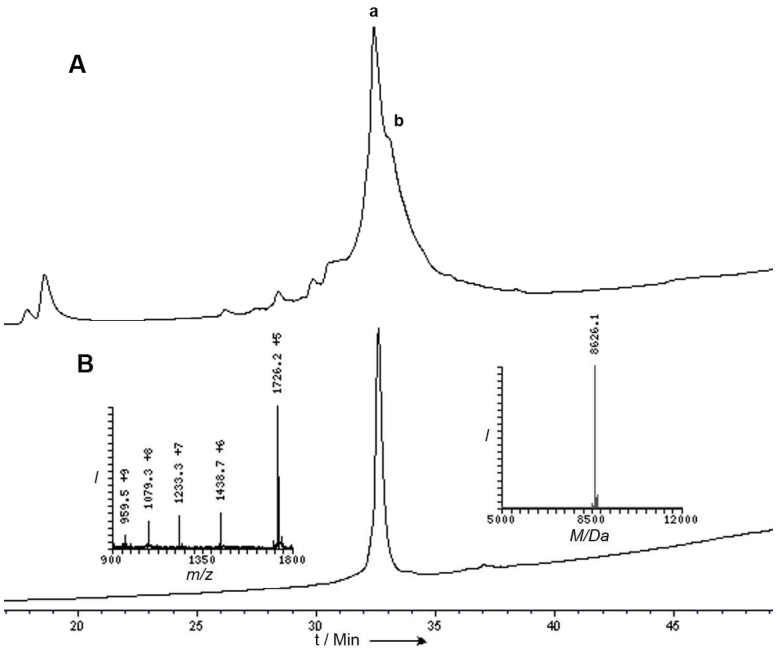

Figure 4: Analytical HPLC/ESI-MS of the crude peptide before treatment with MPA (A). Peak a corresponds to the desired product with the observed mass 8653.7 Da (calcd 8652.9 Da); peak b is the piperidine adduct (+67 Da) with the observed mass 8720.8 Da. B) Purified **Ub[1]** after MPA treatment with the observed mass 8626.1 Da (calcd 8625.8 Da).

Analytical data for the polymerization of Ub^{48} and Ub^{63} under denaturation conditions:

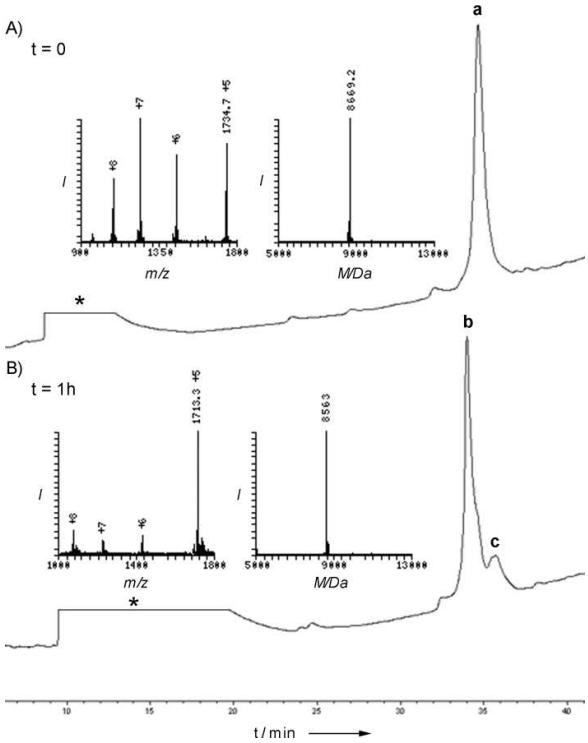

Figure 5: Analytical HPLC/ESI-MS of the polymerization reaction of **Ub^{48}** under denaturation conditions. A) Analytical HPLC analysis (t=0) of the polymerization reaction; peak a corresponds to **Ub^{48}** monomer with the observed mass 8669.2 Da (calcd 8667.8 Da); B) analytical HPLC analysis of the polymerization reaction after 1 hour. Peak b corresponds to the cyclization product with the observed mass 8563 Da (calcd 8561.8); peak c with unidentified mass and Peak * corresponds to thiol additives.

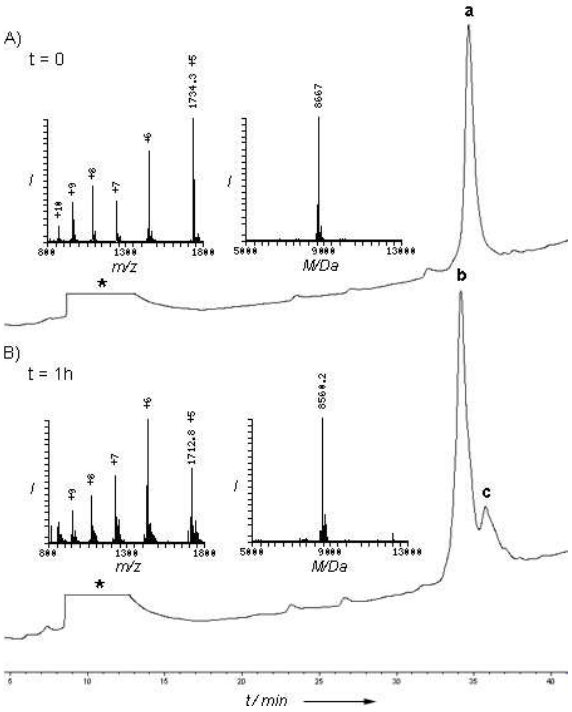

Figure 6: Analytical HPLC/ESI-MS of the polymerization reaction of **Ub**^{63} under denaturation conditions. A) Analytical HPLC analysis (t=0) of the polymerization reaction; peak a corresponds to **Ub**^{63} with the observed mass 8667 Da (calcd 8667.8 Da); B) analytical HPLC analysis of the polymerization reaction after 1 hour. Peak b corresponds to the cyclization product with the observed mass 8560.2 Da (calcd 8561.8); peak c with unidentified mass and peak * corresponds to thiol additives.

Mass spectrometry data of the isolated products from the polymerization reaction of Ub monomers under folding conditions:

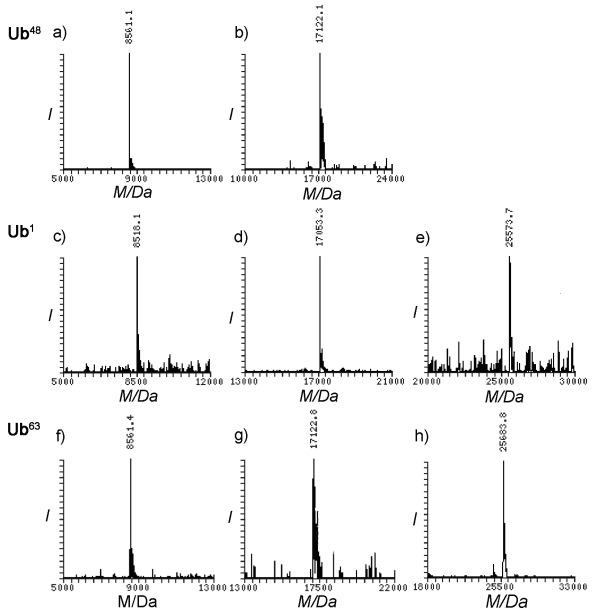

Figure 7: ESI-MS of polymerization of **Ub^{48}**, **Ub^{1}** and **Ub^{63}** under folding conditions: a) **cy-Ub^{48}** with the observed mass 8561.1 Da (calcd 8561.8 Da); b) cyclized **Ub^{48}** dimer with the observed mass 17122.1 Da (calcd 17122.6 Da); c) **cy-Ub^{1}** with the observed mass 8518.1 Da (calcd 8519.9 Da); d) hydrolyzed thioester product of **Ub^{1}** dimer with the observed mass 17053.3 Da (calcd 17055.6 Da); e) hydrolyzed thioester product of **Ub^{1}** trimer with the observed mass 25573.7 Da (calcd 25574.4 Da); f) **cy-Ub^{63}** with the observed mass 8561.4 Da (calcd 8561.8 Da); g) cyclized **Ub^{63}** dimer with the observed mass 17122.8 Da (calcd 17122.6 Da); h) cyclized **Ub^{63}** trimer with the observed mass 25683.8 Da (calcd 25683.4 Da).

Synthesis of UBA2 binding domain:

The synthesis of peptide UBA2 was carried out on Rink amide resin (0.44 mmol/g, 0.1 mmol scale). The amino acids were coupled manually using 4 eq of AA/8 eq of DIEA/4 eq of HCTU to the initial loading of the resin. Fmoc removal was achieved using 20% piperidine with 5-10-5 min cycles.

Pseudoproline dipeptides Ala-Ser and Leu-Ser were manually coupled at positions Ala25-Cys26 and Leu38-Ser39 junctions using 2.5 eq of Fmoc-Ala-Ser($\psi^{Me,Me}$Pro)-OH and Fmoc-Leu-Ser($\psi^{Me,Me}$Pro)-OH respectively for 2 h.

Cleavage from the resin: The resin-bound peptide was washed with DMF, methanol, DCM and dried. The cleavage cocktail (TFA, H_2O, Triisopropylsilane, 95:2.5:2.5)

was added to the dried resin and the reaction mixture was shaken for 2.5 h at room temperature. The combined filtrate was added drop-wise to a 10-fold volume of cold ether, centrifuged, the precipitated crude peptide was dissolved in acetonitrile-water (1:1) and was further diluted to ~30% with water and lyophilized. The HPLC analysis was carried out on a C18 analytical column using a gradient of 5-60% B over 30 min.

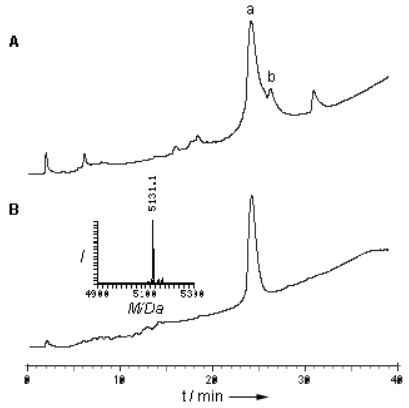

Figure 8: Analytical HPLC / ESI-MS of the crude **(A)** and pure **(B)** of UBA2 binding domain; peak a corresponds to the desired product with the observed mass 5131.1 Da (calcd 5130.7 Da); peak b corresponds to an unidentified mass.

Table 1: Summary of the different polymerization product of the three monomers under various conditions.

Conditions / Monomer	Denaturation	Folding	UBA2	
			+	-
Ub^{48}	Cy-Ub^{48}	Cy-Ub^{48}, Cy-$Ub^{48}2$, $Ub^{48}3$, Cy-$Ub^{48}3$, $Ub^{48}4$*	Cy-Ub^{48}, Cy-$Ub^{48}2$, $Ub^{48}3$, traces of $Ub^{48}4$*	Cy-Ub^{48}, Cy-$Ub^{48}2$, $Ub^{48}3$, Cy-$Ub^{48}3$, $Ub^{48}4$*
Ub^{1}	Cy-Ub^{1} (42%), $Ub^{1}2$ - $Ub^{1}10$*	Cy-Ub^{1}, $Ub^{1}2$, $Ub^{1}3$, $Ub^{1}4$ - $Ub^{1}10$*	No difference in the presence and absence of UBA2 (see products list of folding conditions)	
Ub^{63}	Cy-Ub^{63}	Cy-Ub^{63}, Cy-$Ub^{63}2$, $Ub^{63}3$, Cy-$Ub^{63}3$, $Ub^{63}4$ and longer chains*	No difference in the presence and absence of UBA2 (see products list of folding conditions)	

* cyclized/ uncyclized stat not determined.