Supporting Information

Rapid Optimization of Labeled Ubiquitinated Peptides for Monitoring Deubiquitinases Activities

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GENREAL METHODS:

SPPS: Peptides were synthesized manually in syringes equipped with teflon filters, purchased from Torviq, or by automated peptide synthesizer (CS336X, CSBIO) using standard Fmoc protocol conditions. Coupling was done by using 4 fold excess of HCTU and amino acids, while using 8 fold excess of DIEA to the initial loading of the resin. Deprotection of the Fmoc protecting group was achieved by treating the resin with 20% piperidine in DMF (3x3min). Global deprotection and cleavage of the peptide from the resin was done by treating the resin with a mixture of 95:2.5:2.5 TFA: triisopropylsilane: H₂O for two hours at room temperature. The crude peptide was precipitated in anhydrous Et₂O, centrifuged, dissolved in HPLC buffer and lyophilized. Coupling of the MCA fluorophore was assisted by a CEM Discover Microwave reactor.

High Performance Liquid Chromatography (HPLC): Analytical reverse phase HPLC was performed on a Thermo instrument (Spectra System p4000) system using Jupiter C4 column (5 μm, 300 Å, 150 × 4.6 mm). Preparative reverse-phase HPLC was performed on a Waters instrument system using a Jupiter C4 column (5 μm, 300 Å, 250 × 22.4mm). The flow rates used were 1.2 mL/min (analytical) and 20 mL/min (preparative). The linear gradients used to elute the bound peptides were integrated from buffer A (water with 0.1% (v/v) trifluoroacetic acid [TFA]) and buffer B (acetonitrile with 0.1% (v/v) TFA).

Mass Spectrometry: Electrospray ionization mass spectrometry (ESI–MS) was performed on a LCQ Fleet mass spectrometer (Thermo Scientific) with an ESI source.

Reagents: Resin, protected amino acids, 7-methoxycoumarin-4-acetic acid (MCA), HCTU, HATU, HBTU and HOBT were purchased from Novabiochem. Bovine serum albumin was purchased from Calbiochem. TRIS and EDTA were purchased from Sigma. DMF was purchased
in biotech grade. 2,4-Dinitrophenyl (Dnp) was purchased from AK-Scientific. USP2 (catalytic domain) and USP7 (full-length) were obtained from Dr. Shira Albeck (The Weizmann Institute of Science, Rehovot, Israel). Commercial reagents were used without further purification.

**Synthesis of fragment I:** The synthesis of fragment I was carried out on Rink amide resin (0.27 mmol/g, 0.1 mmol scale) as described before in general methods (SPPS), with the following modifications: 4 fold of 3-Fmoc-4-diaminobenzoic acid (Fmoc-Dbz) was initially double coupled to the resin using 4 fold excess of HBTU/HOBt and 8 fold excess of DIEA to the initial loading of the resin. Then, 6 fold of Fmoc-Val-OH was double coupled using 6 fold HATU and 9 fold excess of DIEA to the initial loading of the resin. Asp at position 52 was modified to the labeled Fmoc-Asp(Dnp)-OH and thiazolidine (Thz) amino acid was coupled as a protected form of Cys.

**Synthesis of fragment II:** The synthesis of fragment II was carried out on Rink amide resin (0.6 mmol/g, 0.1 mmol scale) as described before in general methods (SPPS). Fmoc-K(ivDde)-OH was introduced at the desired position in the peptide sequence. Subsequently, the free amine was coupled to MCA assisted by microwave irradiation at 60 °C for 20 - 120 min, using 5-fold excess of MCA, HOBt and DIC to the initial loading of the resin. Next, the ivDde group was deprotected by applying 5% hydrazine in DMF (4x20min), followed by SPPS of the Cys-Ub(72-76) segment. Finally, global deprotection and cleavage from the resin was achieved as described in the general methods. This procedure was followed for all substrates that were tested.

**Synthesis of fragment IV:** fragment IV synthesized as described before.1

**Ligation of fragments I and II:** The ligation of fragment I (1.1 eq.) and fragment II (1 eq.) was carried out in presence of tris(2-carboxyethyl)phosphine (TCEP, 15 eq.) and 4-
mercaptophenylacetic acid (MPAA, 30 eq.) in 6 M Gn·HCl, 200 mM phosphate buffer, pH~7.2 solution at 37 °C for 7 hr, followed by methoxylamine (30 eq.) and TCEP (15 eq.) treatment in 37 °C, at pH 4 to unmask the thiazolidine to give fragment III in 28-39% isolated yield.

**Ligation of fragments III and IV:** The ligation of fragment III (1 eq.) and fragment IV (1 eq.) was carried out in presence of TCEP (15 eq.) and MPAA (30 eq.) in 6 M Gn·HCl, 200 mM phosphate buffer, pH~7.2 solution at 37 °C for 2 hr, to give product V in 38-51% isolated yield.

**Figure S1:** Analytic HPLC and mass spectrometry analyses (ESI-MS) of the pure substrates 2-7. The predicted masses are: 2 - 9667.6 Da, 3 - 9681.6 Da, 4 - 9610.5 Da, 5 - 9638.5 Da, 6 - 9592.5 Da, 7 - 9626.5 Da.
Figure S2: Analytic HPLC and mass spectrometry analysis (ESI-MS) of the pure substrates 8-11. The predicted masses are: 8 – 9596.5 Da, 9 – 9652.6 Da, 10 - 9686.6 Da, 11 – 9695.6 Da.

DUBs activity assay: Fluorescent measurements were performed on an Infinite M200 fluorescence plate reader (TECAN). In general, 34 μl of 70 nM stock solution of USP2 or USP7 (50 mM Tris, 0.5 mM EDTA, 1 mM DTT and 0.5 mg/ml ovalbumin, pH 7.5) were added to a Nunc 96-well black assay plate and were incubated for 10 min to ensure full reduction of the enzymes, for maximal activity. To start the enzymatic reaction, 66 μl of substrate (under K_M value) were added to each well and the fluorescence emission intensity (Eex = 325 nm, Eem = 445 nm) was measured to reveal the effect of the mutated substrates on DUBs activities.

Kinetic characterization of USP2 and USP7: To obtain the kinetic parameters of USP2 and USP7 with the different substrates we performed fluorescence measurements as described for DUBs activity assay with various concentrations of the substrates (1-25 μM for USP2 and 0.5-4 μM for USP7). The initial velocities of product formation were fitted to Michaelis–Menten equation to determine K_M and k_cat for USP2 and catalytic efficiencies for USP7 using SigmaPlot 2000 software.

References: