New Discovery Platform Tools for Deubiquinating Enzymes (DUBs)

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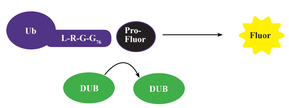
Introduction

Ubiquitination is a reversible process, and the removal of Ubiquitin (Ub) from modified proteins is mediated by deubiquitinating enzymes (DUBs). DUBs maintain optimal levels of cellular Ub by recycling Ub attached to inappropriate targets, removing and disassembling poly-Ub chains, and preprocessing proteins degraded by the proteasome.

Despite their importance, substrate specificity and kinetic characteristics of most DUBs toward isopeptide-linked Ubiquitin remains poorly defined due to the lack of appropriate tools to monitor activity in real time and at sub-micromolar concentrations. Most DUBs have been kinetically characterized using Ub-fluorophore adducts, and so the signal due to DUB activity was generated by hydrolysis of mono-Ub as opposed to the cleavage of an isopeptide bond found in the native substrate. We developed novel FRET-based di-ubiquitin substrates which provide real time measurement of true isopeptide bond cleavage. The substrates are capable of identifying differences between catalytic domain and full-length enzyme versions of DUBs including USP7 and USP8. Furthermore, our results indicate that the FRET-based di-ubiquitin substrates are more physiologically relevant and also provide the ability to screen chain linkage specificity of different DUBs.

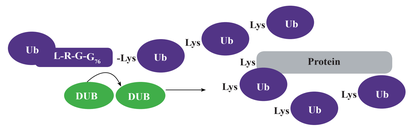
Existing Deubiquinating Enzyme Tools

C-Terminal Cleavage Releases Fluorescent Product (Ub-AMC/AFC/R110)



Pros: Sensitive, Continuous, Fluorogenic (UV/Vis)
Cons: Non-Isopeptide Bond, Not Suitable for All Enzymes

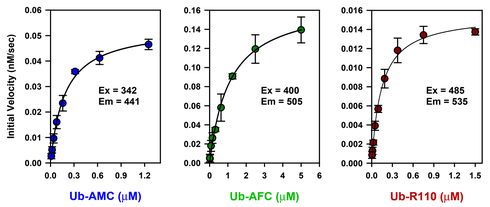
Ub Chains (Isopeptide Bond Cleavage)



Pros: True Isopeptide Bond
Cons: Non-Continuous, Low Sensitivity, Time Consuming Assay Required (HPLC, Capture or Gel Based)

Ubiqui-Flour Substrates for Assay & HTS

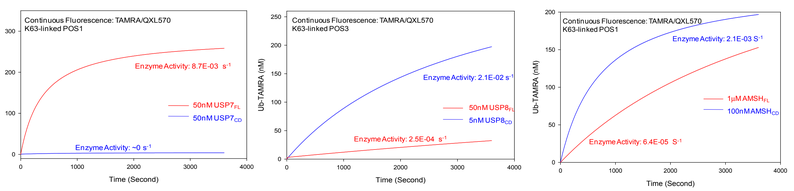
Catalyzed Hydrolysis of Ubiqui-Fluor Substrates: Kinetic Constants



Ubiqui-Fluor substrates were produced by the covalent attachment of 7-amino-4-methylcoumarin (AMC), 7-amino-4-trifluoromethylcoumarin (AFC), or rhodamine 110 (R110) to the terminal glycine of ubiquitin. Each substrate was incubated with 2 pM UCHL3 in a buffer containing 50 mM HEPES ph 8, 5 mM DTT, 0.5 mM EDTA, and 0.2 mg/ml BSA. Assays were performed at 25oC in a Corning non-binding 384 well plate. Data points represent the mean and S.D. of 4 samples after background subtraction. Data were fit with the hyperbolic Michaelis-Menten plot to derive the kinetic constants shown below each plot.

Catalytic Domain vs. Full Length: USP7, USP8 and AMSH

Identification of Activity Difference Between DUB_{CD} and DUB_{FL}



K63-linked di-Ub POS1 (300 nM) was incubated with USP7CD and USP7FL (50nM). K63-linked di-Ub POS3 (300nM) was incubated with USP8CD (5nM) and USP8FL (50nM). K63-linked (300nM) di-Ub POS1 was incubated with AMSHCD (100nM) and AMSHFL (1mM) The assay was monitored in 50mM HEPES, 150mM NaCl, and 2mM DTT and at ex544 nm, em572 nm for 60 minutes. USP7FL demonstrated strong activity against K63-linked di-Ub substrate, whereas USP7CD showed little detectable cleavage. The catalytic domain of USP8 and AMSH demonstrated much stronger activity against K63-linked FRET di-Ub substrates than the full length version. This data is consistent with our native unlabeled di-Ub gel-based assay results, which further indicate that the novel FRET-based di-Ub substrates are capable of detecting specific isopeptide cleavage activity of DUBs.

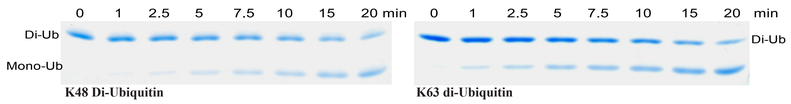
Comparison of DUB Activity Between Catalytic Domain and Full Length

Enzyme Activity	K63 POS1	K63 POS2	K63 POS3	Native K63 Di-Ub
$USP7_{FL}$>>$USP7_{CD}$	∞	∞	∞	∞
$USP8_{CD}$>>$USP8_{FL}$	36	50	81	24
$AMSH_{CD}$>>$AMSH_{FL}$	32	11	10	N/A

The activity differences of three enzymes were compared and shown in the table (fold difference). Note: USP7CD does not show any activity against all three substrates, the value is close to ∞.

DUB Profiling Using Di-Ubiquitin Substrates with All Linkages

Representative Gel Illustrating USP5 Hydrolysis of Di-Ub Substrates: Kinetic Constants



Different linkage of 10μM di-ubiquitin substrates (K48 and K63) were incubated with 1nM USP5 in a buffer containing 50 mM HEPES pH 7.5, 150mM NaCl, and 2 mM DTT. Assays were performed at 25˚C and samples were taken at different time points for gel analysis. Densitometry was applied to quantify the appearance of mono-ubiquitin and linear regression fit was applied to obtain initial cleavage velocity. The enzyme kinetic activity is calculated by the initial velocity divided by enzyme concentration.

Linkage Specificity of Di-Ub Substrates for Different DUB Enzymes (Kinetic Parameters)
Rates of Hydrolysis of Di-Ub for Each DUB in Order of Preference (High to Low)

Linkage	USP2CD	Linkage	USP5	Linkage	USP7CD	Linkage	USP7FL	Linkage	USP8CD
K11 Di-Ub	0.21	K11 Di-Ub	1.79	K6 Di-Ub	no	K6 Di-Ub	0.26	K6 Di-Ub	0.62
K48 Di-Ub	0.18	K63 Di-Ub	1.73	K11 Di-Ub	no	K11 Di-Ub	0.12	K33 Di-Ub	0.46
K63 Di-Ub	0.18	K48 Di-Ub	1.61	K27 Di-Ub	no	K48 Di-Ub	0.08	K48 Di-Ub	0.43
K6 Di-Ub	0.13	K6 Di-Ub	1.60	K29 Di-Ub	no	K27 Di-Ub	0.07	K11 Di-Ub	0.28
K33 Di-Ub	0.13	K33 Di-Ub	1.28	K33 Di-Ub	no	K33 Di-Ub	0.05	K27 Di-Ub	0.26
K27 Di-Ub	0.06	K29 Di-Ub	1.06	K48 Di-Ub	no	K29 Di-Ub	0.05	K63 Di-Ub	0.25
K29 Di-Ub	0.06	K27 Di-Ub	0.36	K63 Di-Ub	no	K63 Di-Ub	0.05	K29 Di-Ub	0.15

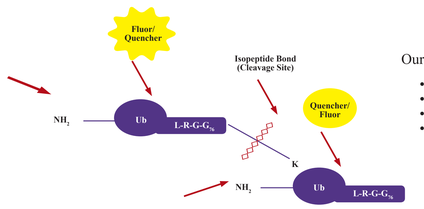
Linkage	USP8FL	Linkage	A20CD	Linkage	A20FL	Linkage	AMSHCD	Linkage	AMSHFL
K6 Di-Ub	0.025	K48 Di-Ub	0.001	K48 Di-Ub	0.0001	K63 Di-Ub	yes	K63 Di-Ub	0.0004
K11 Di-Ub	0.014	K11 Di-Ub	yes	K6 Di-Ub	no	K6 Di-Ub	no	K6 Di-Ub	no
K48 Di-Ub	0.013	K6 Di-Ub	no	K11 Di-Ub	no	K11 Di-Ub	no	K11 Di-Ub	no
K33 Di-Ub	0.011	K27 Di-Ub	no	K27 Di-Ub	no	K27 Di-Ub	no	K27 Di-Ub	no
K29 Di-Ub	0.011	K29 Di-Ub	no	K29 Di-Ub	no	K29 Di-Ub	no	K29 Di-Ub	no
K63 Di-Ub	0.010	K33 Di-Ub	no	K33 Di-Ub	no	K33 Di-Ub	no	K33 Di-Ub	no
K27 Di-Ub	0.008	K63 Di-Ub	no	K63 Di-Ub	no	K48 Di-Ub	N/A	K48 Di-Ub	no

Conclusions: Linkage specificity was demonstrated using di-Ub substrates. Catalytic domain and full length enzymes (DUBs) demonstrated different activity against di-Ub substrates.

- USP2, USP5, $USP7_{FL}$, $USP8_{CD}$, and $USP8_{FL}$ disassemble all Ub chains regardless of linkage type.
- AMSH only digests K63-linked di-Ub, whereas $A20_{CD}$ only digests K11- and K48-linked di-Ub.
- $USP7_{CD}$ can not cleave all seven di-Ub substrates whereas $USP7_{FL}$ has activity.
- $USP8_{CD}$ is much more active in cleaving di-Ub substrates as compared to $USP8_{FL}$.
- $A20_{CD}$ is also slightly more active than $A20_{FL}$.

FRET-Based Di-Ubiquitin Substrates

Schematic of FRET-Based Di-Ub Substrates. Customizable Substrates with True Isopeptide Bonds Between Ub Moieties



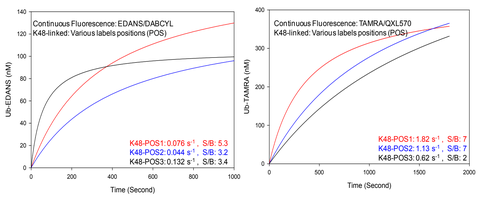
= Custom Options

Our FRET di-ubiquitin substrate platform provides:

- True isopeptide bonds
- Different fluor labels and tags
- Continuous and sensitive assay
- Multiple linkages

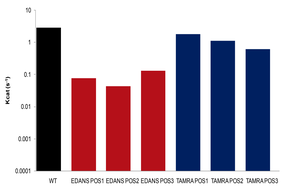
FRET Substrate Optimization: A Case Study

Optimization K48-Linked Di-Ub Substrates for USP5



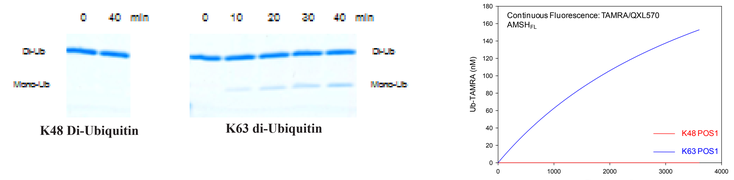
Left: USP5 catalyzed hydrolysis of 3 different K48-linked di-Ub substrates with different sites of labeling (EDANS and DABCYL). Substrates (250nM) were incubated with 5nM USP5 and the hydrolysis was monitored at ex336nM, em485nM for 17 minutes.

Right: USP5 catalyzed hydrolysis of 3 different K48-linked di-Ub substrates with different sites of labeling (TAMRA and QXL570). Substrates (400nM) were incubated with 0.5nM USP5 and the hydrolysis was monitored at ex544 nM, em572 nM for 30 minutes.



Comparison of FRET-based substrates (different labels and positions) with native unlabeled di-Ub in USP5 activity. The activity was calculated by the initial cleavage velocity divided by enzyme concentration.

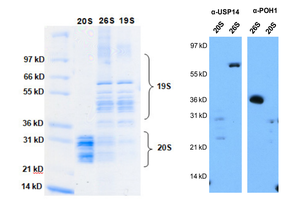
Identification of Linkage Specificity Using Fret Substrates



AMSH (Associated Molecule with the SH3-domain of STAM) is involved in K63-linked chain cleavage, as shown using our native, unlabeled, di-Ub substrates. 5mM of K48- and K63-linked di-Ub substrates were incubated with 2mM AMSHFL at 25oC. 300nM K48-linked di-Ub POS1 and K63-linked di-Ub POS1 were incubated with 1mM AMSHFL in 50mM HEPES, 150mM NaCl, and 2mM DTT. The assay was monitored at ex544 nm, em572 nm for 60 mins. No detectable activity was found for K48-linked di-Ub POS1, whereas AMSHFL showed activity against the K63-linked di-Ub POS1. Our data demonstrates that linkage specificity of particular DUBs can be detected by our FRET-based di-Ub substrates.

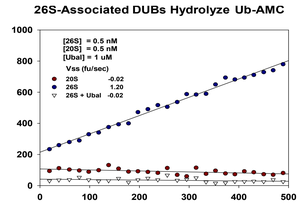
26S & 19S Associated DUB Activity

Purification and Characterization of Human 26S Proteasome



Stable human embryonic kidney cells, expressing a tagged component of the 26S complex, were used as the source for human 19S and 26S. Purified human 19S, 20S and 26S (2μg) were separated by 4-20% SDS-PAGE. Western blot analysis confirms presence of the 19S intrinsic DUB POH1, and 19S-associated DUB USP14.

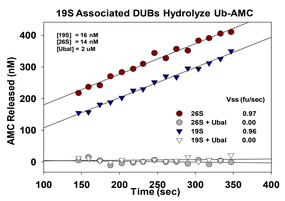
26S-Associated DUBs Hydrolyze Ub-AMC



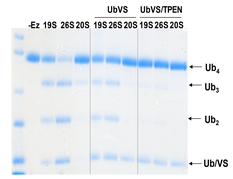
Enzymes (0.5 nM 20S and 26S) were incubated in the presence and absence of Ub-aldehyde (1mM) in 25mM Hepes pH 7.5, 100mM NaCl, 2mM MgATP, 0.5mM EDTA, 2mM DTT. The hydrolysis of Ub-AMC (1mM) was measured kinetically using a calibrated fluorimeter (λem:340, λex:465) at 37ºC.

Ub-VS-Insensitive cleavage of Ub Chains is Due to the 19S Subunit POH1.

19S Associated DUBs Hydrolyze Ub-AMC



Enzymes (16nM 19S or 14nM 26S) were incubated in the presence and absence of Ub-aldehyde (2mM) in 25mM Hepes pH 7.5, 100mM NaCl, 2mM MgATP, 2mM DTT. Ub-AMC (400nM) hydrolysis was measured kinetically using a calibrated fluorimeter (λem:340, λex:465) at 37ºC.



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