Supporting Information

Inhibiting the Protein Ubiquitination Cascade by Ubiquitin-Mimicking Short Peptides

Bo Zhao,[†,‡] Chan Hee J. Choi, [†,‡] Karan Bhuripanyo,[†] Eric Villhauer,[†] Keya Zhang,[†] Hermann Schindelin,[§] and Jun Yin[†,*]

[†]Department of Chemistry, University of Chicago, Chicago, IL 60637

[§]Rudolf Virchow Center for Experimental Biomedicine and Institute for Structural Biology, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany

Table S1. PISA analysis of the binding of the UB-mimicking peptides to Uba1.

Peptides	Interface area ($Å^2$)	ΔG (kcal/mol)	$K_{1/2}$ * (μM)	ΔG based on $K_{1/2}$ ** (kcal/mol)
P1 (^{70}VLRLRGG^{76})	704	-4.3	-	-
P2 (^{70}VWRFHGG^{76})	679	-6.0	342	-4.7
P3 (^{70}VQRYWGG^{76})	714	-5.7	426	-4.6
P4 (^{70}VYRFYGG^{76})	692	-6.3	141	-5.2

*Measured by ATP-PPi exchange from Table 1.
**Calculated based on the equation $\Delta G = 2.3\ RT \log_{10} K_{1/2}$, T= 298 K.

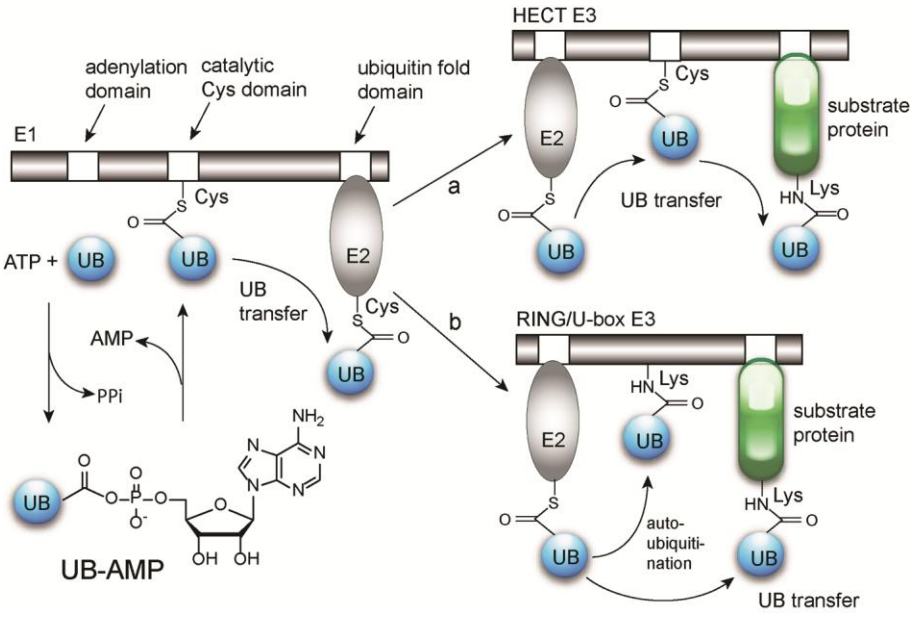

Figure S1. The E1-E2-E3 enzymatic cascade that catalyzes UB transfer to the substrate proteins for UB modification. Path a, UB transfer through HECT-type E3 enzymes to the substrate proteins. A covalent thioester intermediate is formed between UB and the HECT domain of E3 before UB is transferred to the substrate proteins. Path b, UB transfer from E2 directly to the substrate proteins by RING or U-box E3s. The autoubiquitination of RING/U-box E3s during UB transfer is also shown.

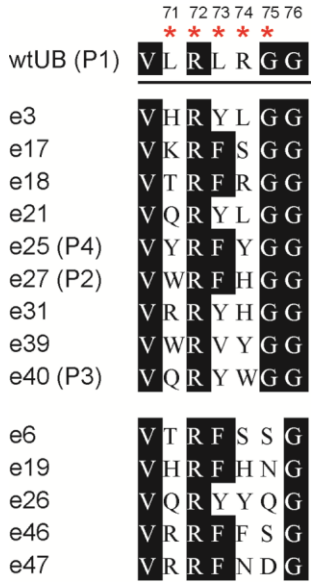

Figure S2. Alignment of the C-terminal sequences of the phage selected UB clones that are reactive with the human E1 enzymes Ube1 and Uba6. The red stars designate residues that were randomized in the UB library. This figure was adapted from a recent study on E1 specificity with UB C-terminal sequences.[1]

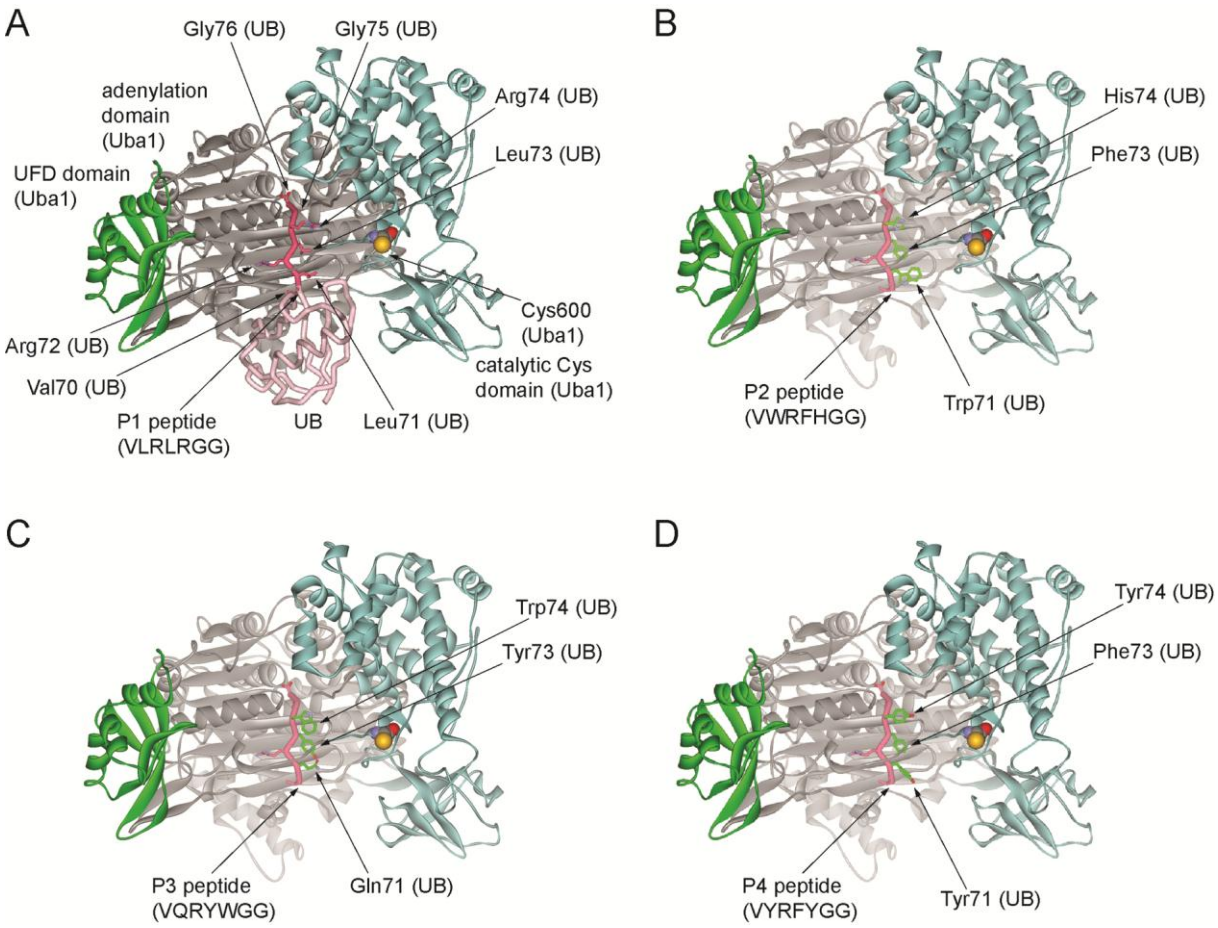

Figure S3. Modeled structures of UB-mimicking peptides bound to the yeast E1, Uba1. (A) Full length UB bound to Uba1 with the portion of the P1 peptide at the UB C-terminus colored in red (PDB ID 3CMM).[2] The adenylation domain of Uba1 is colored in grey, the catalytic Cys domain in cyan and the ubiquitin fold domain (UFD) in green. UB is colored in pink. The catalytic Cys residue of Uba1 (Cys600) is shown in a CPK model. Residues of the P1 peptides are also shown. The structure shows that E1 not only binds to the C-terminal peptide of UB, but also interacts with the hydrophobic patch of UB composed of residues Leu8, Ile44 and Val70, and a polar surface of UB involving Lys11, Thr12, Gln31 and Asp32.[2] (B), (C) and (D) show the modeled structures of the UB-mimicking peptides P2, P3 and P4 bound to Uba1, respectively. The peptides are colored in red and the mutated residues in the peptides are in green. Numbering of the peptide residues follows the full length UB protein.

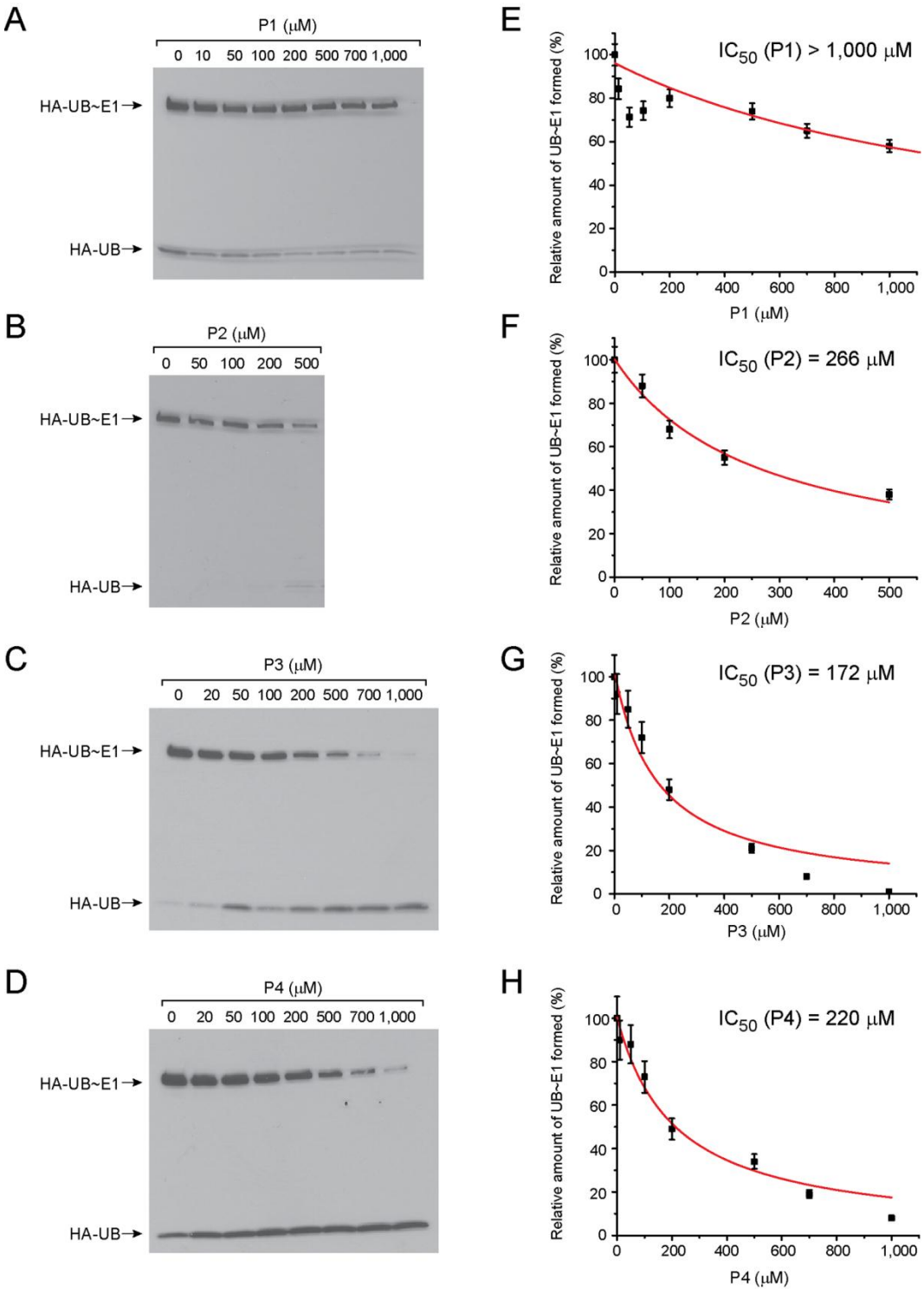

Figure S4. Inhibition of UB~E1 thioester formation by P1 with the C-terminal sequence of wtUB and the UB-mimicking peptides P2, P3 and P4. (A) - (D), Western blots of the UB transfer reactions to Ube1. 1 μM of UB was incubating with varying concentrations of peptides P1 (A), P2 (B), P3 (C) and P4 (D) for

competitive transfer to 0.3 μM of Ube1. The amounts of UB~E1 thioester formed in the reactions were quantified by measuring the intensities of the thioester bands on the Western blots of (A)-(D). The ratio of the amount of UB~E1 formed with and without the peptides were plotted against concentrations of the peptides in (E)-(H) to determine the IC_{50} values of the peptides.

Peptide P1

Sequence: VLRLRGG

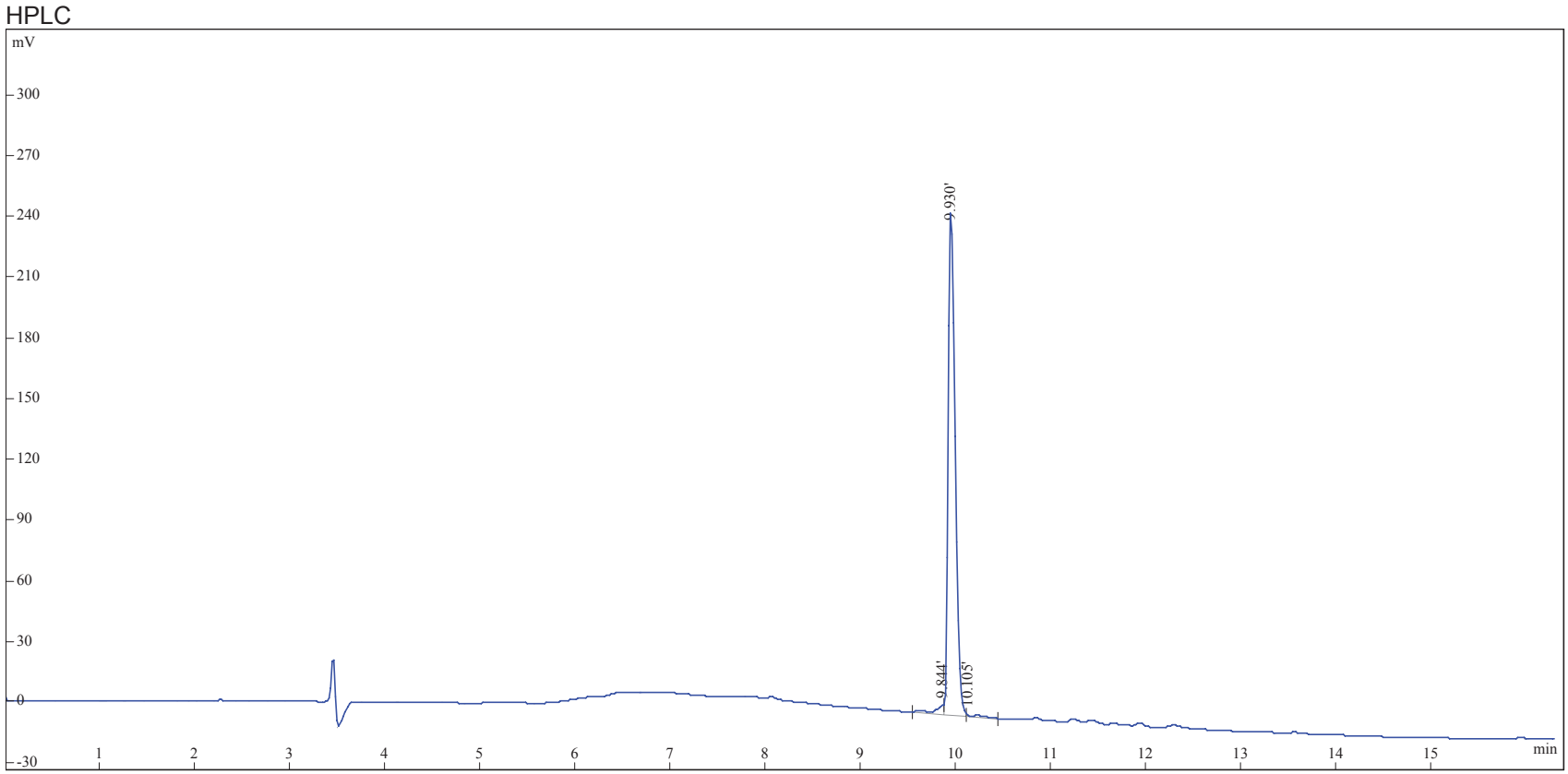

Figure S5. HPLC analysis of the P1 peptide.

Peptide P1

Sequence: VLRLRGG

M.W [M+H$^+$]: 769.95

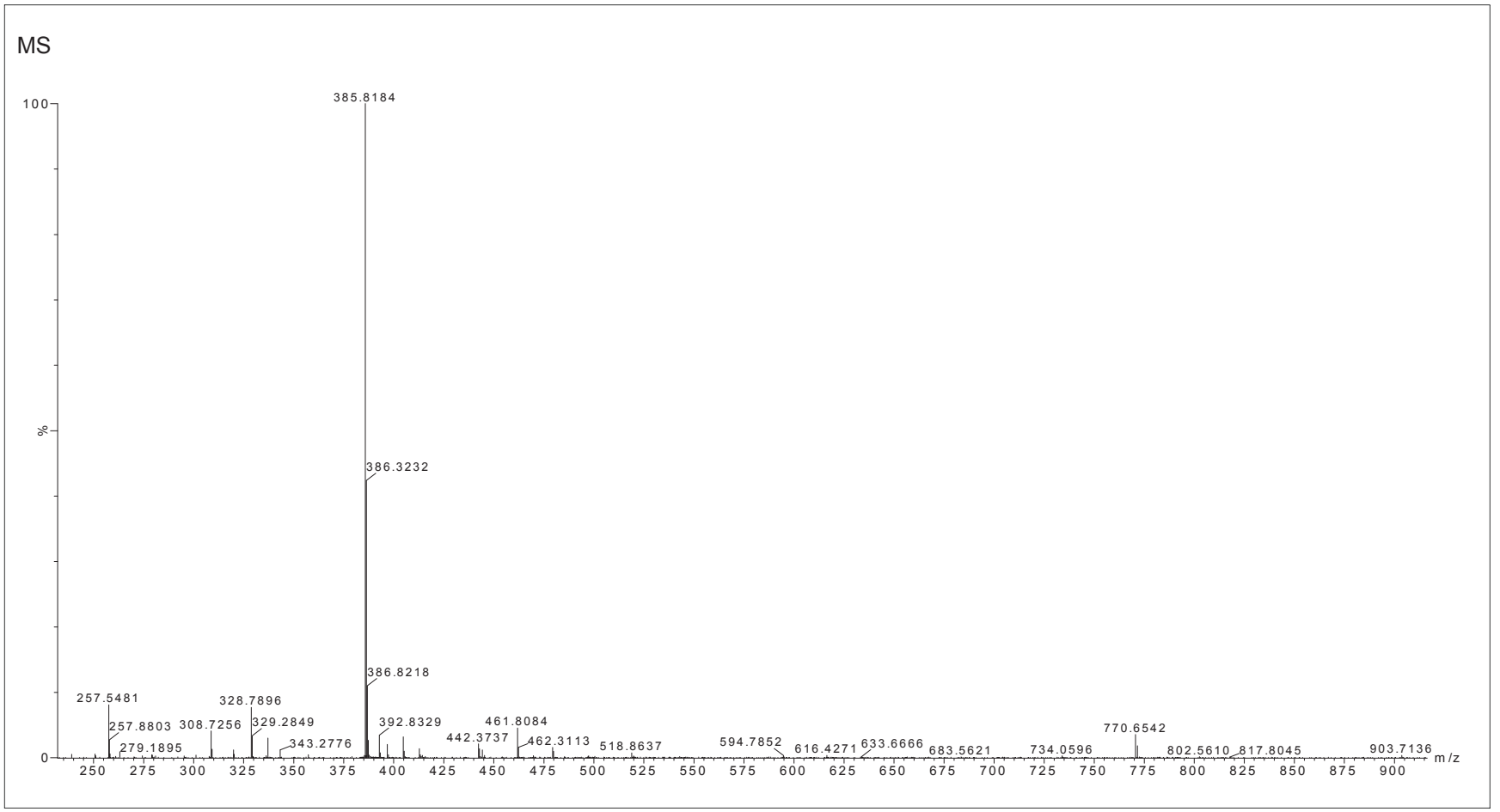

Figure S6. MALDI analysis of the P1 peptide.

Peptide P2

Sequence: VWRFHGG

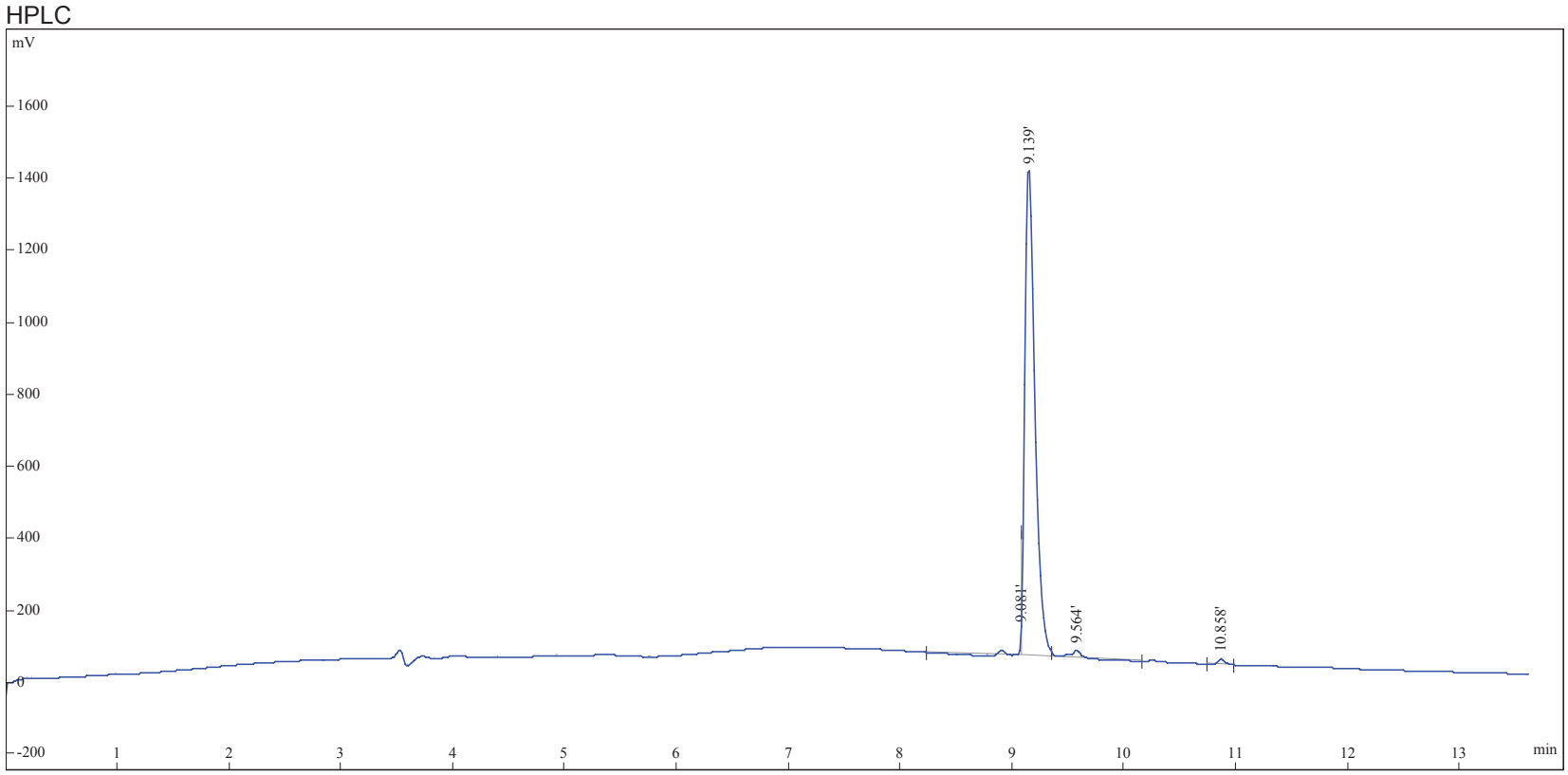

Figure S7. HPLC analysis of the P2 peptide.

Peptide P2

Sequence: VWRFHGG

M.W [M+H$^+$]: 858.96

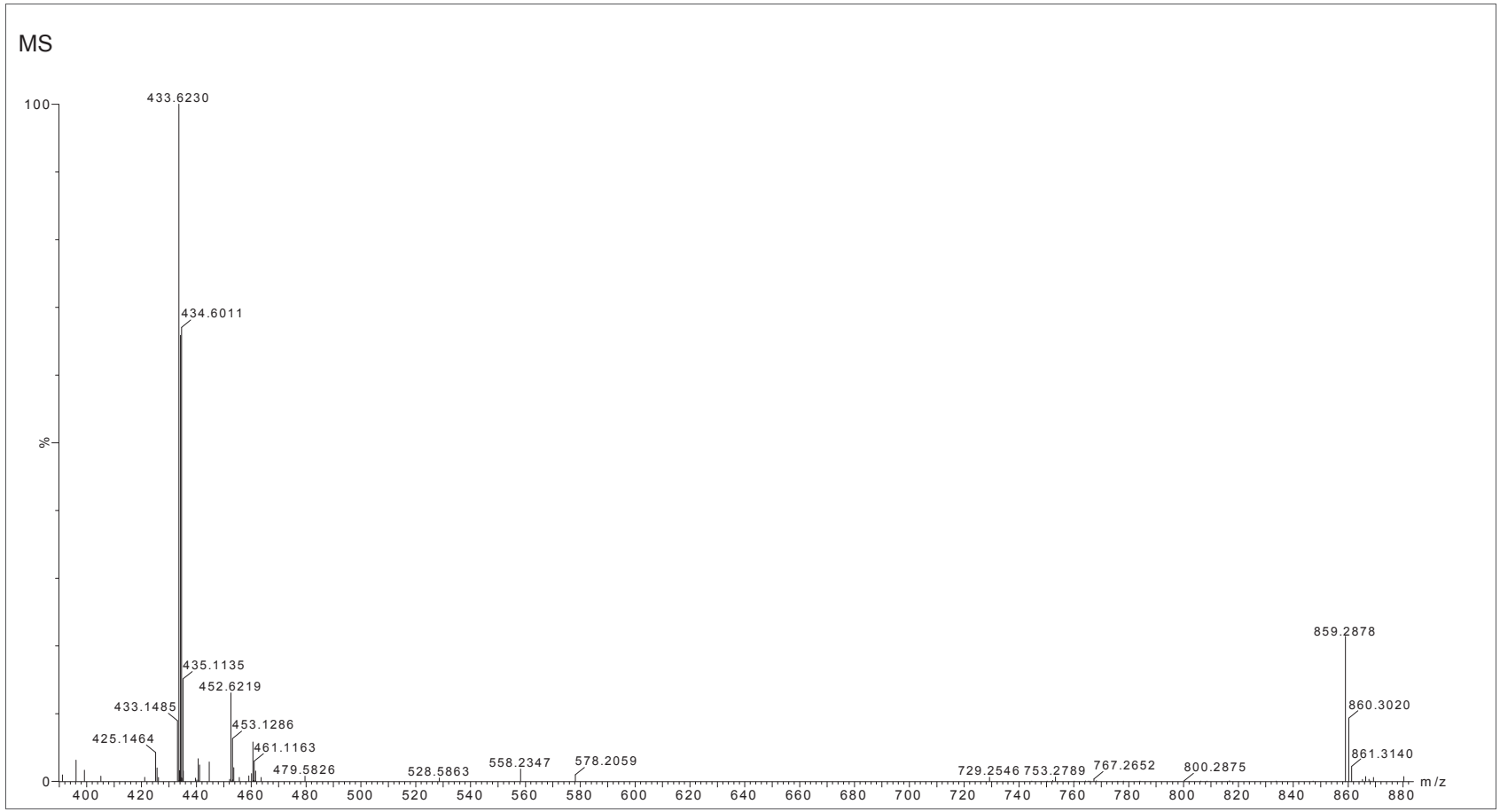

Figure S8. MALDI analysis of the P2 peptide.

Peptide P3

Sequence: VQRYWGG

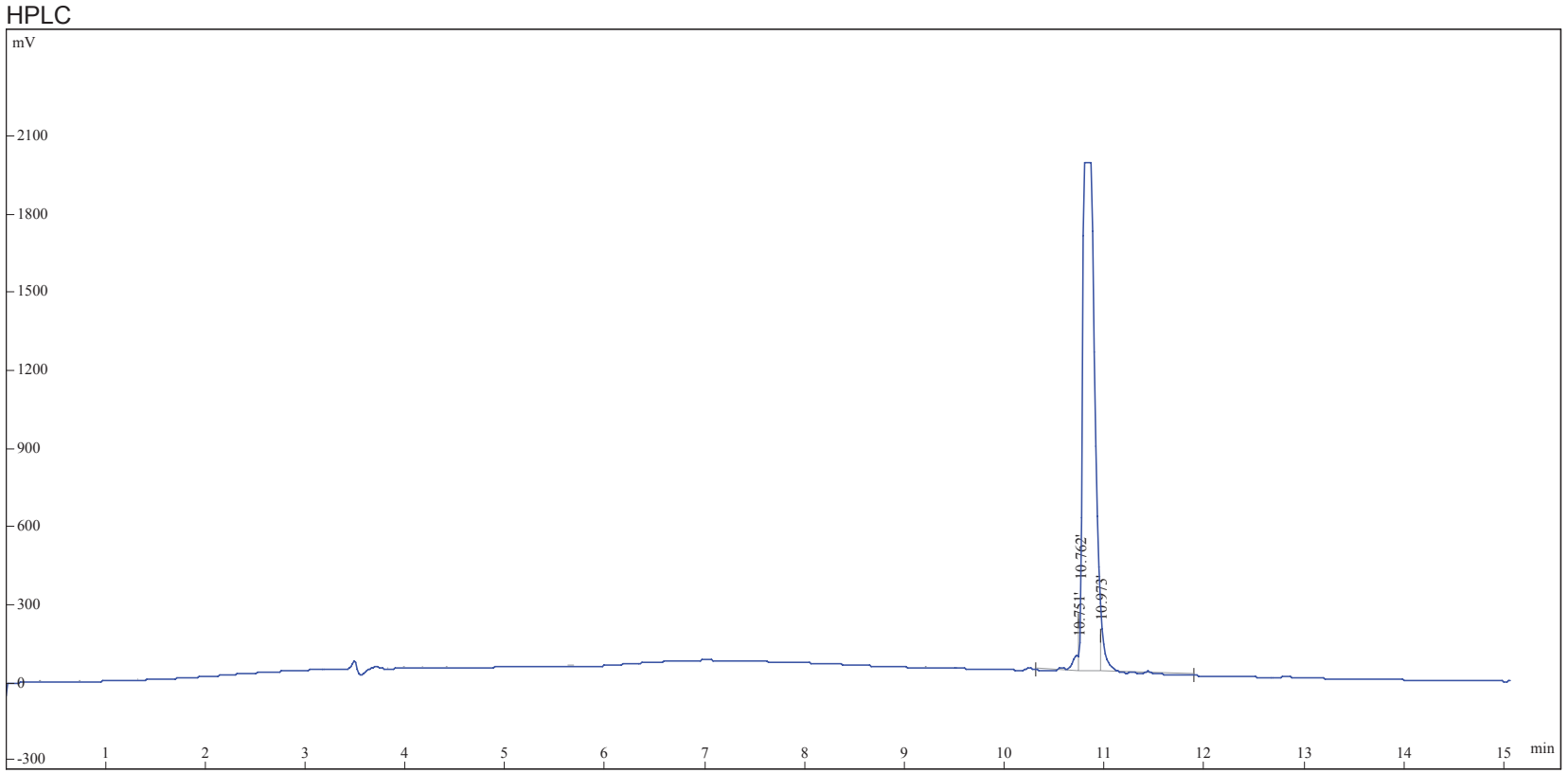

Figure S9. HPLC analysis of the P3 peptide.

Peptide P3

Sequence: VQRYWGG

M.W [M+H$^+$]: 864.97

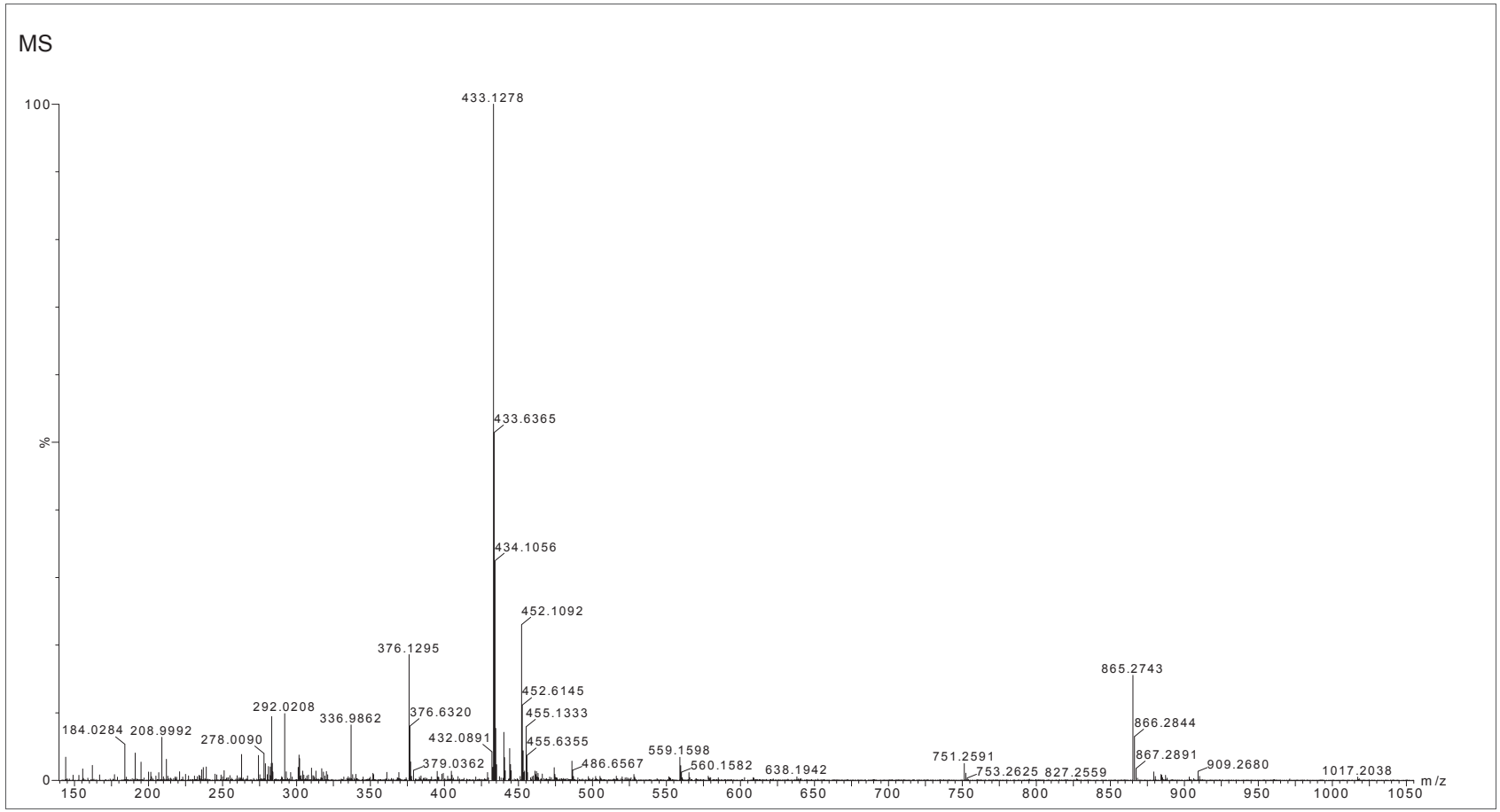

Figure S10. MALDI analysis of the P3 peptide.

Peptide P4

Sequence: VYRFYGG

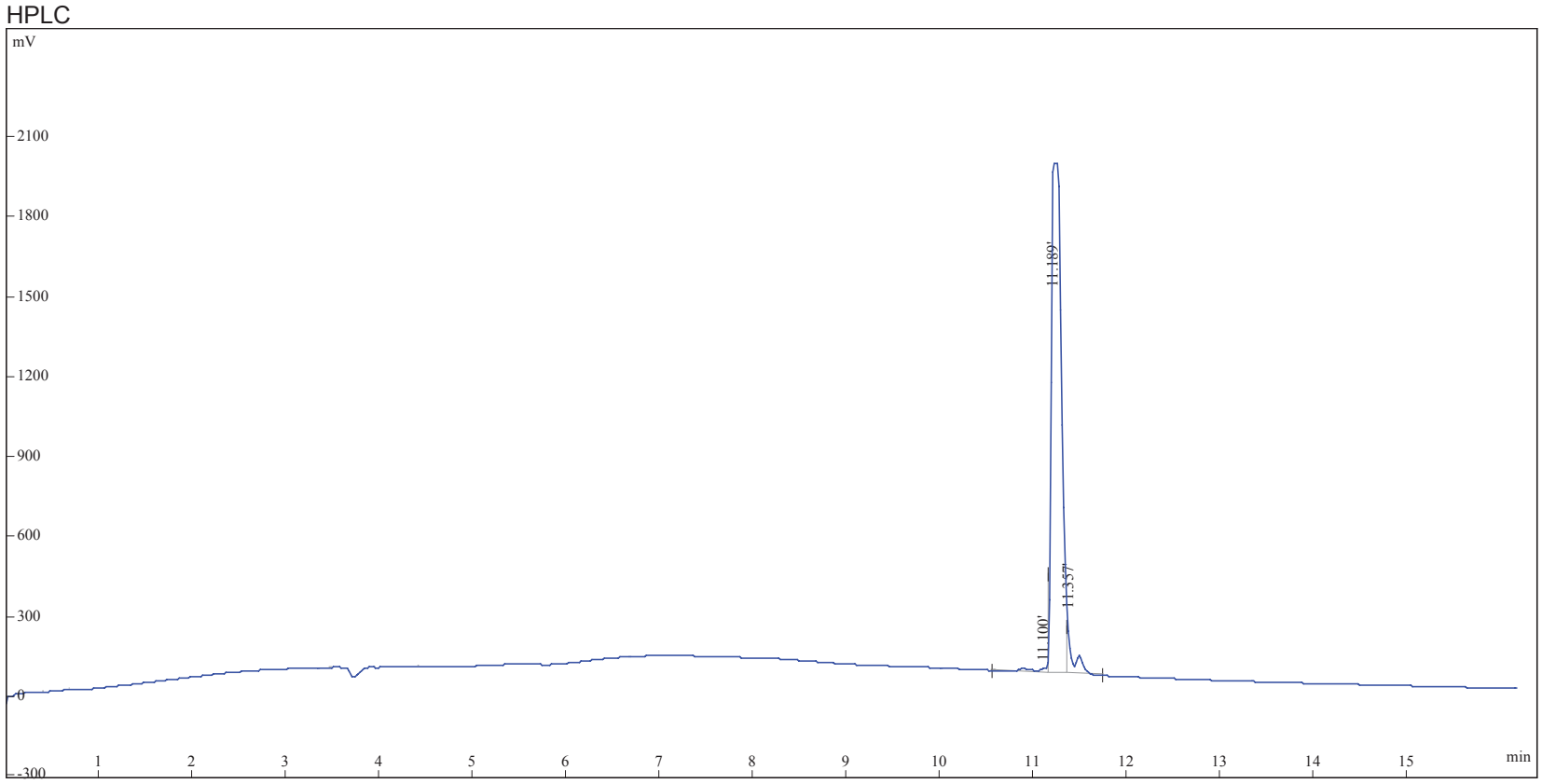

Figure S11. HPLC analysis of the P4 peptide.

Peptide P4

Sequence: VYRFYGG

M.W [M+H$^+$]: 860.98

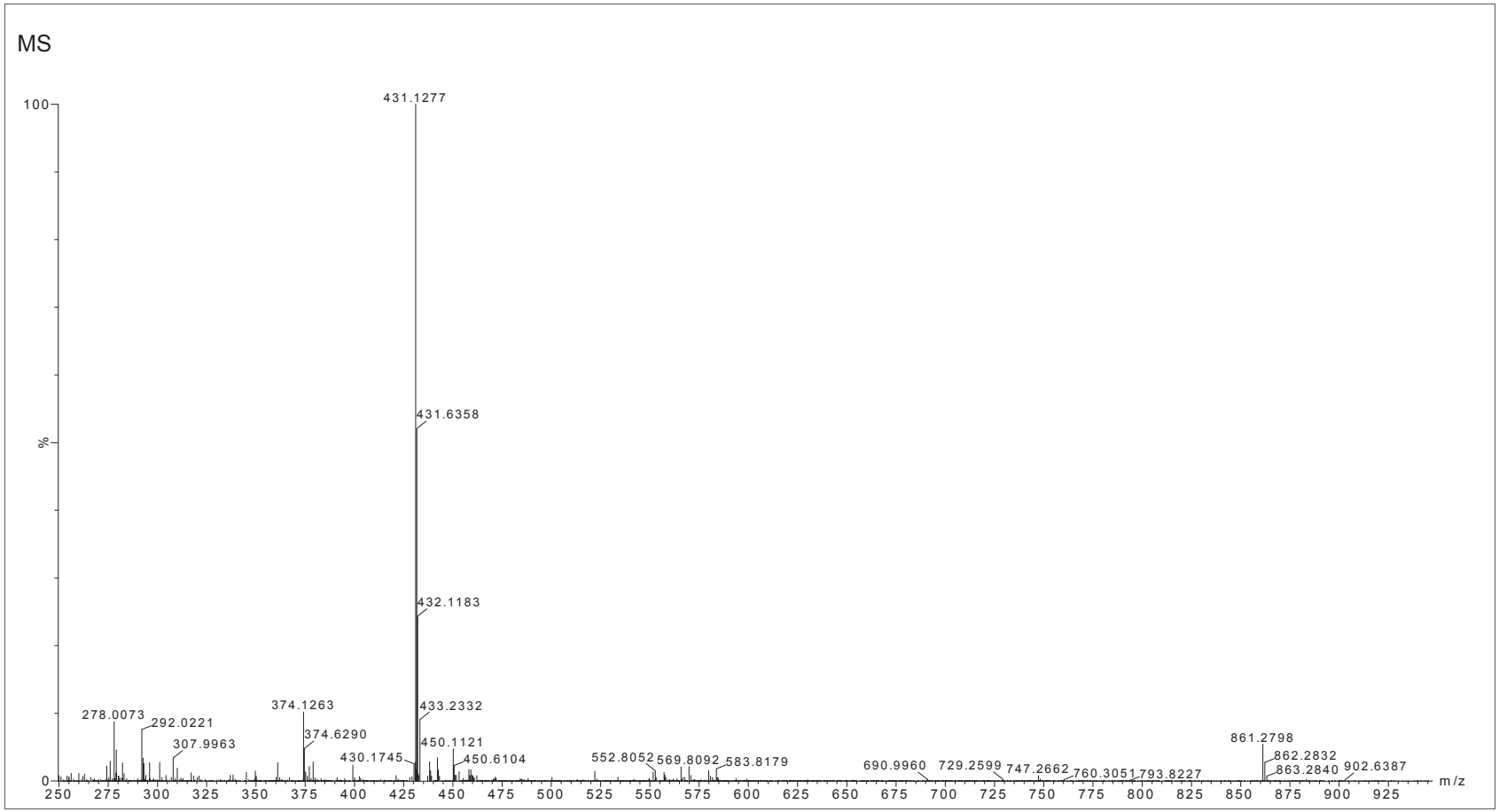

Figure S12. MALDI analysis of the P4 peptide.

Experimental Procedures

General

Unless otherwise indicated, all reagents were obtained from commercial sources and were used without further purification. All solutions and buffers were sterilized by either autoclaving or filtration. Kits for isolating DNA plasmids, and the Ni-NTA agarose resin for protein purification were from Qiagen. Restriction endonucleases were from New England Biolabs. Taq DNA polymerase was from Promega. HPLC purification of the peptide and biotin-peptide conjugates was carried out on a POLARIS BioInert Gradient LC System (Varian, Walnut Creek, CA) with a reverse phase Nucleodur C-18 column of 250 mm in length, 21 mm i.d. and 10 mm particle size (Phenomenex, Torrance, California). Biotin-peptide conjugates were analyzed by MALDI-TOF spectra acquired with a Voyager DE PRO MALDI mass spectrometer (PerSpective Biosystems, Framingham, MA) using 2,5-dihydroxybenzoic acid as the matrix.

Biotin conjugation of the peptides

Heptamer peptides with the C-terminal sequences of wtUB and UB variants were ordered from EZBiolab (Carmel, Indiana). The peptides were further purified by HPLC to be more than 95% pure. The results of HPLC and MALDI analysis of the peptides were shown in Figures S5-S12.

Heptamer peptide P1 (2 mg, 2.598 μmol) was added to a solution of N-hydroxysuccinimidyl-6'-(biotinamido)-6-hexanamido hexanoate (NHS-LC-LC-biotin, Pierce) (4.4 mg, 7.751 μmol) in 50 mM sodium phosphate buffer (pH 7.0, 300 μL) and DMSO (300 μL). The reaction was allowed to proceed by stirring overnight at room temperature. The reaction mixture was then purified by HPLC with a gradient of 5-85% acetonitrile in 0.1% TFA/water at a flow rate of 10 mL/min over the course of 25 minutes. Biotin was conjugated to the N-terminal amino groups of the peptides P2, P3 and P4 with the same procedure. The purified biotin-peptide conjugates were lyophilized, and their identities were confirmed via MALDI-TOF mass spectrometry (positive mode): calculated for biotin-P1 (VLRLRGG), $C_{55}H_{99}N_{17}O_{12}S$: 1222.55 Da, found: 1223.21 Da; calculated for biotin-P2 (VWRFHGG), $C_{63}H_{91}N_{17}O_{12}S$: 1310.57 Da, found: 1311.08 Da; calculated for biotin-P3 (VQRYWGG), $C_{62}H_{92}N_{16}O_{14}S$: 1317.56 Da, found: 1317.57 Da; calculated for biotin-P4 (VYRFYGG), $C_{64}H_{92}N_{14}O_{14}S$: 1313.57 Da, found: 1313.64 Da.

Biotin conjugation to UB.

The 11 residue ybbR tag was fused to the N-terminus of UB and expressed in the pET21b plasmid.[1,3] Sfp catalyzed ybbR tag labeling with biotin-CoA followed a reported protocol.[4] 100 μL labeling reaction was set up with 20 μM ybbr-UB, 25 μM biotin-CoA, 1 μM Sfp in a buffer containing 10 mM $MgCl_2$ in 50 mM HEPES (pH 7.5). The reaction was allowed to proceed for 1 hour at 30˚C. The attachment of biotin-phosphopantetheinyl group to UB was confirmed by MALDI-TOF spectra.

Protein expression and purification.

The pET plasmids for the expression of HA-UB, ybbR-UB, Ube1, Ubc1, UbcH5a, UbcH7, E6AP HECT domain and CHIP were reported previously.[1] The expression plasmids were transformed into

BL21(DE3)pLysS chemically competent cells (Invitrogen) and plated on the LB-agar plates with appropriate antibiotics. Protein expression and purification followed the protocol provided by the vendor of the pET expression system (Novagen) and the Ni-NTA agarose resin (Qiagen).

ATP-PP_i exchange assays.

The initial velocities of E1-catalyzed UB and peptide activation were followed by the ATP-PPi exchange assay. [5] Typically 50 μL reactions were set up containing varying concentrations of wtUB or UB-mimicking peptides, 0.05 μM Ube1, 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, and 1 mM ATP. The reactions were initiated by the addition of 1 mM sodium [^{32}P]pyrophosphate (4.6 Ci/mol). The reactions were incubated at room temperature and quenched at various time points by addition of 0.5 mL of a suspension of activated charcoal (1.6% (w/v) charcoal, 0.1 M tetrasodium pyrophosphate, 0.35 M perchloric acid) to each of the reactions. The charcoal was pelleted by centrifugation. Each charcoal pellet was washed three times with 1 ml 2% trichloroacetic acid. Finally the charcoal pellet was resuspended in 0.5 ml water and the suspension was added to 3.5 ml Ultima Gold LSC-cocktail (PerkinElmer). The radioactivity bound to charcoal was determined by liquid scintillation counting. To measure the kinetics of the wtUB activation by Ube1, initial velocities of ATP-PPi exchange were determined with concentrations of UB varying from 0.05 μM to 5.0 μM. To measure the kinetics of UB-mimicking peptide activation by Ube1, the concentrations of the peptides were varied from 10 μM to 1,000 μM. The kinetic data were fitted to the Michaelis-Menten equation with the data analysis software Origin.

Western blot analysis of peptide loading onto the E1, E2 and E3 enzymes.

The conditions of the peptide transfer reactions to E1, E2 and E3 enzymes in Figure 1 and 2 are as follows. Figure 1A: 5 μM biotinylated peptide and 1 μM Ube1 were incubated with 1 mM ATP, 10 mM $MgCl_2$, and 50 μM DTT in TBS buffer (20 mM TrisHCl, 150 mM NaCl, pH 7.5) for 1 hour at room temperature before SDS-PAGE and Western blot analysis. Figure 1B, 5 μM biotin-labelled peptide, 1 μM Ube1, 3 μM E2 (Ubc1, UbcH5a, or UbcH7) were incubated with 1 mM ATP, 10 mM $MgCl_2$, 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE and Western blot analysis. For peptide transfer to the E3 enzymes in Figure 2, the reaction condition was the same as in Figure 1C with the use of 1 μM UbcH7 and 5 μM the HECT domain of E6AP in Figure 2A, and 1 μM UbcH5a and 5 μM CHIP in Figure 2B. For control reactions in Figure 1A and 2A, 5 μM biotin-labeled UB was used instead of biotin-labelled peptides.

To analyze peptide transfer reactions by Western blot, 20 μL of the reaction was loaded on a 4-15 % SDS-PAGE gel (Bio-Rad). After electrophoresis, the protein bands were electroblotted onto a piece of polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with 3% BSA in TBS buffer (pH 7.5) for 1 hour followed by incubation with 3% BSA in TBS buffer (pH 7.5) containing 1:10,000 diluted 1 mg/mL streptavidin-horse radish peroxidase conjugate (Pierce) for 1 hour. The membrane was then washed 5 times by TBS-T buffer (0.05% (v/v) Tween 20, 0.05% (v/v) Triton X-100 in TBS, pH 7.5) and 5 times by TBS buffer (pH 7.5) followed by detection with the ECL luminescent detection kit (GE Healthcare).

Peptide inhibition of UB transfer to the E1-E2-E3 cascade.

The conditions for the inhibition of UB transfer to E1 by the peptides (Figure 3A) were as follows. Varying concentration of the peptides of 10, 20 and 50 μM were incubated with 1 μM Ube1 in the presence of 1 mM ATP and 10 mM $MgCl_2$ in TBS buffer for 10 minutes at room temperature. 5 μM HA-UB was then added and the reaction mixtures were incubated for another hour to allow UB transfer to the E1 enzyme. The reactions were then analyzed by SDS-PAGE and Western blot. The conditions for inhibition of UB transfer to the E3 enzymes were as follows. Figure 3B: Varying concentrations of the P3 or P4 peptide at 5, 10, 20, 50 and 100 μM were incubated with 0.5 μM Ube1, 0.5 μM UbcH5a and 2 μM CHIP for 10 minutes in TBS buffer containing 1 mM ATP, 10 mM $MgCl_2$ and 50 μM DTT. After preincubation with the peptides, 5 μM HA-UB was added and the CHIP ubiquitination reaction was allowed to proceed for 1 hour. Figure 3C: Varying concentration of the P3 and P4 peptide at 5, 10, 20, 50, 100 and 200 μM were incubated with 1 μM Ube1, 1 μM UbcH5a and 3 μM HECT domain of E6AP for 10 minutes before the addition of 5 μM HA-UB to initiate the UB transfer to the HECT domain. The UB transfer reaction was allowed to proceed for another hour before SDS-PAGE and Western blot analysis. To probe for HA-UB conjugated proteins, the blot was developed by sequential incubation with 3% BSA in TBS buffer (pH 7.5) containing 1:500 diluted 200 μg/mL anti-HA antibody (Santa Cruz Biotechnology) and 1:10,000 diluted anti-mouse antibody - horseradish peroxidase conjugate (Pierce) for 1 hour, respectively.

Measuring the IC_{50} values of peptide inhibition of UB~E1 thioester formation.

Peptides P1, P2, P3 and P4 of varying concentrations (10, 50, 100, 200, 500, 700, 1000 μM) were incubated with 1 μM HA-UB and 0.3 μM Ube1 in 50 μL reactions containing 10 mM $MgCl_2$, 1 mM ATP and TBS buffer. The reaction was allowed to proceed for 15 seconds at room temperature to measure the initial velocities of the UB transfer reaction to E1. The reaction mixtures were analyzed by SDS-PAGE and Western blots probed with the anti-HA antibody. The intensities of the protein bands corresponding to HA-UB~E1 conjugates were quantified with the Molecular Imager (Bio-Rad FX Pro Plus) and the associated software. The ratio of the amount of UB~E1 formed in the reactions with and without the peptide inhibitors were calculated and plotted against the concentrations of the peptides. The IC_{50} values were calculated by the Origin program as the concentration of the peptide inhibitors that afforded 50% inhibition for the formation of UB~E1 conjugate.

Modeling of the Uba1-peptide complexes.

Based on the crystal structure of Uba1 from *Saccharomyces cerevisiae* in complex with ubiquitin bound to the adenylation active site (PDB entry 3CMM, Molecule 2 consisting of chains B and D),[2] residues 1-70 of ubiquitin were deleted resulting in the Uba1-P1 peptide complex. The corresponding P2-P4 complexes were generated by *in silico* mutagenesis with COOT.[6] All altered residues could be inserted in a preferred rotamer conformation and no changes to the main chain were incorporated. The interfaces between Uba1 and the P1-P4 complexes were analyzed with the Protein Interfaces, Surfaces and Assemblies (PISA) server provided by the European Bioinformatics Institute (EBI).[7]

References

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