Supporting Information

Stabilized β-Hairpin Peptide Inhibits Insulin Degrading Enzyme

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Contents

1. General Information

Abbreviations

IPTG, isopropyl β-D-1-thiogalactopyranoside

PMSF, phenylmethanesulfonyl fluoride

2. Experimental Section

Confocal laser-scanning microscopy images

HeLa cells were seeded in 24-well plates on coverslips and allowed to grow overnight in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco), medium supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL) and 1% penicillin/streptomycin (pen/strep, Gibco) at 310 K with 5% CO_2 in a humidified incubator. HeLa cells were treated with 5 μM FITC-labelled peptides in FBS-free medium for 2 h at 310 K. The peptide solution was removed and cells were washed with phosphate-buffered saline (PBS) and 0.4% trypan blue. The cells were fixed in 4% (wt/vol) paraformaldehyde in PBS for 15 min at 298K and then washed with PBS for 3 times. The coverslips were mounted on slides upside down with histology mounting medium containing DAPI (Sigma). Samples were dried at room temperature and imaged using a confocal microscope (Olympus).

Cellular uptake of B35-FITC experiment

HeLa cells were cultured for 2 days and treated with 50 μM **B35**-FITC peptides for 2 h. The peptide solution was removed and cells were washed with phosphate-buffered saline (PBS) and 0.4% trypan blue. Cells were then exposed to trypsin (0.25%, Gibco) for 3 min at 37 °C, washed with PBS, and repeated freeze-thaw cycles. After centrifugation, the soluble peptide in the supernatant was analyzed by LC-MS. LC-MS analysis was performed on LC-MS 8030 (SHIMAZU LC-MS 2020 mass-spectrometer, Agilent Zorbax SB-Aq: 4.6 × 250 mm, 495 nm).

1D NMR and 2D NMR experiments[1]

1D NMR and 2D NMR spectra of peptide **B35** were collected on a Bruker Avance III 500MHz spectrometer with a cryo probe in H_2O:CD_3CN(v/v) = 6:4. Watergate pulse sequence with gradients were used for water suppression in 1D and 2D NMR spectrum. 2D 1H-1H TOCSY and NOESY data were collected with mixing time of 100 ms and 300 ms, respectively. 2D 1H-1H TOCSY and NOESY spectra were collected with a 12 ppm spectra width and 2048 × 200 complex points. All the data were processed with TopSpin® and analyzed with NMRViewJ software.

Expression and purification of wild type IDE and IDE-E111Q proteins[2-3]

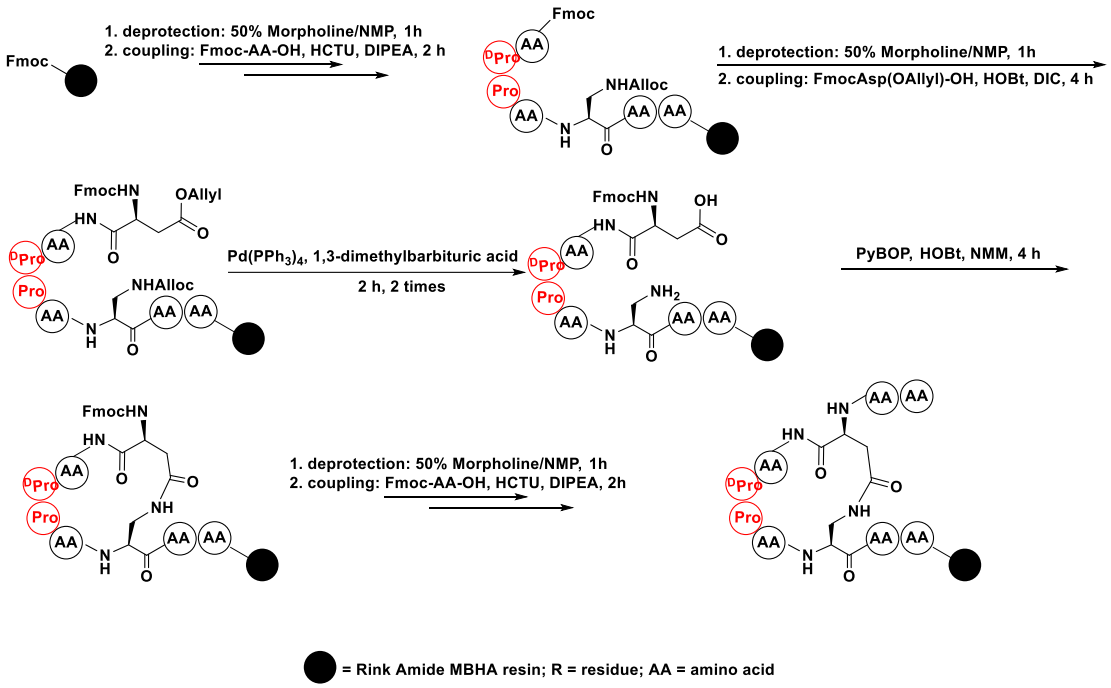
The IDE wide type strain *IDE-pProEX* was the kind gift of Prof. Wei-Jen Tang (Ben-May Institute for Cancer Research, The University of Chicago) and expressed in *E. coli* strains Rosetta (DE3). Mutant IDE-E111Q constructs were generated by amplifying the full vector wild type *IDE-pProEX* with the primers showed in Table S1. The mutant strain IDE-E111Q mutation was expressed in *E. coli* strains Rosetta (DE3). Protein expression was induced by adding 0.5 mM IPTG at OD_{600} 0.6-0.8 After overnight growth at 289 K with shaking for 16-20 h, cells were collected by centrifugation and re-suspended in 25-30 mL of Ni-NTA buffer A (20 mM Tris-HCl pH7.6, 5 mM imidazole, 100 mM NaCl, 1 μM β-mercaptoetnanol, 1 mM PMSF), then lysed by sonication (273 K). Cell debris was removed by centrifugation (13,000 rpm, 277 K, 30 min) and then the supernatant was filtrated with filter membrane (0.45 μm). The supernatant proteins were loaded to a Ni-NTA column (GE LifeScience), then the column were washed with 3 × bed volume of 0 %, 5 % and 10 % (v/v) buffer B (20 mM Tris-HCl pH 7.6, 150 mM imidazole, 50 mM NaCl, 1 μM β-mercaptoetnanol, 1 mM PMSF). Then the protein was eluted with a 40%-100 % gradient of buffer B. The fractions were collected, concentrated, and further purified with source-Q column of anion exchange chromatography (GE Life

Science) using a gradient program with buffer C (50 mM Tris-HCl pH8.0, 1 µM β-mercaptoetnanol, 1 mM PMSF) and buffer D (50 mM Tris-HCl pH8.0, 1 M NaCl, 1 µM β-mercaptoetnanol, 1 mM PMSF). The corresponding protein fractions were further purified with a Gel filtration column of size exclusion chromatography with buffer E (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM TCEP).

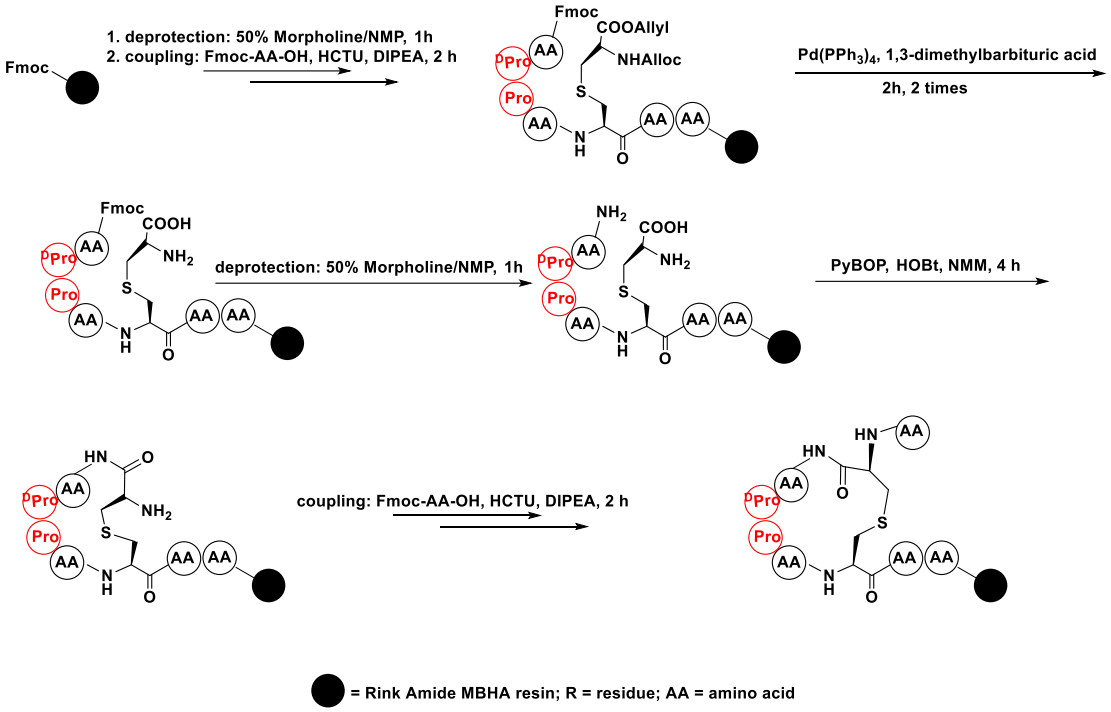
IDE-insulin degrading experiment

To confirm the wild type IDE protein can degrade insulin *in vitro*, 20 µg wild type IDE and 10 µg insulin were co-incubated in 310 K for 10 min or 40 min, then the mixture was centrifuged and the supernatant were analyzed with HPLC.

3. Schemes, Figures and Tables



Scheme S1. Synthesis of ^{D}Pro-^{L}Pro template and covalent macrocyclization to construct the β-sheet peptides using manual Fmoc/tBu solid-phase peptide synthesis (SPPS). Cyclization was performed on the resin using PyBOP/HOBt/NMM (2:2:2.4 eq) in DMF with N_2 bubbling.



Scheme S2. Synthesis of ^{D}Pro-^{L}Pro template and thioether bond to construct the β-sheet peptides using manual Fmoc/tBu solid-phase peptide synthesis (SPPS). Cyclization was performed on the resin using PyBOP/HOBt/NMM (2:2:2.4 eq) in DMF with N_2 bubbling.

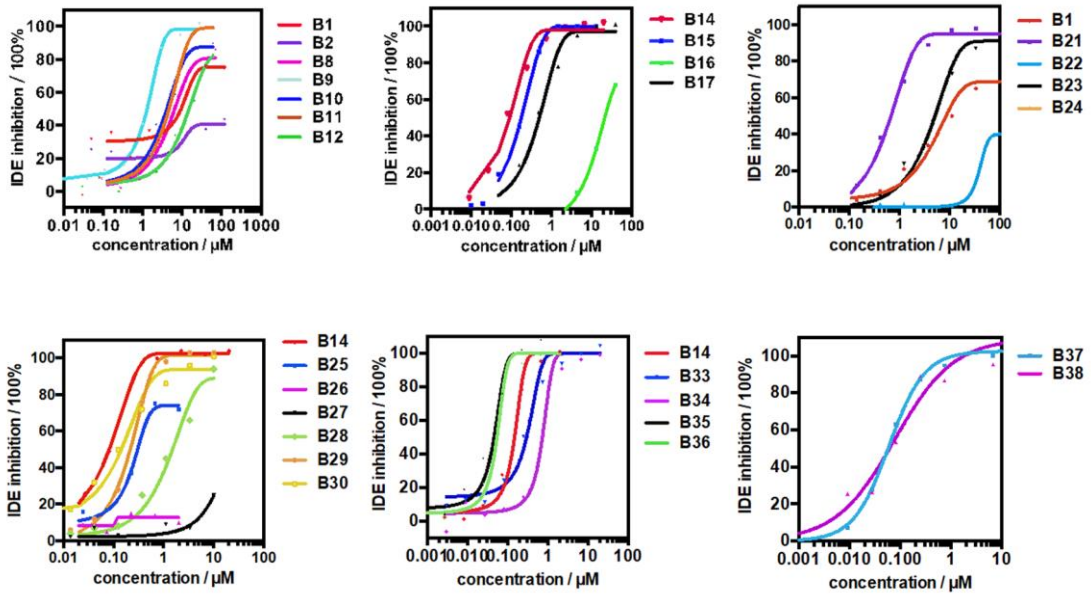


Figure S1. Protease assay results using the reported fluorophore/quencher-tagged peptide substrate Mca-RPPGFSAFK(Dnp)-OH (R&D). Sequence of these peptides were listed in **Table S2**.

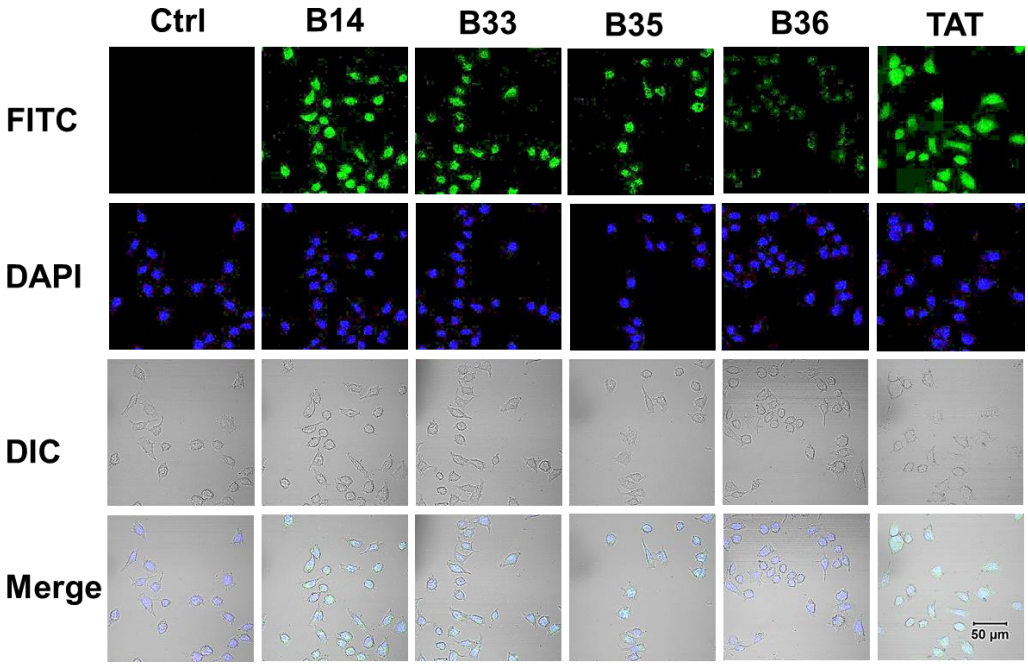


Figure S2. Confocal laser-scanning microscopy images of HeLa cells incubated with DMSO (Ctrl), **B14**-FITC, **B33**-FITC, **B35**-FITC, **B36**-FITC and **TAT**-FITC (5 μM) at 310 K for 2 h. Cells were washed with PBS and 0.4% trypan blue, fixed in 4% paraformaldehyde, stained with DAPI, and imaged using a confocal microscope (Olympus). Scale bar: 50 μm.

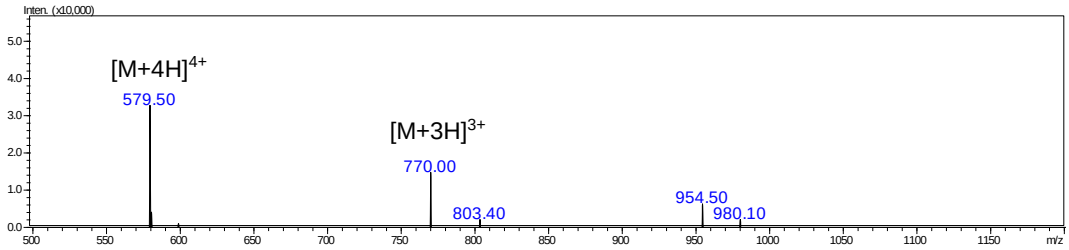


Figure S3. Cellular uptake of **B35**-FITC conjugate. HeLa cells were cultured for 2 days and treated with 50 μM **B35**-FITC peptides for 2 h. Cells were washed with PBS and 0.4% trypan blue, then exposed to trypsin (0.25%, Gibco) for 3 min at 37 °C. Collected cells and repeated freeze-thaw cycles. After centrifugation, the soluble peptide in the supernatant was analyzed by LC-MS. **B35**-FITC conjugate was confirmed by fragment ion peak of 770.00 $[M+3H]^{3+}$ and 579.50 $[M+4H]^{4+}$.

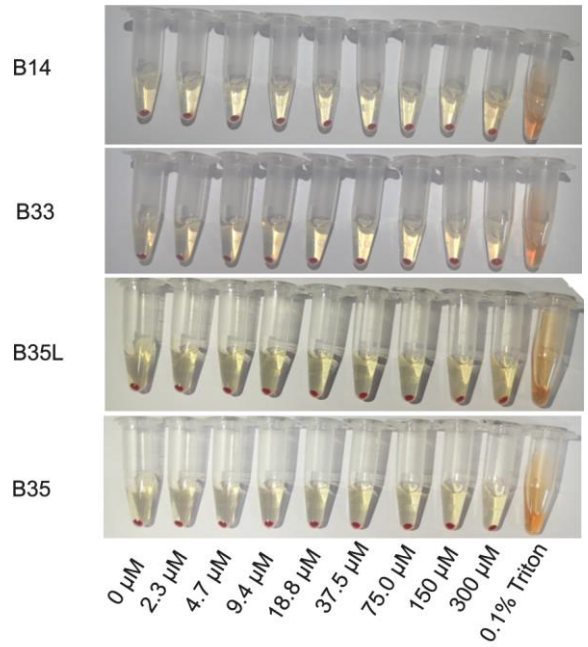


Figure S4. Hemolytic activity assay of peptides in fresh mouse blood. The corresponding relative supernatant of different peptides incubated with mouse's red blood cells in hemolytic activity assay. 0.1% Triton X 100 acted as positive control. All the peptides did not exhibit obvious hemolysis at concentrations below 300 μM.

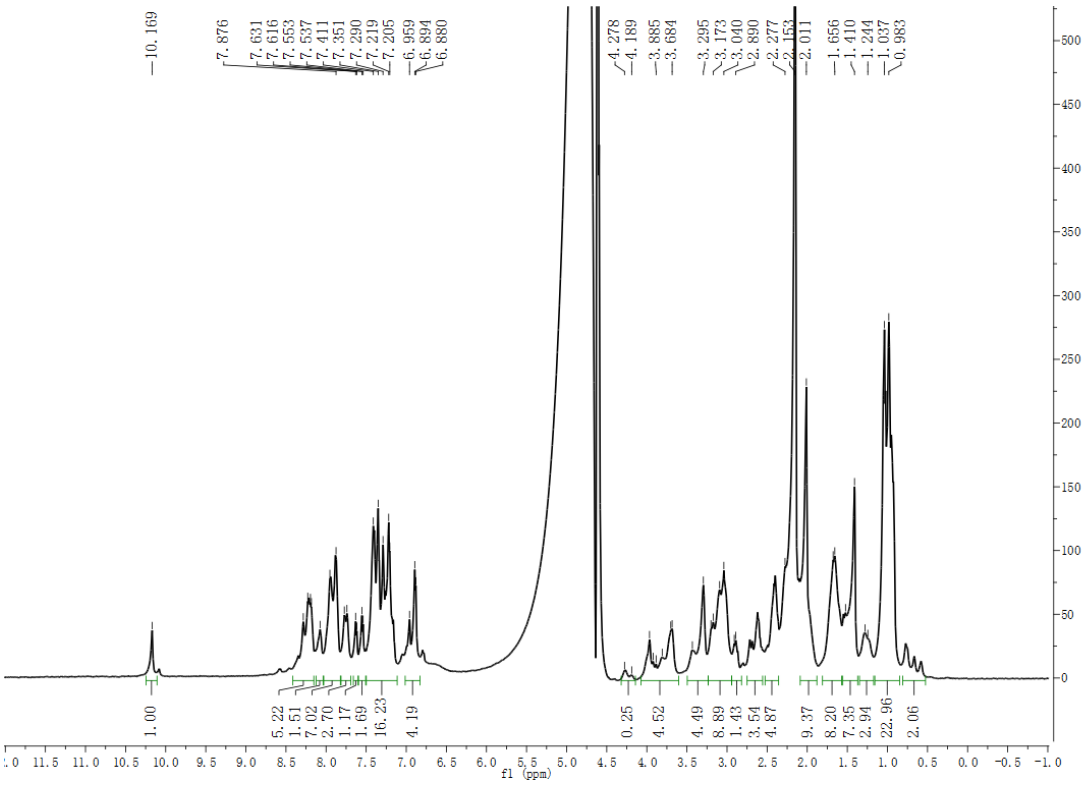


Figure S5. 1H NMR spectra of peptide **B35** (H_2O:CD_3CN(v/v) = 6:4, 298K, 500 MHz, mixing time: 300 ms).

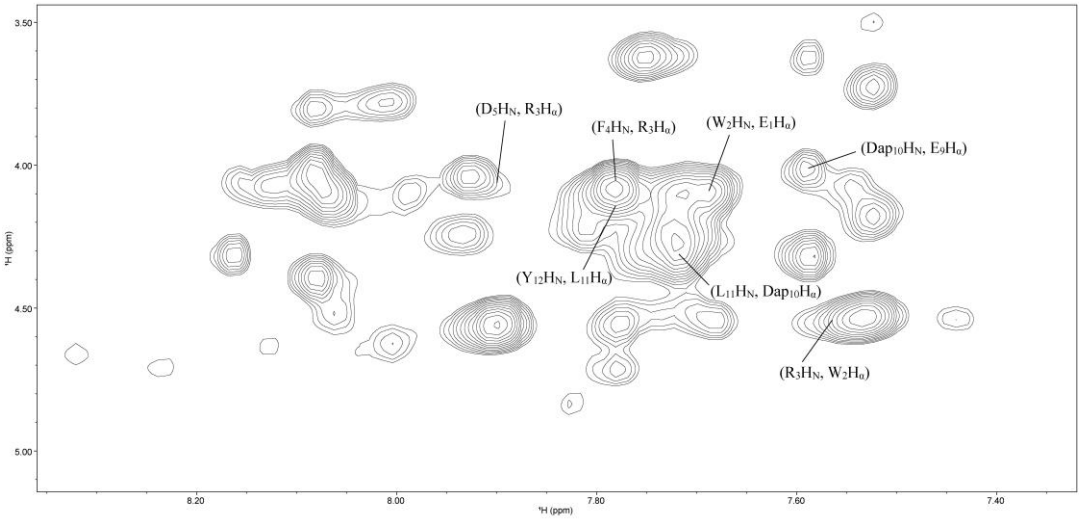


Figure S6. HN-H**α**C**α** region of 2D-NOESY spectrum for peptide **B35** (H_2O:CD_3CN(v/v) = 6:4, 298K, 500 MHz, mixing time: 300 ms). HN-H**α**C**α** cross-peaks were indicated and labelled by one letter amino acid codes and their sequential numbers from N to C terminal in **B35**. There are the **α**N(i, i + 2) cross-peaks between the residue Arg3-Asp5, **α**N(i, i + 1) cross-peaks in residues Glu1-Phe4 and Glu9-Tyr12.

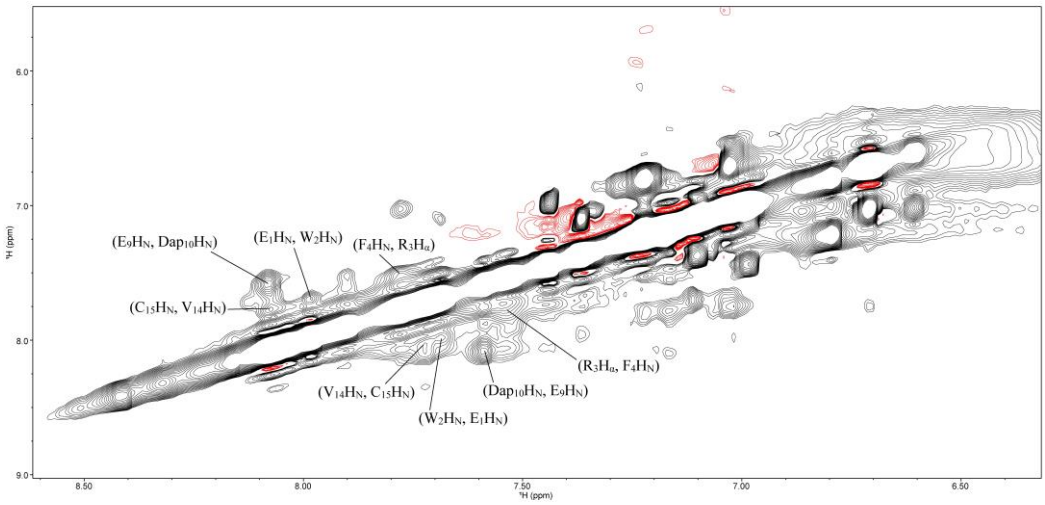


Figure S7. HN-HN region of 2D-NOESY spectrum for peptide **B35** (H_2O:CD_3CN(v/v) = 6:4, 298K,

500 MHz, mixing time: 300 ms). There are the NN(i, i + 1) cross-peaks in residues Glu1-Phe4, Glu9-Dap10 and Val14-Cys15 clearly.

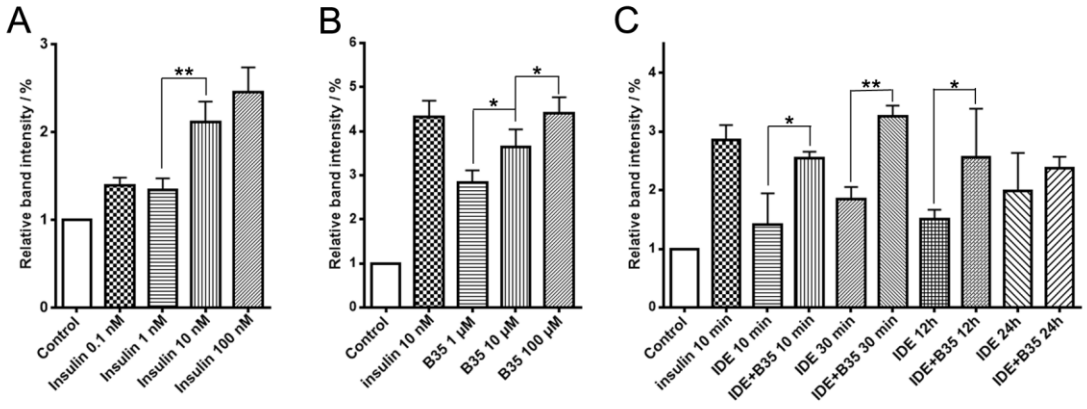


Figure S8. The corresponding relative band intensity of Western blot results in Figure 3A-3C. (A) Western blot analysis of pAKT induced by different concentrations of insulin. (B) Effects of different concentrations of peptide **B35** on the block of IDE inhibition to insulin signaling in skeletal muscle cells. (C) The inhibition efficiency of peptide **B35**. Skeletal muscle cells were treated with 100 nM IDE and 100 μM **B35** at various time points (10 min, 30 min, 12 h and 24 h). Different regions of corresponding spots were measured. Data were shown as mean ± SEM. SEMs shown as error bars in the figures. Statistics were carried out using a two-tail Student's t-test with GraphPad Prism 6.0. The significance levels were shown as *$P < 0.05$ and **$P < 0.01$.

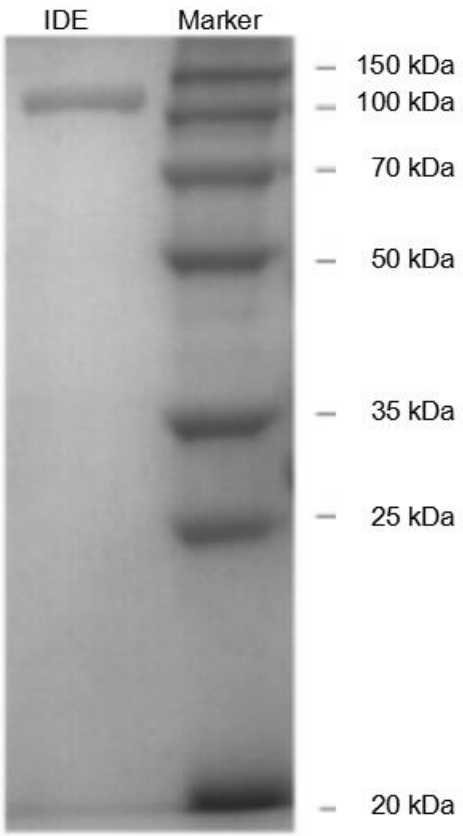


Figure S9. The 12 % (wt/vol) SDS-PAGE gel of purified wild type IDE. The protein was > 90 % pure with molecular weight about 113 kDa.

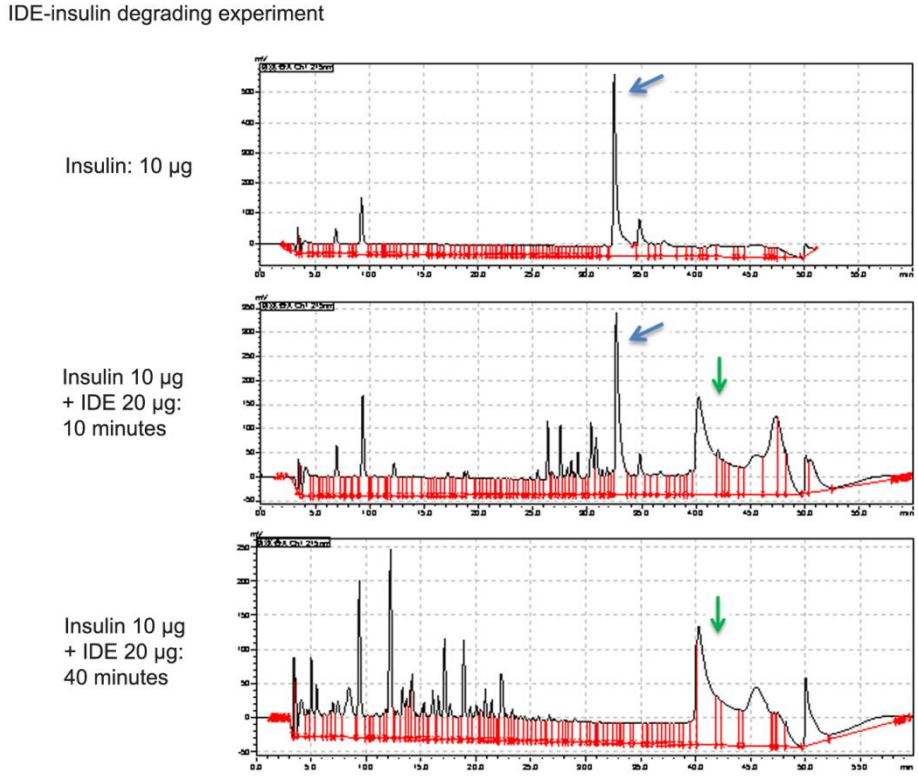


Figure S10. The HPLC plots of IDE-insulin degrading experiment. 20 μg wild type IDE and 10 μg insulin were co-incubated in 310 K for 10 min or 40 min, then the mixture were centrifuged and the supernatant were analysised with HPLC. The purified wild type IDE can degrade insulin completely in 40 min.

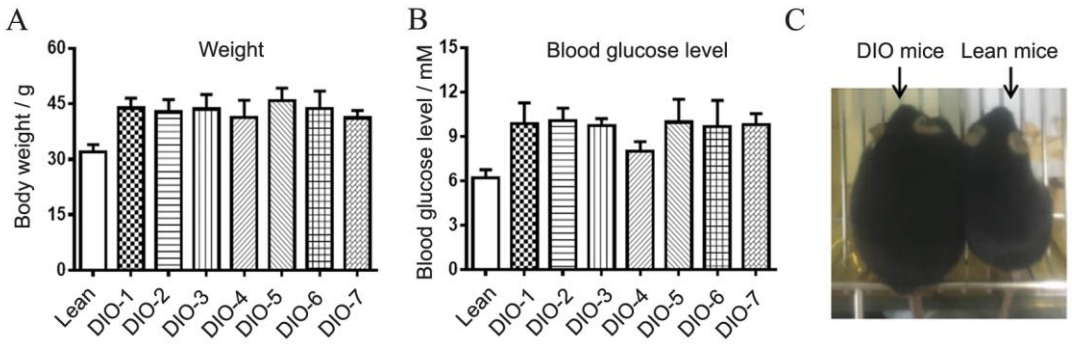
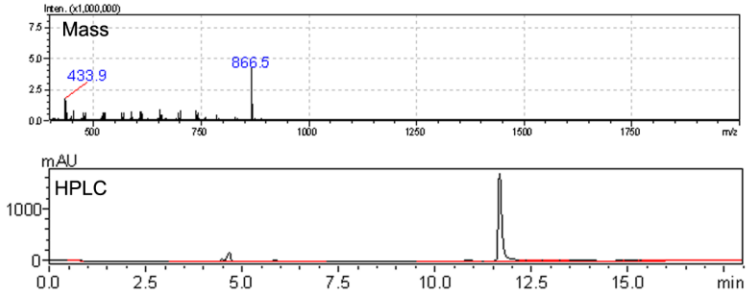


Figure S11. Construct the diet-induced obese mice (DIO mice) model. (A and B) Using high-fat diet (45 kcal % fat) to feed C57BL/6J mice, and finally inducing the mouse's weight of 40 g and a high glucose level about 10. Lean mice were fed with normal diet and acted as negative control. Data were shown as mean ± SEM. SEMs shown as error bars in the figures. (C) The bodily form difference between DIO mice (left) and lean mice (right).

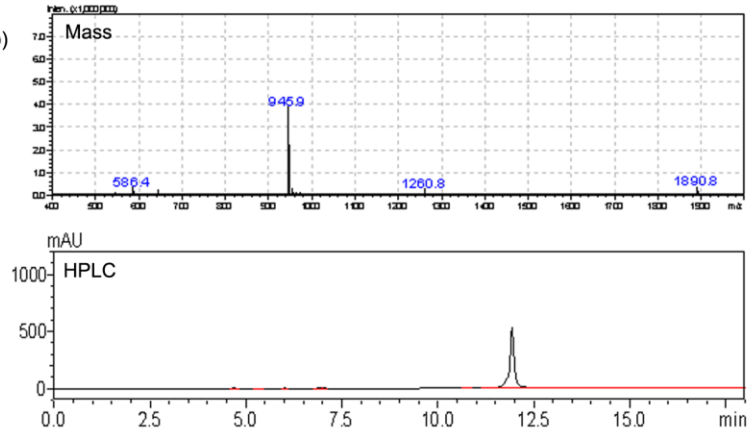
B1: H-EALYLVCG-NH_2

Calculated mass 865.4,
Found mass 866.5 $[M+H]^+$



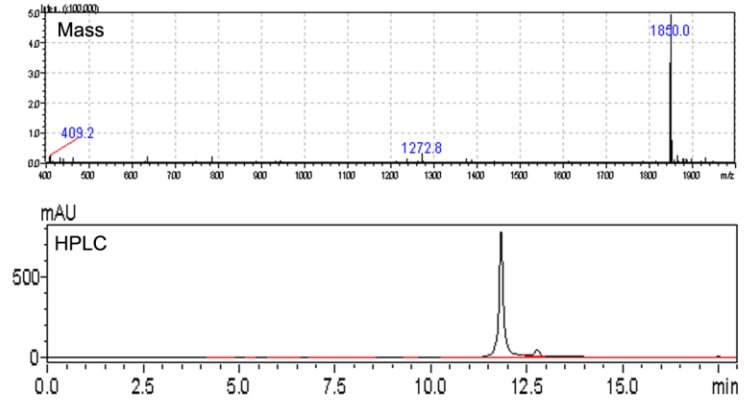
B35: Ac-EWRF-cyclo(DG^{D}P^{L}PEDap)
-LYLVCG-NH_2

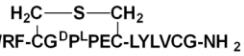
Calculated mass 1888.9,
Found mass 1890.8 $[M+H]^+$;
945.9 $[M+2H]^{2+}$



B35L: Ac-EFLWAG^{D}P^{L}PYA
-CVGRLE-NH_2

Calculated mass 1848.9,
Found mass 1850.0 $[M+H]^+$





B37: H-EWRF-CG^{D}P^{L}PEC-LYLVCG-NH_2

Calculated mass 1835.9,
Found mass 919.2 $[M+2H]^{2+}$

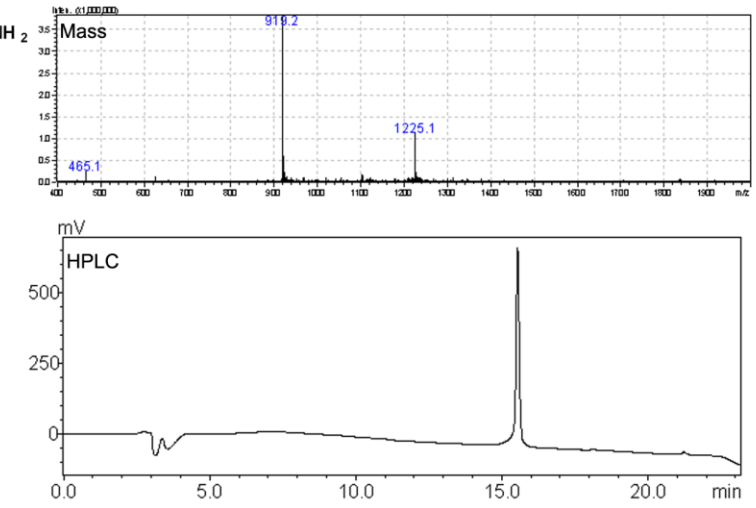


Figure S12. Mass spectrums and analytical HPLC plots. Peptide **B1**, **B35**, **B35L** and **B37** were showed

as representative examples. All the Mass data were listed in the **Table S2**. All the final peptides were ≥ 95% purity detected by HPLC and mass spectrometry.

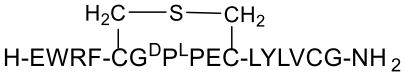
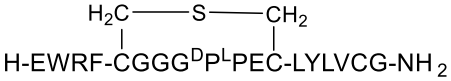
Table S1. Primers for IDE-E111Q mutation in this study.

Primers	Sequence(5'-3')
IDE-E111Q-F	GCTGGCTTAAGTCATTTTTGTCAACATATGCTTTTTTTGG
IDE-E111Q-R	TTGACAAAAATGACTTAAGCCAGCAATATTTGGAGGA

Table S2. Mass data and purity of the peptides.

Peptide	Sequence	Calculated mass	Found mass	Purity (%)
B1	H-EALYLVCG-NH_2	865.4	866.5$[M+H]^+$ 433.9$[M+2H]^{2+}$	97.8
B2	H-EALYLVFG-NH_2	909.5	910.5$[M+H]^+$ 455.9$[M+2H]^{2+}$	97.5
B3	H-YLAE^{D}P^{L}PEAYL -NH_2	1163.6	1164.6$[M+H]^+$ 583.0$[M+2H]^{2+}$	96.8
B4	H-GCVLYLAE^{D}P^{L}PEALYLVCG-NH_2	1908.0	955.7$[M+2H]^{2+}$	96.2
B8	H-EALYLVCG^{D}P^{L}PGCVLYLAE-NH_2	1908.0	954.6$[M+2H]^{2+}$	96.5
B9	H-EALYLVCG^{D}P^{L}PEALYLVCG-NH_2	1808.9	604.2$[M+3H]^{3+}$	97.1
B10	H-GCVLYLAE^{D}P^{L}PEALYLVCG-NH_2	2037.0	1019.8$[M+2H]^{2+}$	97.5
B11	H-DALYLVCGD^{D}P^{L}PEALYLVCG-NH_2	2009.0	1005.7$[M+2H]^{2+}$	96.6

B12	H-DALYLVCG^{D}P^{L}PEALYLVCG-NH_2	1893.9	948.4$[M+2H]^{2+}$	96.8
B13	H-EFLYLVCG^{D}P^{L}PEALYLVCG-NH_2	1984.0	993.5$[M+2H]^{2+}$	96.1
B14	H-EWRF^{D}P^{L}PEALYLVCG-NH_2	1677.8	1679.0$[M+H]^{+}$ 840.4$[M+2H]^{2+}$	97.2
B15	H-FVNQH^{D}P^{L}PEALYLVCG-NH_2	1684.8	1686.5$[M+H]^{2+}$ 843.8$[M+2H]^{2+}$	96.2
B16	H-EWRF-NH_2	635.3	636.3$[M+H]^{+}$	98.2
B17	H-EWRF^{D}P^{L}PEWRF-NH_2	1447.7	1449.8$[M+H]^{+}$ 725.1$[M+2H]^{2+}$ 483.9$[M+3H]^{3+}$	96.1
B19	H-EWRFAAEALYLVCG-NH_2	1625.8	814.2$[M+2H]^{2+}$	96.5
B21	H-EWRF^{D}P^{L}PEALYLV-NH_2	1517.8	760.2$[M+2H]^{2+}$	97.2
B22	H-RF^{D}P^{L}PEALYLV-NH_2	1202.7	602.6$[M+H]^{+}$ 1203.8$[M+2H]^{2+}$	97.6
B23	H-EWRF^{D}P^{L}PEALY-NH_2	1305.7	1307.7$[M+H]^{+}$ 654.4$[M+2H]^{2+}$	96.8
B24	H-RF^{D}P^{L}PEALY-NH_2	990.5	991.7$[M+H]^{+}$ 496.5$[M+2H]^{2+}$	97.2
B25	H-cyclo(DWRF^{D}P^{L}PEALYLVDap)G-NH_2	1628.9	815.8$[M+2H]^{2+}$	95.8
B26	H-cyclo(DEWRF^{D}P^{L}PEALYLVDap)G-NH_2	1757.9	1759.8$[M+H]^{+}$ 880.3$[M+2H]^{2+}$	96.2

B27	H-FRWE-NH_2	635.3	637.4$[M+H]^+$	97.6
B28	H-LVCG^{D}P^{L}PFRWE-NH_2	1201.6	1202.6$[M+H]^+$ 602.1$[M+2H]^{2+}$	95.8
B29	H-LYLVCG^{D}P^{L}PFRWE-NH_2	1477.8	740.3$[M+2H]^{2+}$	96.6
B30	H-EALYLVCG^{D}P^{L}PFRWE-NH_2	1677.8	1678.6$[M+H]^+$ 840.0$[M+2H]^{2+}$	95.8
B31	H-EWRFAib^{D}AEALYLVCG-NH_2	1639.8	1640.7$[M+H]^+$ 821.0$[M+2H]^{2+}$	96.6
B32	H-FRWEAib^{D}AEALYLVCG-NH_2	1639.8	1642.0$[M+H]^+$ 821.7$[M+2H]^{2+}$	97.5
B33	H-EWRFGGEALYLVCG-NH_2	1597.8	799.8$[M+2H]^{2+}$	96.8
B34	H-EWRFGGGGEALYLVCG-NH_2	1711.8	1712.8$[M+H]^+$ 857.0$[M+2H]^{2+}$	96.5
B35	Ac-EWRF-cyclo(DG^{D}P^{L}PEDap)LYLVCG-NH_2	1888.9	1890.8$[M+H]^+$ 945.9$[M+2H]^{2+}$	97.5
B35L	Ac-EFLWAG^{D}P^{L}PYACVGRLE-NH_2	1847.9	1850.0$[M+H]^+$	96.4
B36	H-EWRF-cyclo(DGGG^{D}P^{L}PEDap)LYLVCG-NH_2	1960.9	1962.1$[M+H]^+$ 981.7$[M+2H]^{2+}$	97.2
B37		1835.9	919.2$[M+2H]^{2+}$	98.5
B38		1949.9	1951.6$[M+H]^+$ 976.0$[M+2H]^{2+}$	97.6

(Abbreviations: Ac, acetyl; Dap, 2,3-diaminopropionic acid.)

Table S3. Chemical shift assignments of **B35** (H_2O:CD_3CN(v/v) = 6:4, 298K, 500 MHz, mixing time: 300 ms) backbone atoms.

Amino acid No.	**HN**	**Hα**	**Sidechain HN**
E1	7.98	4.09	unobserved
W2	7.68	4.55	unobserved
R3	7.55	4.07	7.01
F4	7.78	4.57	unobserved
D5	7.90	4.53	unobserved
G6	8.06	3.81	unobserved
P7	unobserved	unobserved	unobserved
P8	unobserved	unobserved	unobserved
E9	8.08	4.02	unobserved
Dap10	7.59	4.32	7.75
L11	7.71	4.10	unobserved
Y12	7.78	4.57	unobserved
L13	7.70	4.21	unobserved
V14	7.73	4.10	unobserved
C15	8.07	4.01	unobserved
G16	8.01	3.78	unobserved

References

(1) Lin, H.; Jiang, Y.; Hu, K.; Zhang, Q.; He, C.; Wang, T.; Li, Z. An in-tether sulfilimine chiral center induces β-turn conformation in short peptides. *Org. Biomol. Chem.* **2016**, *14*, 9993-9999.

(2) Zheng, W.; Liang, Y.; Zhao, H.; Zhang, J.; Li, Z. 5,5'-Methylenedisalicylic acid (MDSA) modulates SarA/MgrA phosphorylation by targeting Ser/Thr phosphatase Stp1. *Chembiochem*. **2015**, *16*, 1035-1040.

(3) Shen, Y.; Joachimiak, A.; Rosner, M. R.; Tang, W. J. Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. *Nature* **2006**, *443*, 870-874.