Supporting Information

Development of myostatin inhibitory D-peptides to enhance the potency increasing skeletal muscle mass in mice

*Kentaro Takayama,[§, †] Keisuke Hitachi,[#] Hideyuki Okamoto,[§] Mariko Saitoh,[§] Miki Odagiri,[§] Rina Ohfusa,[§] Takahiro Shimada,[§] Akihiro Taguchi,[§] Atsuhiko Taniguchi,[§] Kunihiro Tsuchida,[#] and Yoshio Hayashi[§,] **

[§]*Department of Medicinal Chemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan.*

[†]*Department of Environmental Biochemistry, Kyoto Pharmaceutical University, Yamashina, Kyoto 607-8414, Japan.*

[#]*Division for Therapies against Intractable Diseases, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan.*

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1. Materials

Reagents and solvents including SB431542 were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Tokyo Chemical Industries (Tokyo, Japan), and Watanabe Chemical Industries (Hiroshima, Japan). All materials were used as received. Sterile 100-mm dishes, 96-well white-wall plates, and 96-well clear-wall poly-D-Lys-coated plates were purchased from BD Biosciences (Franklin Lakes, NJ), Corning (Cambridge, MA), and Thermo Fisher Scientific (Waltham, MA), respectively. Fetal bovine serum (FBS) were purchased from Nichirei Bioscience Inc. (Tokyo, Japan) and Sterile Dulbecco's Modified Eagle's Medium (DMEM) from Nacalai Tesque (Kyoto, Japan). Plasmids, FuGENE HD and Dual-Luciferase Reporter Assay System for cell-based assay were purchased from Promega (Madison, WI). Recombinant human/mouse/rat myostatin was purchased from Merck Millipore (Billerica, MA). Trypsin and α-chymotrypsin from bovine pancreas were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human/mouse/rat GDF-11, human/mouse/rat activin A, human TGF-β1 and mouse myostatin-derived recombinant prodomain protein were purchased from R&D Systems (Minneapolis, MN).

2. Synthesis of peptide derivatives

The protected peptide-bound resins of new D-peptides were synthesized using Fmoc-NH-SAL resin (54 mg, 0.02 mmol). Fmoc-amino acids (0.1 mmol) were sequentially coupled using the *N*,*N*'-diisopropylcarbodiimide (DIPCI, 0.2 mmol)-1-hydroxybenzotriazole (HOBt, 0.1 mmol) method for 2 h in DMF (1.0 mL) after removing each Fmoc group with 20% piperidine in DMF (1.5 mL, 20 min). To obtain crude peptides, the resins were treated with TFA-*m*-cresol-thioanisole-EDT (4.3 mL, 40:1:1:1, v:v:v:v) for 150 min at rt. Purified peptides as the TFA salts were obtained by preparative RP-HPLC in a 0.1% aqueous TFA-CH_3CN system. The purity check of each synthesized D-peptide was performed by RP-HPLC analysis using a C18 reverse-phase column [4.6 x 150 mm; COSMOSIL 5C18-AR-II for MID-35 or COSMOSIL 5C4-AR-300 for **7c-ri**, MID-36 and MID-39] with a binary solvent system: a linear gradient of CH_3CN (25-45%, 40 min for **7c-ri** and MID-35, or 25-45%, 30 min for MID-36 and MID-39) in 0.1% aqueous TFA at a flow rate of 1.0 mL/min, detected by UV at 220 nm. Yields of all products obtained as a white powder were calculated as TFA salts. HRMS (TOF MS ES+) was recorded on a micromass LCT. Analytical data of synthetic D-peptides are shown below.

Peptide **7c-ri**: Yield of 10%; HRMS *m*/*z* $[M+H]^+$ found 2253.3545 (calcd. for $C_{114}H_{174}N_{29}O_{19}$ 2253.3541); HPLC purity 99.9% (t_R = 18.73 min).

MID-35: Yield of 21%; HRMS *m*/*z* $[M+H]^+$ found 2350.4646 (calcd. for $C_{118}H_{185}N_{34}O_{17}$ 2350.4657); HPLC purity 99.5% (t_R = 14.85 min).

MID-36: Yield of 20%; HRMS *m*/*z* $[M+H]^+$ found 2237.3816 (calcd. for $C_{112}H_{176}N_{33}O_{16}$ 2237.3816); HPLC purity 100% (t_R = 14.73 min).

MID-39: Yield of 13%; HRMS *m*/*z* $[M+H]^+$ found 2164.3867 (calcd. for $C_{107}H_{177}N_{32}O_{16}$ 2164.3864); HPLC purity 99.8% (t_R = 13.45 min).

3. Luciferase reporter assay

As previously reported,[19,21,24] HEK293 cells were subcultured in DMEM containing 10% FBS and nonessential amino acids at 37 °C under 5% CO_2. Cells were seeded at 2.0×10^4 cells per well in 96-well clear-wall poly-D-Lys-coated plates and then incubated for 24 h. After the transfection of reporter (pGL4.48[luc2P/SBE/Hygro]) and control (pGL4.74[hRluc/TK]) plasmids using FuGENE HD, the culture medium incubated for 24 h was exchanged to serum-free DMEM for 8 h starvation. The stock solutions of respective synthesized peptides were diluted by addition of DMEM containing human/mouse/rat myostatin, human/mouse/rat GDF-11, human/mouse/rat activin-A or human TGF-β1 [final concentration; 8 ng/mL (0.32 nM), 8 ng/mL (0.32 nM), 8 ng/mL (0.32 nM) or 2.5 ng/mL (0.1 nM), respectively], and incubated for 20 min at rt. Cells were treated with a peptide solution and incubated for 4 h at 37 °C under 5% CO_2, and then washed with PBS. According to manufacturer's protocol for the Dual-Luciferase Reporter Assay System (Promega), the preparation of cell lysates and the measurement of the luciferase activities were carried out. Mouse myostatin-derived recombinant propeptide (prodomain) or SB431542 was used as a positive control and underwent the same manipulation, arriving at a final concentration of 10 nM or 5 μM, respectively. Each experiment was carried out in triplicate and values represent means ± SD (N = 3). To determine IC_{50} values against myostatin, GDF-11, activin-A and TGF-β1, MID-35 was dissolved at concentrations of 0.025–2 μM, 0.08–6 μM, 0.025–18 μM and 0.025–18 μM, respectively, and MIPE-1686 was dissolved at the same concentration except for TGF-β1 where it was 0.074–54 μM. The inhibitory activities of peptides were determined in triplicate at each concentration. Curve fitting and the calculation of IC_{50} values were performed using KaleidaGraph 4.5.

4. Analysis of stability against isolated enzymes

The peptide (50 μM) was incubated in trypsin from bovine pancreas (1 μg/mL) on 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij-35 at 37 °C for 400 min, or in α-chymotrypsin from bovine pancreas (2 μg/mL) in 100 mM Tris buffer (pH 7.5) containing 10 mM $CaCl_2$ at 37 °C for 400 min. After the incubation, an aliquot of the peptide solution (20 μL) was sampled and mixed with 0.1% TFA-containing H_2O-CH_3CN (4:1) solution (80 μL). Then, 20 μL of this solution was analyzed by RP-HPLC bearing a C_{18}-bound reverse-phase column (4.6 x 150 mm; COSMOSIL 5C18-AR-II) with a binary solvent system: a linear gradient of CH_3CN (25–40%, 30 min) in 0.1% aqueous TFA (flow rate = 1.0 mL/min), UV spectroscopy (220 nm). The appeared new fragments were identified on an LCMS-2020 mass spectrometer (Shimadzu, Kyoto, Japan). Statistical analysis for the comparison of peak areas was performed using a paired samples *t*-test (Figure S2).

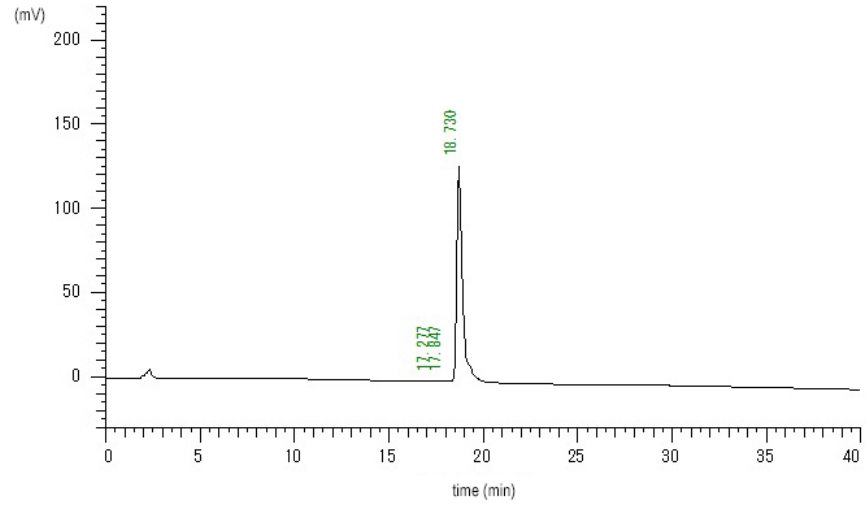
5. Intramuscular administration of peptides into anterior tibialis of mice

Animal studies were approved by the Animal Research Committee of Fujita Health University. The peptide solution (0.75 mM in saline, 40 μL) and a saline (control, 40 μL) were injected intramuscularly into left and right tibialis anterior muscle of 8-week-old male C57BL/6J mice, respectively (at day 0). At day 28, the muscles were collected and weighed. Statistical

analysis was performed using a paired samples *t*-test (Figures 5A and 5B) or a Welch's *t*-test with a F-test (Figure 5C).

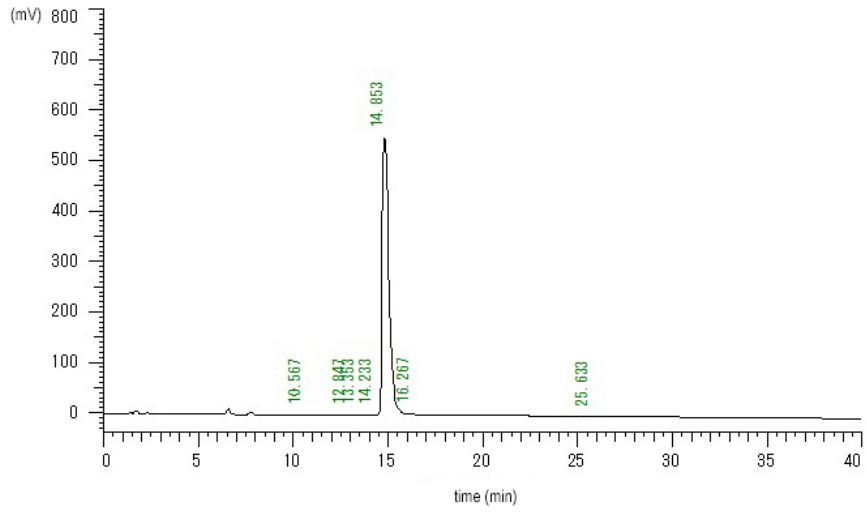
Analytical HPLC chromatograms

peptide **7c-ri**:



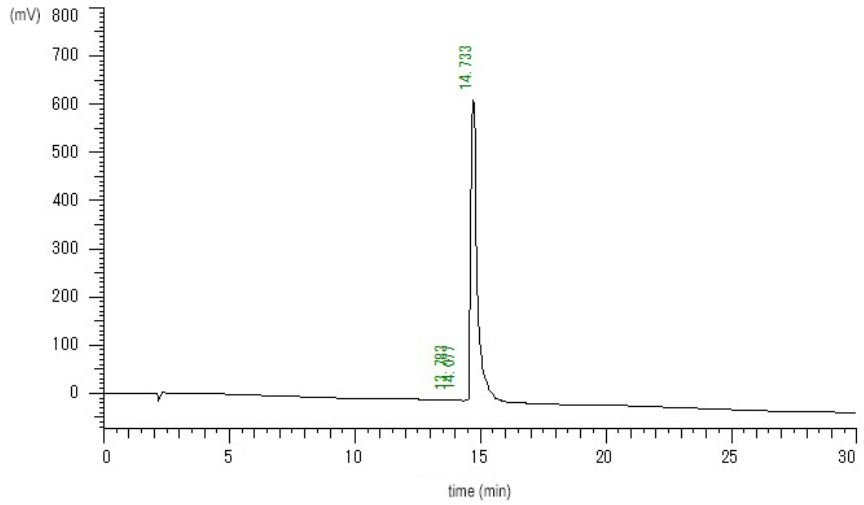
NO	RT	AREA	CONC	BC
1	17.277	1495	0.065	BB
2	17.847	887	0.038	BB
3	18.73	2313580	99.897	BB
		2315962	100	

MID-35:



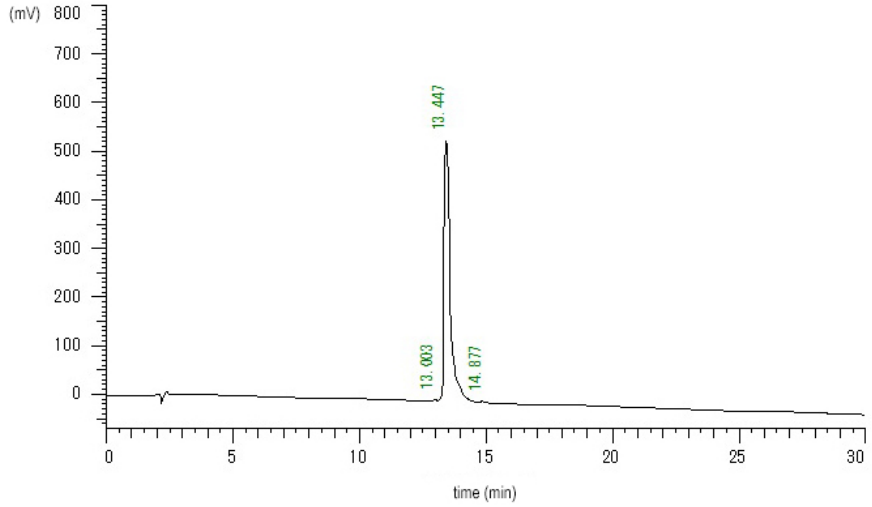
NO	RT	AREA	CONC	BC
1	10.567	1879	0.016	BB
2	12.847	989	0.008	BB
3	13.353	6763	0.057	BB
4	14.233	18249	0.155	BV
5	14.853	11729940	99.501	VV
6	16.267	27877	0.236	TBB
7	25.633	3110	0.026	BB
		11788807	100	

MID-36:



NO	RT	AREA	CONC	BC
1	13.783	813	0.009	BB
2	14.077	624	0.007	BB
3	14.733	9526849	99.985	BB
		9528286	100	

MID-39:



NO	RT	AREA	CONC	BC
1	13.003	17537	0.222	BV
2	13.447	7876720	99.586	VV
3	14.877	15191	0.192	TBB
		7909448	100	

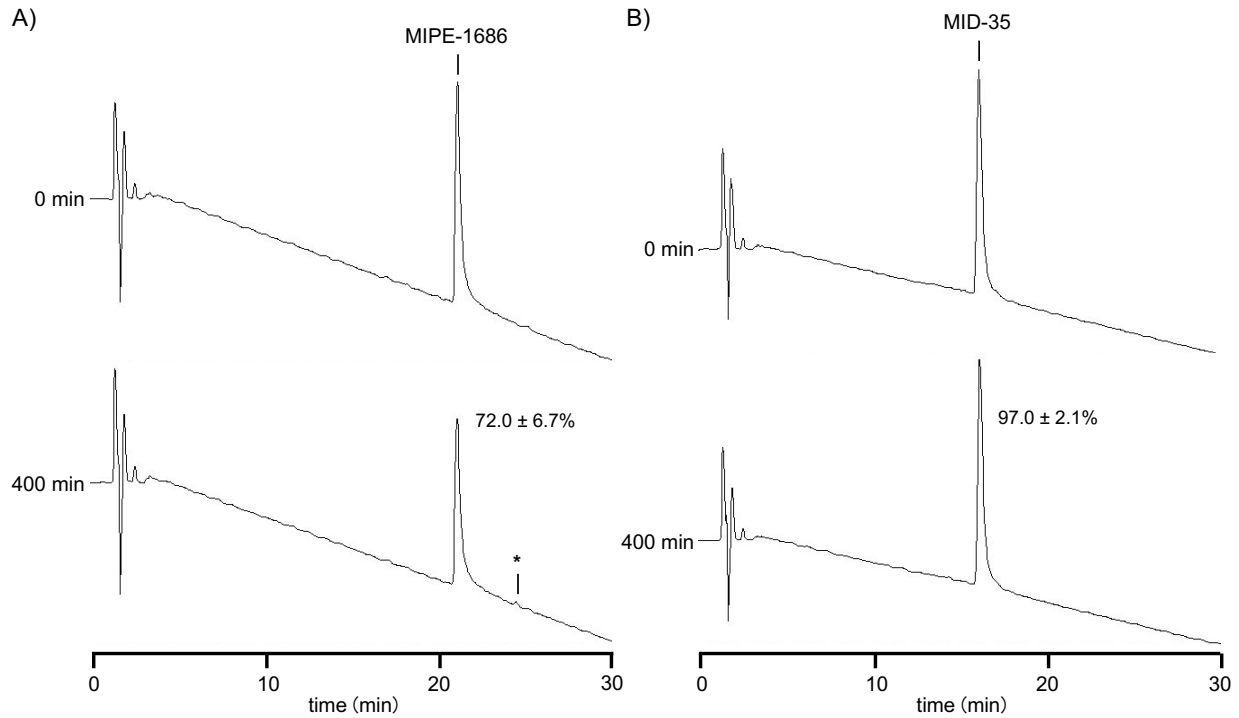


Figure S1. Representative analytical RP-HPLC chromatograms of MIPE-1686 (A) and MID-35 (B) incubated in a solution of α-chymotrypsin from bovine pancreas (2 μg/mL) on 100 mM Tris buffer (pH 7.5) containing 10 mM $CaCl_2$ at 37 °C for 400 min. Column, COSMOSIL 5C18-AR-II (4.6 x 150 mm); binary solvent system, a linear gradient of CH_3CN (25–40%, 30 min) in 0.1% aqueous TFA; Flow, 1.0 mL/min; detection, UV 220 nm. Asterisks mean unknown peaks.

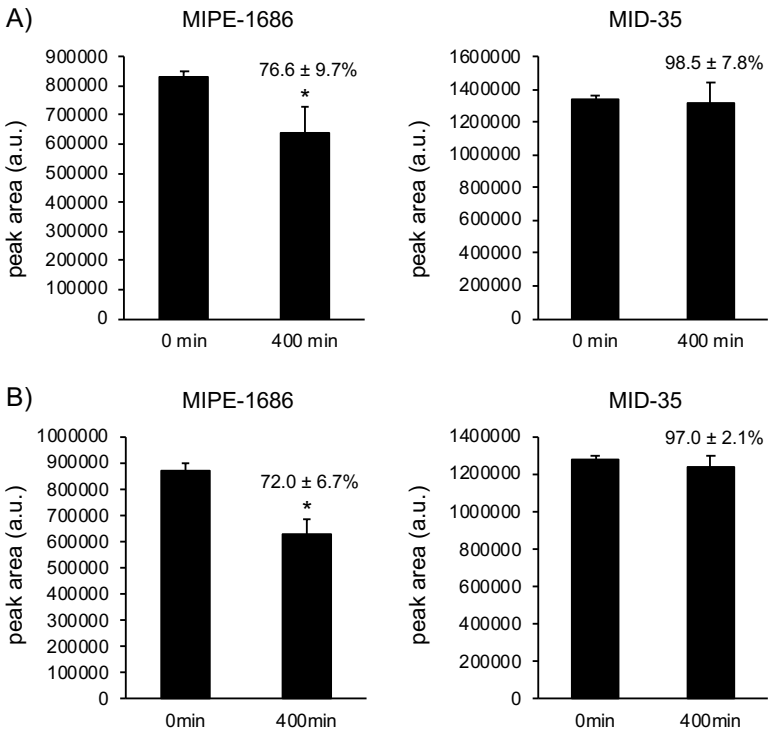


Figure S2. The comparisons of the analytical HPLC peak area of intact MIPE-1686 and MID-35 between 0 and 400 min incubation in the solution of (A) trypsin from bovine pancreas (1 μg/mL) and (B) α-chymotrypsin form bovine pancreas (2 μg/mL) at 37 °C. Results are presented as mean values ± SD (N = 3). The survived rate of intact peptide after 400 min incubation are represent on the top of each bar. *$p < 0.05$

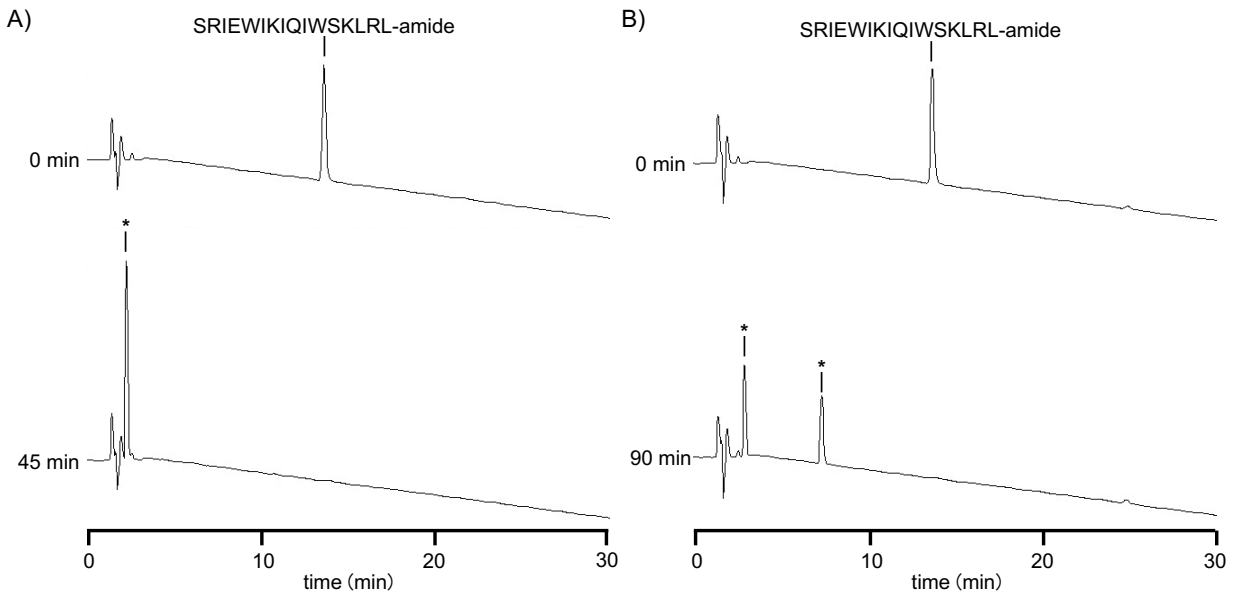


Figure S3. Analytical RP-HPLC chromatograms of the myostatin inhibitory L-peptide (SRIEWIKIQIWSKLRL-amide; **4b** in ref. 24, **2** in ref. 28) incubated in the solutions of (A) trypsin from bovine pancreas (1 μg/mL) on 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij-35 and (B) α-chymotrypsin from bovine pancreas (2 μg/mL) on 100 mM Tris buffer (pH 7.5) containing 10 mM $CaCl_2$ at 37 °C for 45 and 90 min, respectively. Column, COSMOSIL 5C18-AR-II (4.6 x 150 mm); binary solvent system, a linear gradient of CH_3CN (25–40%, 30 min) in 0.1% aqueous TFA; Flow, 1.0 mL/min; detection, UV 220 nm. Asterisks mean degraded peptide peaks (unidentified).