*Pyropia yezoensis* peptide PYP1-5 protects against dexamethasone-induced muscle atrophy through the downregulation of atrogin1/MAFbx and MuRF1 in mouse C2C12 myotubes

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**Abstract.** Skeletal muscle atrophy refers to the decline in muscle mass and strength that occurs under various conditions, including aging, starvation, cancer and other cachectic diseases. Muscle atrophy caused by aging, known as sarcopenia, primarily occurs after 50 years of age. Muscle atrophy-related genes, including atrogin1/muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1), are expressed early in the muscle atrophy process, and their expression precedes the loss of muscle mass. The present study investigated the potential anti-atrophic effects of the *Pyropia yezoensis* peptide PYP1-5. The MTS assay did not detect cytotoxic effects of PYP1-5 on C2C12 mouse myoblast cells. Subsequently, the anti-atrophic effects of PYP1-5 on skeletal muscle cells was examined by treating C2C12 myotubes with 100 µM dexamethasone (DEX) and/or 500 ng/ml PYP1-5 for 24 h. Compared with the control, myotube diameter was reduced in DEX-treated cells, whereas PYP1-5 treatment protected against DEX-induced muscle atrophy. MAFbx and MuRF1 protein and mRNA expression levels were detected by western blot analysis and reverse transcription-quantitative polymerase chain reaction, respectively. The results demonstrated that PYP1-5 significantly reduced the expression of atrogin1/MAFbx and MuRF1. Therefore, data from the present study suggest that PYP1-5 inhibits the expression of atrogin1/MAFbx and MuRF1 in C2C12 cells, and these characteristics may be of value in the development of anti-atrophy functional foods.

**Introduction**

*Pyropia yezoensis*, a marine red alga previously known as *Porphyra yezoensis*, is a commercially important seaweed in a number of Asian countries, including Korea, China, and Japan. *P. yezoensis* is a crucial source of bioactive substances that contain large quantities of essential proteins, vitamins and minerals. *P. yezoensis* has potentially beneficial biological effects, including anti-inflammatory, antioxidant, anticancer, anti-fatigue and anti-aging (1.2).

Aging is a multifactorial process that is facilitated by a decline in neuromuscular functions and stress tolerance, which results in tissue degeneration and malfunction, most notably in the skeletal muscles. Age-related muscle atrophy and a reduction in skeletal muscle mass and strength is a condition known as sarcopenia (from the Greek for ‘lack of flesh’). Sarcopenia leads to muscle weakness and greatly affects physical activity and the quality of life of elderly individuals (3,4); sarcopenia is common in elderly people, and is estimated to occur in 5-13% of people aged 60-70 years and in 11-50% of those ≥80 years (5). The mechanisms of sarcopenia development are actively being studied and involve both intrinsic and extrinsic factors, including the decline of satellite cell activation, altered hormonal status, contraction-induced injury, cellular vacuolization, autophagy, apoptosis and increased oxidative stress (6).

C2C12 mouse skeletal muscle cells are an in vitro model that is widely used to study the factors that regulate muscle growth, proliferation and differentiation (7). The differentiation of myotubes can be induced by 2% fetal bovine serum (FBS). Previous studies have demonstrated that C2C12 myoblasts cultured in a growth-factor-deficient state causes the mononucleated myoblasts to exit the cell cycle, which activates the expression of genes that promote myoblast fusions and the formation of multinucleated myotubes (8,9). During this process, changes in cell shape along with cell fusion occur, and myotube phenotypes can be observed within 5-6 days. In addition, when muscle fiber formation (myogenesis) begins, myogenic transcriptional regulatory factors belonging to the MyoD family, including MyoD, myogenin, Myf4 and Myf5, are activated (10). Amongst these, myogenin has an important role within the MyoD family as it regulates the differentiation of single nucleated myoblasts into multinucleated myofibers (11).

Glucocorticoids are important in the development of muscle atrophy in humans and animals (12). In both in vivo and in vitro experiments, muscle atrophy is induced by...
peptide and muscle atrophy

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β3

5% CO2

Inc.) at a temperature of 37˚C in a humidified atmosphere of 100˚C; flow rate, 0.4 ml/min (isocratic ANC:DW=8:2, 0.1% trifluoroacetic acid (TFA); Peptron, Inc., Daejeon, Korea). The molecular mass of PYP1-5 was confirmed with the Class-VP software package, version 6.14 (Shimadzu Corporation). The molecular mass of PYP1-5 was determined by focusing on its effects on the expression of atrogin1/MAFbx and MuRF1.

Materials and methods

P. yezoensis peptide synthesis. The N-terminal 15 residues of P. yezoensis peptide PYP1-5 (1-15) (D-P-K-G-K-Q-Q-A-I-H-V-A-P-S-F; designated as PYP1-5) were synthesized by Peptron (Daejeon, Korea). PYP1-5 purification was performed using a Prominence High-Performance Liquid Chromatography apparatus (Shimadzu Corporation, Kyoto, Japan) with a Capcell Pak C18 column (column dimensions, 150x4.6 mm; particle size, 2.7 µm; Shiseido Co., Ltd., Tokyo, Japan) in 0.1% trifluoroacetic acid (TFA; v/v in water), and an elution gradient of 10%-70% acetonitrile (0-20% acetonitrile for 2 min; 20-50% acetonitrile for 10 min; 50-80% acetonitrile for 2 min) in 0.1% TFA, a flow rate of 1.0 ml/min and UV detection at 220 nm; analysis was conducted with the Class-VP software package, version 6.14 (Shimadzu Corporation). The molecular mass of PYP1-5 was confirmed to be 1,622 Da (Fig. 1) using an HP 110 Series LC/MSD mass spectrometer [ionization mode, positive; nitrogen flow, 7 l/min; high vacuum, 1.3E-5 torr; neb press, 40 psi; quadrupole temperature, 100˚C; flow rate, 0.4 ml/min (isocratic ANCDW=8:2, 0.1% TFA); Peptron, Inc., Daejeon, Korea].

Cell culture. C2C12 mouse skeletal muscle cells (ATCC no. CRL-1772; American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at a temperature of 37˚C in a humidified atmosphere of 5% CO2. The cells were cultured to 70-80% confluence in 100 mm dishes, and the medium was replaced every 2 days. Induction of differentiation. C2C12 myoblasts were grown to 70-80% confluence in culture dishes at 37˚C, trypsinized and seeded (4x104 cells/well) into 6-well culture plates for experiments. Cells were grown to 70-80% confluence in DMEM supplemented with 10% FBS at 37˚C for 24 h, at which time the medium was replaced with DMEM containing 2% FBS to induce myotube differentiation; the medium was replaced every 2 days. Cells were allowed to differentiate for 6 days, at which point 90% of the cells had fused into myotubes.

Treatment with DEX and PYP1-5. Following 6 days of differentiation, C2C12 myotubes were treated with 1, 10, 50 or 100 µM DEX at 37˚C for 24 h for concentration screening. The C2C12 myotubes were subdivided into four groups: i) the control group, in which cells were incubated in serum-free medium (SFM; DMEM containing 100 µ/ml penicillin and 100 µg/ml streptomycin); ii) the DEX group, in which cells were treated with 100 µM DEX; iii) the DEX+PYP1-5 group, in which cells were treated with 100 µM DEX and 500 ng/ml PYP1-5; and iv) the PYP1-5 group, in which cells were treated with 500 ng/ml PYP1-5. All groups were incubated at 37˚C for 24 h, prior to harvesting cells for experiments.

MTS assay. Cell viability was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation assay (Promega Corporation, Madison, WI, USA), which is based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into a formazan product that is soluble in tissue culture medium, according to the manufacturer’s protocol. Cells (1.5x104 cells/well) were seeded in 96-well plates in 100 µl DMEM supplemented with 10% FBS and were allowed to attach at 37˚C for 24 h. Attached cells were maintained in SFM at 37˚C for 4 h and were subsequently treated with or without PYP1-5 (125, 250, 500 and 1,000 ng/ml) for 24 h. MTS solution (10 µl) was added and the cells were incubated at 37˚C for 30 min; the absorbance of each well was measured at 490 nm using a SpectraMax 340 PC Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Experiments were performed in triplicate.

Measurement of myotube diameter. Images of myotube cultures were captured with a phase contrast microscope set to x100 magnification at 1, 2, 4, 5 and 6 days following induction of differentiation. Images of myotube cultures were also captured following treatment with 100 µM DEX and/or 500 ng/ml PYP1-5 for 24 h. A total of 50 myotube diameters from at least 10 random fields were measured using ImageJ software (version 4.16; National Institutes of Health, Bethesda, MD, USA).

Preparation of whole-cell protein lysates. Cells were allowed to differentiate for 6 days at 37˚C, followed by incubation at 37˚C for 24 h in either SFM (control group) or SFM containing 100 µM DEX (DEX group), 100 µM DEX + 500 ng/ml PYP1-5 (DEX+PYP1-5 group) or 500 ng/ml PYP1-5 (PYP1-5 group). Cells were washed in cold phosphate-buffered saline (PBS) and lysed with extraction buffer [1% NP-40, 0.25% sodium deoxycholate, 1 mM ethylene glycol-bis-(β-aminomethyl ether)-N, N,N’, N’-tetraacetic acid, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5] containing protease inhibitors (1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 200 mM Na2VO4, 1 mM phenylmethylsulfonylfluoride, 1 mM EDTA).
Extracted of nuclear lysates. Cells were treated and harvested as aforementioned, lysed with hypotonic lysis buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.5), 5 mM EDTA, 5 mM MgCl₂ and 5 mM dithiothreitol (DTT)), and incubated for 15 min on ice. NP-40 (2.5%) was added and the cells were lysed for an additional 10 min. Nuclei were collected by centrifugation at 7,310 x g for 15 min at 4°C. Nuclear proteins were resuspended in extraction buffer (10 mM HEPES pH 7.9, 100 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA and 0.2 mM DTT) and incubated for 20 min at 4°C. Nuclear proteins were resuspended in extraction buffer (10 mM HEPES pH 7.9, 100 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA and 0.2 mM DTT) and incubated for 20 min at 4°C. Extracts were centrifuged at 18,341 x g for 10 min, and the protein levels were determined using a Bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The supernatant was then used in western blotting.

Western blot analysis. Proteins (20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked at room temperature for 1 h with 1% bovine serum albumin (BSA) in TBS-T (10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) and then incubated with primary antibodies against Myogenin, MuRF1, MAFbx and GAPDH (Table I; all purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 3 h. Membranes were incubated with secondary horseradish peroxidase-conjugated rabbit anti-goat IgG, goat anti-mouse IgG (both Santa Cruz Biotechnology, Inc.) and goat anti-rabbit IgG (Cell Signaling Technology, Inc., Danvers, MA, USA; Table II) at room temperature for 1 h and bands were detected using an enhanced chemiluminescence western blotting kit (Thermo Fisher Scientific, Inc.). Experiments were performed in triplicate and densitometry analysis was performed using Multi-Gauge software version 3.0 (Fujifilm Life Science, Tokyo, Japan).

Statistical analysis. Multiple mean values were assessed by analysis of variance using SPSS version 10.0 software.
Table III. Oligonucleotide primer sequences used in reverse transcription-quantitative polymerase chain reactions.

| Gene   | GenBank accession no. | Sequence (5’-3’)                  | Amplicon size (bp) |
|--------|-----------------------|-----------------------------------|--------------------|
| MuRF1  | DQ229108.1            | F: TGTCTGGAGGTCCGTTTCG           | 59                 |
|        |                       | R: GTGCCGGTCCATGATCTT            |                    |
| MAFbx  | NM_026346.3           | F: ATGCACACTGGTGCGAGAG           | 168                |
|        |                       | R: TGTAAGCAGACAGCGGTC            |                    |
| GAPDH  | NM_008084.3           | F: ACTCCACTCCGGCAAATCA           | 91                 |
|        |                       | R: CGCTCGGAGATGATGAGT            |                    |

bp, base pairs; MAFbx, muscle atrophy F-box; MuRF1, muscle RING finger 1; F, forward; R, reverse.

Results

Confirmation of C2C12 myotube differentiation. C2C12 skeletal muscle myoblasts were induced to differentiate in a mitogen-poor media such as 2% FBS, fusing to form multinucleate myotubes (20). Fig. 2 illustrates that the cell diameter remained similar following 4, 5 and 6 days of differentiation. In addition, the level of myogenin expression, a factor that regulates terminal differentiation of muscle cells, was similar following 4 and 5 days then increased following 6 days. These results indicate complete differentiation of myoblasts into myotubes at this time (6 days).
DEX-induced muscle atrophy in C2C12 myotubes. MuRF1 and atrogin1/MAFbx protein expression levels were measured in C2C12 myotubes treated with 1, 10, 50 or 100 µM DEX for 24 h (Fig. 3A and B). Treatment with DEX led to increased protein expression levels for MuRF1 and atrogin1/MAFbx, and the levels of expression increased in a dose-dependent manner. These data suggest that DEX may induce muscle atrophy in C2C12 myotubes. In addition, as shown in Fig. 3C, when compared with the control group, the C2C12 myotube was markedly atrophied in the 100 µM DEX-treated group. Therefore, all further experiments were performed following treatment with 100 µM DEX.

Toxicity of PYP1-5 in C2C12 myoblast. Through mass spectrometry, we identified a 1,622 Da compound from P. yezoensis, which we named PYP1-5 (Fig. 1). The MTS assay was used to determine PYP1-5 cytotoxicity to C2C12 myoblasts, which demonstrated no cytotoxic effects following PYP1-5 treatment for 24 h (Fig. 4). Therefore, all further experiments were performed 24 h following treatment with 500 ng/ml PYP1-5, which is the optimum concentration without toxicity as described by Choi et al. (18).

Inhibitory effect of PYP1-5 on DEX-induced myotube atrophy. C2C12 myotubes were allowed to differentiate for 6 days, followed by 100 µM DEX and/or 500 ng/ml PYP1-5 treatment for a further 24 h. As a result, when compared with the control group, the DEX-treated group exhibited a 36% reduction in cell diameter, whereas the PYP1-5-treated group exhibited a 10% increase in cell diameter. In addition, DEX-induced reduction of cell diameter was suppressed by co-treatment with PYP1-5 (Fig. 5A and B). The anti-atrophic effects of PYP1-5 were investigated by examining the protein and mRNA expression levels of the muscle atrophy markers MuRF1 and atrogin1/MAFbx in C2C12 myotubes that were treated with 100 µM DEX and 500 ng/ml PYP1-5.
Figure 5. Effects of PYP1-5 and/or DEX on differentiated C2C12 myotubes. (A) Representative images of C2C12 myotubes following the various DEX and PYP1-5 treatments. (B) Comparison of myotube diameters among the four groups. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. corresponding control group; †P<0.05 vs. corresponding DEX only treatment group. CON, control; DEX, dexamethasone; PYP1-5, N-terminal 15 residues of *Pyropia yezoensis* peptide PYP1-5.

Figure 6. Inhibition of MuRF1 and atrogin1/MAFbx expression in C2C12 myotubes treated with DEX (100 µM) and/or PYP1-5 (500 ng/ml) for 24 h. MuRF1 and atrogin1/MAFbx Protein expression levels were analyzed by (A) western blot and (B) densitometric analysis; GAPDH was used to normalize expression. (C) mRNA expression levels were quantified by reverse transcription-quantitative polymerase chain reaction. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. corresponding control group; †P<0.05 and ‡P<0.01 vs. corresponding only DEX treatment group. CON, control; DEX, dexamethasone; MAFbx, muscle atrophy F-box; MuRF1, muscle RING finger 1; PYP1-5, N-terminal 15 residues of *Pyropia yezoensis* peptide PYP1-5.
for 24 h (Fig. 6). Cells treated with DEX alone exhibited a marked increase in the mRNA and protein expression levels of MuRF1 and atrogin1/MAFbx compared with cells in the untreated control group (Fig. 6). The DEX-induced upregulation of MuRF1 and atrogin1/MAFbx was suppressed by co-treatment with 500 ng/ml PYP1-5. Therefore, PYP1-5 may be able to inhibit atrophy and induce hypertrophy pathways in C2C12 myotubes.

Discussion

Skeletal muscle atrophy refers to the decline in muscle mass and strength that may occur as a result of various conditions, including denervation, injury, glucocorticoid treatment, starvation, cancer, joint immobilization, sepsis and aging (21). The present study focused on the progression of muscle atrophy caused by aging.

In the present study, anti-atrophic effects of the P. yezoensis peptide PYP1-5 were investigated in C2C12 myotubes. C2C12 mouse skeletal muscle cells are commonly used as a model for myotube differentiation to examine the signaling pathways involved in muscle atrophy. The effects of the PYP1-5 on the muscle atrophy in C2C12 myotubes were determined by first culturing C2C12 myoblasts in media containing 2% FBS for 6 days to induce differentiation. Following differentiation, muscle atrophy was induced by treating the cells with DEX. DEX treatment has been previously demonstrated to reduce muscle mass, largely owing to the breakdown of muscle proteins due to upregulated catabolism by the ubiquitin-proteasome system (22,23). Administration of high concentrations of DEX has been revealed to cause muscle atrophy in animals and humans (24). Results from the present study demonstrated that C2C12 myotubes respond to increasing concentrations of DEX (1-100 μM) in a dose-dependent manner, as revealed by the increased expression of MuRF1 and atrogin1/MAFbx protein levels. Prior to determining the protective effects of PYP1-5 on muscle atrophy, the potential toxicity of PYP1-5 on C2C12 myoblasts was determined using an MTS assay, which revealed that PYP1-5 treatment had no cytotoxic effects on C2C12 myoblasts. We next assessed the protective effects of PYP1-5 by measuring myotube diameter. Compared with the control group, the DEX-treated group exhibited a 10% increase in cell diameter. Subsequently, the effects of PYP1-5 on C2C12 myotube atrophy were investigated by examining the expression levels of muscle atrophy markers MuRF1 and atrogin1/MAFbx, as they are upregulated in a number of catabolic conditions (25). Glucocorticoids such as DEX have been demonstrated to promote myosin heavy chain degradation through the activation of the E3 ligase MuRF1 (26). Therefore, the downregulation of MuRF1 and atrogin1/MAFbx expression may inhibit muscle atrophy. Western blot and RT-qPCR analyses in the present study revealed that PYP1-5 treatment led to a decrease in the mRNA and protein expression levels of MuRF1 and atrogin1/MAFbx, suggesting that PYP1-5 may potentially be used in anti-atrophy functional foods.

In conclusion, the present study provides important new information about the influence of PYP1-5 on DEX-induced muscle atrophy. The results provide molecular evidence that the anti-atrophic effects of PYP1-5 are due to the downregulated expression of the muscle-specific E3 ubiquitin ligases MuRF1 and atrogin1/MAFbx. Future studies on the anti-atrophic effects of PYP1-5 are required, and should involve identifying the signaling pathways that may be associated with the anti-atrophic effects, such as insulin-like growth factor 1/Akt and myostatin.

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