Soybean is one of the most abundant plant sources of dietary protein (1, 2). Numerous studies have found that soy protein intake favorably affects obesity and lipid metabolism; soy protein consumption reduces triglycerides and cholesterol in blood and liver (1, 3). Recently, increased attention has been paid to the effects of soybean protein and derived peptides on skeletal muscle. For example, dietary soy proteins are reported to prevent exercise-induced protein degradation of skeletal muscle in normal rats (4) and improve the impaired insulin action in the skeletal muscle of dyslipidemic insulin resistant rats fed a sucrose-rich diet (5, 6).

Skeletal muscle is composed of four types of fibers classified by physiological and biochemical properties in mammals: oxidative slow-twitch type I, oxidative fast-twitch IIA, and glycolytic fast-twitch IIB and IIX/D (7). These fiber types can be distinguished in mammals according to the predominantly expressed isoform of myosin heavy chain (MyHC) (8); MyHC1 (encoded by Myh4), MyHC2A (Myh2), MyHC2X (Myh7), and MyHC2B (Myh1) are preferentially expressed in type I, IIA, IIX/D, and IIB, respectively. Interestingly, recent studies reported that dietary ingredients can affect the fiber type composition in skeletal muscle (9–13). For example, carnitine supplementation prevents obesity-induced type I to type II muscle fiber transition in genetically obese rats (9). Mizunoya et al. reported that dietary intake of crude soy isolavones prepared from soybean-germ significantly increased the proportion of MyHC1 protein contents in rat skeletal muscle (14). Although soy isolavones are thought to be candidates for PPARδ ligands, the mechanisms were expected to be independent of PPARδ (15). These findings raise the possibility that other soy ingredients, in addition to isolavones, may induce oxidative-type myofibers.

Several studies have demonstrated that intact proteins enter the blood circulation via the gastrointestinal tract in healthy humans and rodents (16–21). For example, orally-administered ovalbumin is detected in peripheral blood (16–18). A recent in vitro study using C2C12 cells reported that ovalbumin and ovomucoid stimulate the proliferation of myoblasts and growth of myotubes, whereas ovotransferrin decreases the proliferation (22), possibly suggesting that proteins directly influence physiological events in skeletal muscles. Similarly, soybean proteins are detected in peripheral blood after an oral administration in mice (19, 20). These findings raise the possibility that soybean protein also directly affects muscle cells.

In this study, we investigated using C2C12 myotubes whether soybean protein extracts affect mRNA levels of MyHCs.
Soybean Protein Increases Myosin Heavy Chain Isoforms

Cell culture. C2C12 myoblasts (DS Pharma Biomedical Co. Ltd., Osaka, Japan) were grown prior to assay in high-glucose DMEM (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% FetalClone III (GE Healthcare, Chicago, IL), 100 U/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque, Inc.) at 37°C, 5% CO2 in humidified air. The cells were plated at 20,000 cells/well in 12-well plates and incubated until confluent. To induce myoblast fusion to form myotubes, the medium was replaced with DMEM supplemented with 2% horse serum (HS; Thermo Fisher Scientific Inc., Waltham, MA). Six days after substituting 2% HS medium, the cells were treated with or without the samples prepared by the method described above. for 24 h before harvest to analyze mRNA levels of MyHC isoforms. The extracts were dissolved in phosphate buffered saline (PBS) and sterile filtered using a 0.2 μm PVDF membrane. All wells contained the same volume of PBS.

Real-time PCR analysis. Total RNA was extracted from the cells using Sepazol-RNA I (Nacalai Tesque, Inc.). First-strand cDNA was synthesized from total RNA using the ReverTra Ace® qPCR RT Kit (Toyobo Co. Ltd., Osaka, Japan). mRNA levels were quantified for each primer in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA), and SYBR Premix Ex Taq II (Tli

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Fig. 1. Procedure for test samples. (A) Procedure for preparing soybean protein extracts. Soyaflavone RS was washed with 70% ethanol at 70°C for 40 min twice and dried at 100°C before use. (B) Procedure for digestive enzyme treatment of soybean-germ protein extract (SGE).
RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier’s recommendations. Expression levels of the target genes were normalized to those of the ribosomal protein S17 (RPS17). The primer sequences are the same as in previous studies (13, 30).

Statistical analysis. Dunnett’s test was performed to analyze the differences among the groups using Excel 2013 (Microsoft, Redmond, WA) with Statcel 3 add-in software (OMS, Tokyo, Japan).

Results and Discussion

We found that treatment with Soyaflavone RS extract (SRE) significantly increased the mRNA level of slow-type myosin heavy chain Myh7 (MyHC1) in C2C12 myotubes, whereas the mRNA level of fast-type myosin heavy chain Myh4 (MyHC2B) significantly decreased (Table 1). No significant change was observed in the mRNA levels of Myh2 (MyHC2A) or Myh1 (MyHC2X) (Table 1). Furthermore, we examined the effect of soybean protein extracts prepared from different commercial soy protein products on MyHC expression. Similarly, the treatment of either soybean meal extract (SME) or soybean-germ protein extract (SGE) significantly increased Myh7 expression, whereas expression of Myh4 and Myh1 was significantly decreased by SME treatment (Fig. 2). No significant change was observed in Myh2 expression. These findings suggest that the extract of soy protein contains one or more factors which activate de novo synthesis of oxidative-type myofiber.

Since SGE was found to be most effective in increasing Myh7 expression, we examined the effects of the digestive-enzyme treatment of SGE on Myh7 expression in C2C12 myotubes. As shown in Fig. 3, the treatment of enzyme-treated SGE (ESGE) significantly increased the mRNA level of Myh7, suggesting that its fragments generated by digestive enzymes (less than 5 kDa, Fig. 3A) can increase Myh7 expression. In contrast, no significant change was observed in Myh4 mRNA level after the treatment of ESGE (Fig. 3B). We further examined ESGE treated strongly with the enzymes (ESGE-2); the volume of enzyme solution was double and incubation time was fourfold in each enzyme-treatment step described in Fig. 1B. Interestingly, ESGE-2 failed to increase Myh7 expression significantly (Fig. 3C). These

Table 1. Effects of Soyaflavone RS extract on myosin heavy chain expression in C2C12 myotubes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Soyaflavone RS extract (mg/mL as protein concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>MyH7 (MyHC1)</td>
<td>1.33±0.06</td>
</tr>
<tr>
<td>MyH2 (MyHC2A)</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>MyH1 (MyHC2X)</td>
<td>0.65±0.02</td>
</tr>
<tr>
<td>MyH4 (MyHC2B)</td>
<td>2.97±0.12</td>
</tr>
</tbody>
</table>

All wells contained the same volume of PBS. The cells were treated for 24 h. Data are expressed as mean±SE (n=4). Dunnett’s test was used to analyze differences. *, ** Significant with respect to 0 mg/mL group (* p<0.05; ** p<0.01).

Fig. 2. Effects of soybean protein extracts on mRNA levels of myosin heavy chain isoforms in C2C12 myotubes. Soybean germ extract (SGE), and soybean meal extract (SME) were prepared by the method described in Fig. 1A. SRE, SGE, and SME were added to wells at 1 mg/mL as protein concentration. Data are expressed as mean±SE (n=6). Dunnett’s test was used to analyze differences. * Significant with respect to the saline injection group (* p<0.05; ** p<0.01).
Fig. 3. Effects of enzyme-treated soybean protein extracts on myosin heavy chain 7 expression in C2C12 myotubes. (A) Soybean germ extract (SGE) was digested and filtered by the method described in Fig. 1B. Image of polyacrylamide gel followed by Coomassie brilliant blue stain shows the molecular weight distribution of each sample. (B) The same volume of each sample was added to each well (50 μL sample/1 mL 2% HS medium). Data are expressed as mean±SE (n=4). Dunnett’s test was used to analyze differences. (C) The sample (ESGE-2) was prepared by a modified method; the volume of enzyme solution was double and incubation time was fourfold in each enzyme-treatment step described in Fig. 1B. Data are expressed as mean±SE (n=6). The t-test was used to analyze differences. *,** Significant with respect to saline injection group (*p<0.05; **p<0.01). ESGE, enzyme-treated soybean germ extract.

findings indicate that the factors which activate Myh7 transcription are digestible macromolecules, probably protein/peptides, but not low molecular. Previous studies show the intestinal absorption of protein and peptide. For example, soy-derived bioactive peptide lunasin (5.5 kDa) is absorbed through the gastrointestinal barrier and reaches the target tissues (19). Orally administered ovalbumin (45 kDa) is detected in the peripheral blood of humans and rodents (16–18). Therefore, oral intake of soybean protein extracts used in this study may increase oxidative-type myofibers in vivo.

A previous study in rats (14) showed that dietary crude soy isolavones prepared from soybean-germ (Soyallavone HG, containing 12.2% protein) significantly increase the MyHC1 proportion in the extensor digitorum longus. Although the concentrations of isolavones were not measured in this study, the samples probably contain a small amount of isolavones. However, a low-molecular fraction of SGE separated by Omega Membrane (3K) failed to increase Myh7 expression, whereas the high-molecular fraction of SGE caused a significant increase (unpublished data). It is therefore unlikely that the isolavones cause the increase of Myh7 expression in this study.

The isoforms of MyHC can be used as a marker to distinguish the type of myofibers. Thus, we analyzed their mRNA levels in this study. However, the mRNA and protein levels of MyHCs do not always coincide (31). Further studies using this method to analyze the protein levels of MyHCs (32) are required.

Type I fibers are mitochondria-rich and mainly utilize oxidative phosphorylation for energy production. In addition to studies using rodent models (9, 33), the proportion of type I fibers is reported to decrease in obese subjects (34, 35). Interestingly, the supplementation of carnitine and niacin prevents obesity-induced muscle fiber transition (9, 33), suggesting a new mechanism for improving obesity-related conditions. Therefore, the data in this study raise the possibility that soybean proteins exert favorable effects on obesity and lipid metabolism via an increase of oxidative muscle fiber, in addition to other well-known mechanisms (1).

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