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Effects of soy protein hydrolysates on antioxidant activity and inhibition of muscle loss

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Abstract

Peptides show biological activity, and are more easily digested than complex proteins. In the present work, we evaluated the effects of soy hydrolysate on skeletal muscle. Soy protein isolate (SPI) was hydrolysed using 2% Alcalase (SPHA) and Flavourzyme (SPHF) at pH 8 for 3 h at 60°C, and at pH 7 for 3 h at 55°C, respectively. Antioxidant properties (total phenolic content and DPPH activity) and inhibition of muscle loss (myogenin, myosin heavy chain [MyHC], creatine kinase, and myostatin) by the SPI hydrolysates in C2C12 cells were compared with those of SPI. Alcalase produced more hydrolysed soy oligopeptides than Flavourzyme. Enzymatic hydrolysis increased the levels of essential amino acids, particularly in SPHF (2,466.85 mg/L) as compared to SPI (56.08 mg/L). The total phenolic contents of hydrolysates increased from 12.02 mg GAE/g in SPI to 22.87 and 18.67 mg GAE/g in SPHA and SPHF, respectively. The IC_{50} value of DPPH activity decreased four times after hydrolysis (SPI: 124.38, SPHA: 32.18, and SPHF: 30.21 mg/mL). SPHA and SPHF treatments increased the expression levels of both MyHC1 and MyHC3, as well as creatine kinase activity. A significant increase in MyHC3 expression was observed in SPHF at 10 µg/mL. Soy hydrolysates (SPHA: 93.5% and SPHF: 61.0%) induced a greater decrease in the expression of myostatin, a muscle reduction marker, than SPI (30.4%). In conclusion, soy hydrolysates may inhibit muscle loss, showing particularly better effects when Alcalase is used for hydrolysis.

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Introduction

Peptides are specific fragments from different kinds of protein sources that are biologically involved in bodily functions and health promotion (Kilara and Panyam, 2003). Depending on the amino acid composition and sequence, these peptides may have different effects on hormones, nerves, cell growth, and the immune system (Korhonen and Pihlanto, 2006). The enzymatic hydrolysis of proteins is an effective method widely used to produce bioactive peptides with multifunctional properties (Panyam and Kilara, 1996). In the case of whey protein (Nakayama *et al*., 2019), protein hydrolysate has been reported to stimulate muscle protein synthesis more effectively than the original protein. Alcalase and Flavourzyme have been mostly used to hydrolyse different plant proteins, such as rice or soy protein, to produce bioactive peptides (Ahmadifard *et al*., 2016; Gomes and Kurozawa, 2020). Soy protein is an excellent plant source of dietary proteins that includes all essential amino acids, and particularly rich in leucine (Waters *et al*., 2010). In particular, soy peptides derived from the enzymatic hydrolysis of soy protein have been reported to exhibit multifunctional beneficial effects such as anti-inflammatory, antioxidant, and antihypertensive activities (Chatterjee *et al*., 2018).

Sarcopenia is defined as the progressive loss of skeletal muscle mass and function (Dionyssiotis, 2019). It is associated with physical disability, frailty, obesity, and cachexia in the elderly population (Cruz-Jentoft *et al*., 2010). Skeletal muscle formation is a multistep process related to the myogenic differentiation of muscle cells (Gao *et al*., 2018). Myogenic differentiation is related to the coordinated activation of muscle-specific gene expression and the cessation of cell division, thereby resulting in the formation of terminally differentiated myotubes (Novitch *et al*., 1996). Determining the total levels of

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myotube-specific proteins such as myogenin and myosin heavy chain (MyHC), or the activity of creatine kinase (CK), can provide an overall and unbiased measurement of myogenic differentiation (Veliça and Bunce, 2011). Myostatin (MSTN), a member of the transforming growth factor β family, is a negative regulator of skeletal muscle that inhibits protein biosynthesis, and enhances protein degradation (McPherron *et al*., 1997). MSTN is used as a marker of muscle degradation or muscle wasting in the diagnosis of sarcopenia (Baczek *et al*., 2020). An imbalance in the rate of protein synthesis and degradation is the main cause of muscle loss, which is further influenced by food intake and physical activity (Gao *et al*., 2018). After sarcopenia was first recognised as an independent condition by the ICD-10-CM code in 2016, there has been an increasing interest in preventing the loss of muscle mass by either improving muscle protein synthesis or preventing muscle protein degradation using nutrients and bioactive compounds (Lee *et al*., 2020).

Protein is an essential nutrient for promoting skeletal muscle mass that is highly dependent on the rate of muscle protein synthesis, which is stimulated by essential amino acids, particularly leucine and branched-chain amino acids (BCAA) (Shimomura *et al*., 2004; Waters *et al*., 2010). Several clinical and epidemiology studies have indicated that the consumption of soy protein can stimulate muscle mass and promote muscle growth (Paul and Mendelson, 2015), that soy-based foods can prevent osteosarcopenia in postmenopausal women (Tang *et al*., 2020), and that consumption of miso can lower the prevalence of sarcopenia in patients with type 2 diabetes (Takahashi *et al*., 2020). However, few studies have investigated the effect of soy protein hydrolysates on muscle loss based on muscle protein synthesis and degradation.

In the present work, we investigated the characteristics of soy protein hydrolysates prepared from soy protein isolates under optimum Alcalase and Flavourzyme hydrolysis conditions, and subsequently determined their effects on the inhibition of muscle loss.

Materials and methods

Soy protein hydrolysate preparation

Hydrolysis was performed following a previously reported method (Hrckova *et al*., 2002) with some modifications related to the enzyme hydrolysis conditions. Soy protein isolates (SPI: protein, 91%; fat, 0.0%; and carbohydrate, 5.4%) hydrolysates of Alcalase (2.4 L FG, Novozymes, Bagsvaerd, Denmark) and Flavourzyme (500 MG, Novozymes) were prepared following their respective optimum conditions, determined by pre-test of hydrolysis optimisation. Soy protein isolates (Eisse Food Co., Ltd., Gyeonggi-do, Korea) were hydrolysed with Alcalase for 3 h at a 5% substrate concentration and 2% enzyme/substrate ratio, at pH 8 and 60°C. Similarly, Flavourzyme (500 MG, Novozymes) hydrolysis of SPI was conducted at a 5% substrate concentration and 2% enzyme/substrate ratio at pH 7 and 55°C. Briefly, SPI dispersions were prepared by heating in an 85°C water bath for 15 min. After cooling to the desired temperature, the optimum pH of the SPI dispersions was adjusted. The enzyme was then added to initiate hydrolysis. Dispersions were adjusted to the optimum pH value in real time, and boiled for 10 min to terminate the enzyme action. SPI hydrolysate supernatants were obtained after centrifugation (VS-24SMTi, Vision S & Tech., Korea) at 2,500 *g* for 10 min. SPI hydrolysates were separated using a 10 kDa molecular weight cut off (MWCO) (Sartorius Stedim Lab Ltd., Stonehouse, UK) by centrifugation (VS-24SMTi) at 5,000 *g* for 20 min. SPI (> 10 kDa) hydrolysed using Alcalase (SPHA) and SPI hydrolysed using Flavourzyme (SPHF) were obtained, freeze-dried (FDU-12AS, AS ONE, Japan), and stored at -20°C until subsequent analyses.

Degree of hydrolysis and SDS-PAGE

The degree of hydrolysis (DH) was measured by determining the number of amino groups using the ninhydrine colorimetry method (Navarrete del Toro and García-Carreño, 2003). The amino group content was calculated using a standard curve of ninhydrine colour reagent and glycine (0 - 20 µg/mL) standard (Samchun Pure Chemical Co., Ltd., Gyeonggi-do, Korea). For protein patterns, SPI and SPI hydrolysates were analysed with SDS-PAGE following the method of Tsumura *et al*. (2005), using a 10% acrylamide separating gel and 5% acrylamide stacking gel. Samples containing 50 µg of protein were prepared under reducing conditions. Dissociating electrophoresis was carried out in a continuous buffer system: 0.025 M Tris-HCl (Sigma Aldrich), 0.192 M glycine (Daejung), and 0.1% SDS (Bio-Rad Laboratories); pH 8.3. Protein molecular weights were estimated using a low-MW marker

(GenDEPOT) with a size range of 6 to 170 kDa. Electrophoresis was performed for 70 min at 35 mA. The separated protein bands were stained with Coomassie R-250 blue (Thermo Fisher Scientific) overnight, and destained with the R-250 destaining solution (Thermo Fisher Scientific).

Amino acid composition

The free amino acids in SPI and SPI hydrolysates were determined following the modified method of Goh *et al*. (2017). Briefly, pre-treated SPI peptides were suspended in borate buffer, and then filtered through a 0.45 µm micropore film. First, primary amino acids were allowed to react, then secondary amino acids were derivatised using phthaldialdehyde (OPA) and fluorenylmethyl-oxycarbonyl chloride (Fmoc) (Agilent Technologies, CA, USA), respectively. The derivatisation process was automatically analysed with HPLC (Dionex Ultimate 3000, Thermo Dionex, USA) equipped with an FL detector (Agilent 1260 Infinity, USA) (emission wavelength 450 nm and excitation wavelength 340 nm for OPA; emission wavelength 305 nm and excitation wavelength 266 nm for Fmoc) and an Inno C_{18} column (4.6 × 150 mm, 5 µm; Youngjin Biochrom, Korea). A gradient of mobile phase A (40 mM sodium phosphate, pH 7) and mobile phase B (10% 3-steamed DW/45% acetonitrile/45% methanol) was employed. The flow rate was maintained at 1.5 mL/min, and the injection volume was 0.5 µL. Calibration curves of amino acid standards (Agilent PN 5061-3330, Agilent Technologies, 17 amino acids) were generated to calculate amino acid concentrations in the samples.

Total phenolic content and DPPH capacity

The total phenolic contents of the SPI and SPI hydrolysates were determined using the Folin-Ciocalteu reagent (Dixit *et al*., 2010). Briefly, 30 µL of the sample extract was added to 120 µL of distilled water, 30 µL of Folin's reagent, and 70 µL of 10% sodium carbonate. The mixture was homogenised (VCX-130PB, Sonics, USA) and incubated (HB-201SF, Hanbaek Scientific Co., Korea) for 2 h at 20°C. The absorbance (725 nm) was monitored using a microplate reader (Multiskan, Thermo Fisher, Waltham, MA, USA), and total phenolic content was expressed as gallic acid equivalent/g. DPPH radical scavenging activity was analysed following the method of Horax *et al*. (2017). Briefly, 100 µL of the sample solution (10 mg/mL in distilled water) was added into 1 mL of DPPH solution (0.05 g/L in 99.5% ethanol), and the absorbance (515 nm) was determined after 30 min of incubation at room temperature. The DPPH radical scavenging capacity (%) was calculated using Eq. 1:

$$DPPH\ radical\ scavenging\ capacity\ (\%) = \left(1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right) x\ 100 \quad \text{(Eq. 1)}$$

where, Abs_{sample} = absorbance of the experimental samples, Abs_{blank} = absorbance of the blank, and $Abs_{control}$ = absorbance of the control.

The results were expressed as the IC_{50} value, which is defined as the concentration of samples required for 50% inhibition. Ascorbic acid in distilled water (2.5 ~ 40 µg/mL) was used as the positive control. The IC_{50} of ascorbic acid was 0.063 ± 0.0001 mg/mL.

C2C12 cell culture

C2C12 cells (mouse myoblasts; American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% foetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/ml penicillin at 37°C, 5% CO_2, 95% air, and 100% humidity (Sung *et al*., 2015). The growth medium was refreshed every two days. Differentiation was induced by culturing in differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum after the C2C12 cells had grown for 5 - 7 passages to 70 - 80% confluence. Fresh differentiation medium was provided to the cells every 48 h. For proliferation assays, C2C12 cells were plated into 96-well plates, and cultured in growth medium (DMEM with 10% FBS) or with a certain concentration (1, 5, 10, 20, or 50 µg/mL) of SPI hydrolysates for 72 h.

Expression of myogenic differentiation marker genes and creatine kinase activity

The expression of myogenic marker was analysed with western blotting (Sung *et al*., 2015). Briefly, the differentiated cells were washed with PBS, and lysed in lysis buffer (40 mM Tris [pH 8.0], 0.5% NP-40, 2 µg/mL aprotinin, 120 mM NaCl, 100 µg/mL phenymethylsulfonylfluoride, 2 µg/mL leupeptin, and complete protease inhibitor), and supernatants were collected by centrifugation. Protein concentrations were measured using Bradford protein

assay (Bio-Rad, Hercules, CA, USA). For denaturation, equal amounts of protein extracts were boiled in sample buffer (Bio-Rad) for 5 min. Proteins were separated with 6 - 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Berlin, Germany). The membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline, with Tween 20 buffer (TBS-T), at room temperature, for 1 h. Finally, the membranes containing the transferred proteins were incubated with primary antibodies specific for myosin heavy chain3 (MyHC3) (sc-53091; Santa Cruz Biotechnology, Dallas, TX), MyHC1 (sc-20641; Santa Cruz Biotechnology), and myogenin (sc-576; Santa Cruz Biotechnology) at 4°C for overnight. After washing three times with TBS-T buffer, the membranes were incubated with secondary antibodies, conjugated to horseradish peroxidase, at room temperature for 1 h. Immunoreactivity was determined using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Creatine kinase activity was evaluated using a CK assay kit (Abnova, Taipei, Taiwan) following the manufacturer's instructions. Cell lysates were obtained using the methods described earlier. Assay reagent (100 μL) was added to 10 μL of the cell lysate in a microplate with an equal amount of protein. The CK activity of untreated cells was set to 100%. CK activity was measured at a wavelength of 340 nm. All assays were performed in duplicate.

Measurement of MSTN expression using quantitative reverse transcription-PCR (qRT-PCR)

Myostatin expression was measured with qRT-PCR (Kim *et al*., 2015). In brief, C2C12 cells were seeded into a 12 well culture plate at a density of 3×10^4 cells/well. After 24 h of incubation at 37°C, 10 μg/mL of the sample was added into each well, while the same amount of dimethyl sulfoxide (DMSO) was added to the control wells. Total RNA was extracted using the TRIzol method (Rio *et al*., 2010), and the amount of RNA was measured using a Taken3 microplate spectrophotometer (Epoch, BioTek, Winooskim, USA). Subsequently, 2 μg of mRNA was converted to cDNA using the cell scripts cDNA synthesis kit (Enzynomics, Daejeon, Korea). Then, the mRNA expression was determined on a real-time PCR machine (Quantstudio3, Thermo Fisher Scientific). The PCR reaction mix comprised a myostatin-specific primer, TOPreal™ qPCR 2X PreMIX (SYBR Green with high ROX; Enzynomics), 40 ng of cDNA, and 10 pmol of each forward and reverse primers (forward primer: GCACTGGTATTTGGCAGAGT, reverse primer: TTCAGCCCATCTTCTCCTGG). *GAPDH* was used as an internal control for normalisation. The cycling conditions were as follows: 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 sec, and amplification at 72°C for 30 sec.

Statistical analysis

Data were reported as mean ± standard deviation, and were analysed by one-way analysis of variance (ANOVA) with multiple comparisons using Duncan's multiple range test. Statistical significance was set at $p < 0.05$. All data analyses were conducted using SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Degree of hydrolysis and protein patterns

Consistent with the known enzyme activity, the Alcalase hydrolysate (28%) had a higher degree of hydrolysis than the Flavourzyme hydrolysate (24%) ($p < 0.05$). The enzymatic activity of Alcalase (3.07×10^4 U/g) was about three times higher than that of Flavourzyme (1.09×10^4 U/g). As compared to those of SPI, the high molecular weight bands of SPI hydrolysates had decreased or disappeared, thus indicating that the hydrolysis of proteins was successful (Figure 1). Alcalase and Flavourzyme have previously been reported to exhibit excellent hydrolysis activity in different plant proteins such as rice or soy protein to produce bioactive peptides (Ahmadifard *et al*., 2016; Gomes and Kurozawa, 2020). Alcalase is a kind of endopeptidase that can hydrolyse peptide bonds inside a large protein with a preference for a large amount of oligopeptides, whereas Flavourzyme is an endopeptidase and exopeptidase complex enzyme that can produce free amino acids and peptides (Sangronis *et al*., 2006). The free amino acid content after hydrolysis is shown in Table 1. The total free amino acid content in SPI hydrolysed with Flavourzyme (SPHF) (3,020.38 mg/L) was higher than that in SPI hydrolysed with Alcalase (SPHA) (498.18 mg/L), as Flavourzyme could break the N-terminal peptide bonds of protein chains to produce much higher amounts of free amino acids (Ambigaipalan *et al*., 2015). Sixteen amino acids were detected in SPI hydrolysates. Similar to

other legume proteins which are deficient in sulphur amino acids (Berrazaga *et al*., 2019), cysteine was not detected; however, methionine content increased from 5.04 mg/L in SPI to 26.92 mg/L in SPHA and 106.49 mg/L in SPHF after hydrolysis. Among the 16 amino acids, the content of leucine mostly increased with hydrolysis from 17.42 mg/L (SPI) to 128.38 mg/L (SPHA) and 872.12 mg/L (SPHF). The content of BCAA increased from 26.11 mg/L (SPI) to 178.76 mg/L (SPHA) and 1,421.6 mg/L (SPHF). The content of essential amino acids increased from 56.08 mg/L (SPI) to 377.03 mg/L (SPHA) and 2,466.85 mg/L (SPHF). The increases in the contents of only certain amino acids after hydrolysis could be attributed to the increase in the number of available amino groups with respect to the degree of hydrolysis (Yu *et al*., 2017). However, the ratios of hydrophobic and hydrophilic amino acids differed with different enzymatic hydrolysis methods (SPI: 4.2, SPHA: 6.7, and SPHF: 5.9). In addition, a relatively higher ratio of leucine and BCAA contents in total amino acids was observed in SPHF (0.29 and 0.47) than in SPHA (0.26 and 0.35), which were higher than those in SPI (0.22 and 0.33).

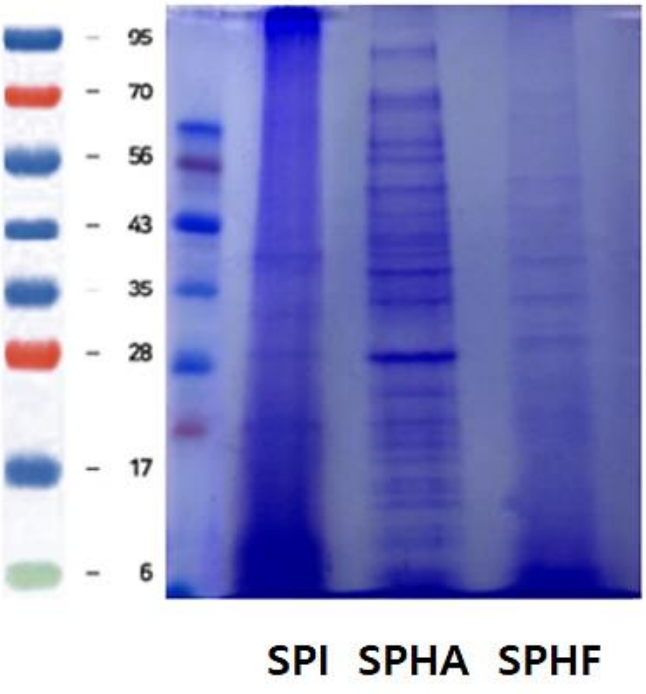

Figure 1. SDS-PAGE profiles of soy protein hydrolysates after hydrolysis with Alcalase or Flavourzyme. SPHA: SPI (> 10 kDa) hydrolysed by Alcalase, SPHF: SPI (> 10 kDa) hydrolysed by Flavourzyme.

Table 1. Free amino acid contents of the soy protein hydrolysed by Alcalase and Flavourzyme.

Amino acid (mg/L)	SPI	SPHA	SPHF
Histidine	0.68	19.82	5.62
Isoleucine	4.44	14.89	280.24
Leucine	17.42	128.38	872.12
Lysine	14.12	68.13	116.07
Methionine	5.04	26.92	106.49
Phenylalanine	10.13	66.82	579.7
Tryptophan	ND	15.57	112.05
Threonine	ND	1.01	125.32
Valine	4.25	35.49	269.24
Sum of EAA	56.08	377.03	2466.85
Alanine	7.68	53.02	218.88
Arginine	1.37	6.88	12.29
Aspartic acid	0.27	1.01	3.54
Cysteine	ND	ND	ND
Glutamic acid	7.26	11.21	239.15
Glycine	1.36	11.29	45.99
Proline	ND	ND	ND
Serine	0.18	0.77	5.12
Tyrosine	4.05	36.97	28.56
Total AA	78.25	498.18	3020.38
Hydrophobic amino acid	50.32	336.81	2372.66
Hydrophilic amino acid	11.76	50.97	401.69
BCAA	26.11	178.76	1421.6

SPHA: SPI (> 10 kDa) hydrolysed by Alcalase, SPHF: SPI (> 10 kDa) hydrolysed by Flavourzyme. ND: not detected.

Total phenolic content and antioxidant capacity

After hydrolysis, the total phenolic content increased from 12.02 mg GAE/g (SPI) to 22.87 mg GAE/g (SPHA) and 18.67 mg GAE/g (SPHF) (Figure 2) ($p < 0.05$), because hydrolysis causes the bound polyphenols to be released in a free state. The DPPH radical scavenging activity showed the same trend as that of the total phenolic content (Figure 2). The IC_{50} value was lower in SPHA (32.18 mg/mL) and SPHF (30.21 mg/mL) than in SPI (124.38 mg/mL) ($p < 0.001$). Similar results have been reported for the casein protein hydrolysate produced by Alcalase (Kumar *et al*., 2016). Enzymatic hydrolysis might lead to changes in protein structures, which may expose more amino acid residues, and lead to the

generation of more stable structures, thus preventing free radical chain reactions (Hall *et al*., 2017). These peptides can serve as potential radical scavengers by donating the protons of their aromatic amino acid residues to free radicals (Horax *et al*., 2017). The antioxidant activity of the hydrolysates increased with an increase in the degree of hydrolysis (Vaštag *et al*., 2011). SPHA (28%) had a higher degree of hydrolysis than the Flavourzyme hydrolysate (24%). Hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val, and Met released through enzyme hydrolysis may exhibit antioxidant capacity through their electron-donating ability (da Silva Lucasa *et al*., 2020). In addition, the changes in specific amino acid sequences and molecular sizes of the individual peptides in protein hydrolysates affect the overall antioxidant capacity of the hydrolysate (Vaštag *et al*., 2011). The increased antioxidant capacity may influence muscle loss because muscle degradation is associated with oxidative stress (Huang *et al*., 2017).

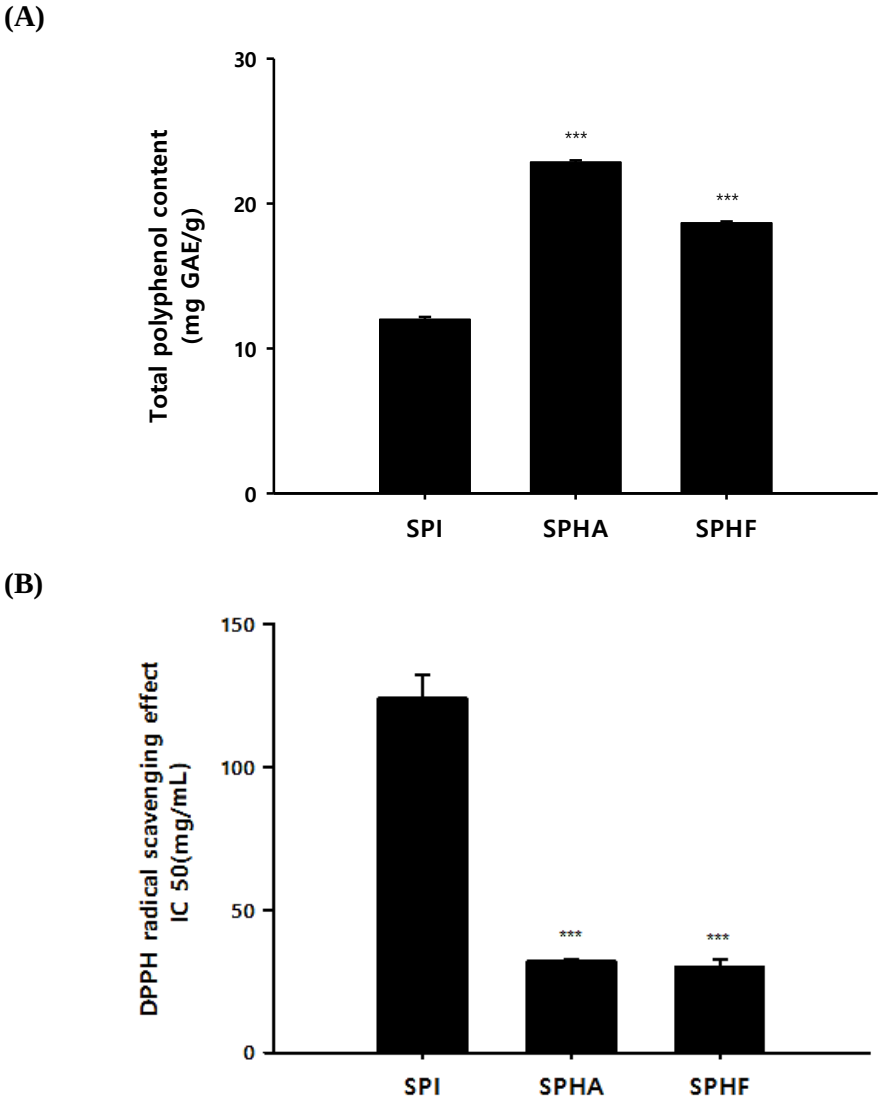

Figure 2. (A) Total phenolic content and **(B)** DPPH capacity. SPHA: SPI (> 10 kDa) hydrolysed by Alcalase, SPHF: SPI (> 10 kDa) hydrolysed by Flavourzyme. ***Significant at $p < 0.001$ as compared to SPI.

Effects of SPI hydrolysates on myogenic differentiation

Regarding C2C12 cells proliferation, a slight concentration-dependent increase in cell proliferation was observed upon treatment with SPHA and SPHF, but with no statistical significance ($p > 0.05$). Myogenin is a muscle-specific transcription factor that controls myogenic determination and myogenic differentiation (Buckingham and Rigby, 2014). MyHC, the major myotube structural protein, is muscle-specific marker differentiated terminally (Novitch *et al*., 1996). In terms of the expression of

myogenin, no significant increase was observed after treatment with soy protein hydrolysates (Figure 3) regardless of the increase in SPHF concentration. However, the expression of both MyHC1 and MyHC3 increased in a concentration-dependent manner (Figure 3). In particular, a significant (p < 0.05) increase in MyHC3 expression was observed after treatment with SPHF at concentrations of 1, 5, and 10 µg/mL. This effect might be related to the increased leucine and BCAA contents (Waters *et al*., 2010; Ahmadifard *et al*., 2016) after hydrolysis, as well as the increased contents of essential amino acids, particularly methionine and lysine (Table 1), which are used for muscle protein synthesis (Berrazaga *et al*., 2019). Furthermore, the polyphenols in soy, particularly isoflavone, have been reported to stimulate muscle growth (Zheng *et al*., 2018) by promoting mitochondrial biogenesis in C2C12 myoblasts (Yoshino *et al*., 2015). Creatine kinase activity is generally defined as an important muscle-specific indicator during the muscle cell differentiation process (Lawson and Purslow, 2000). Treatment with SPHA or SPHF was found to increase CK activity, although without statistical significance (p > 0.05). Although there was no statistical significance, SPHA and SPHF treatments increased CK activity and the expression levels of both MyHC1 and MyHC3.

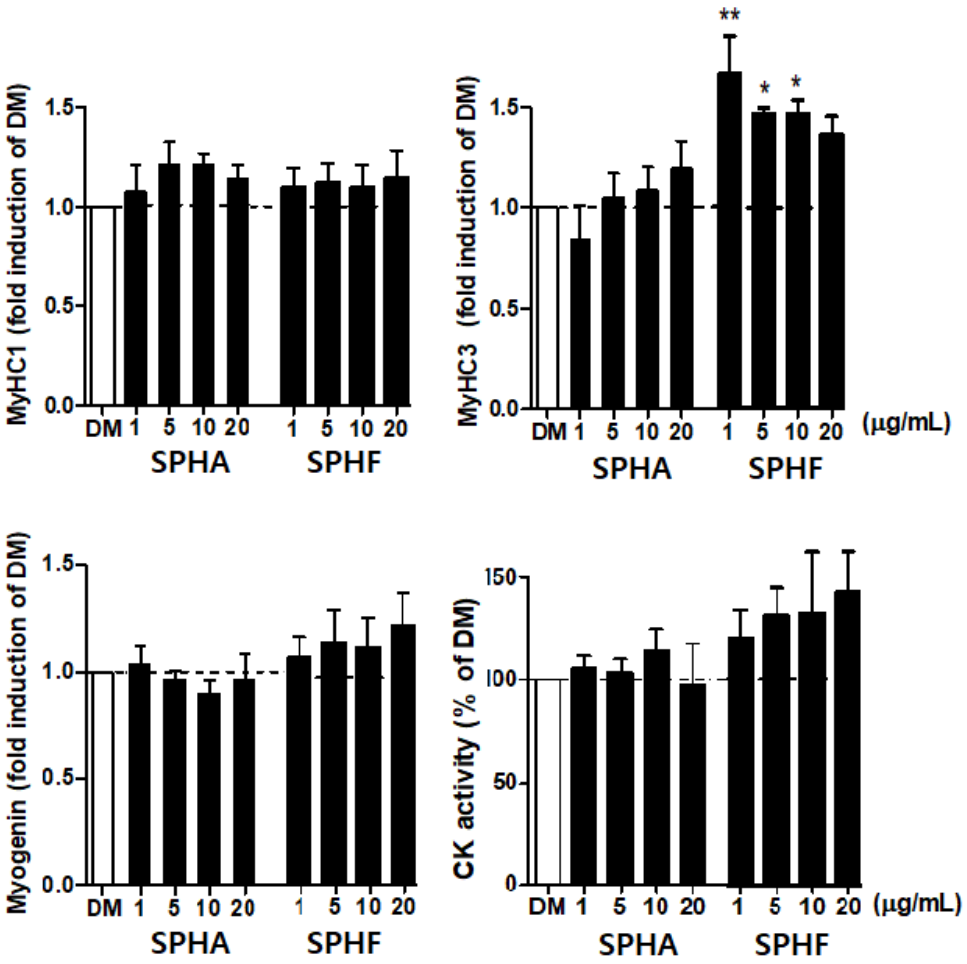

Figure 3. Effect of soy protein hydrolysates on myogenic marker expression and CK activity of C2C12 myotubes. SPHA: SPI (> 10 kDa) hydrolysed by Alcalase, SPHF: SPI (> 10 kDa) hydrolysed by Flavourzyme. *Significant at p < 0.05. **Significant at p < 0.01.

Inhibition of muscle degradation

To determine the inhibition of muscle degradation by SPI, the mRNA expression of myostatin, a muscle degradation marker, was measured (Figure 4). Soybean protein isolate and hydrolysate significantly decreased the expression of myostatin (p < 0.001). When cells were treated with 10 µg/mL of SPI for 24 h, myostatin expression decreased by approximately 30.4% (SPI). After hydrolysis, myostatin expression decreased by

approximately 93.5% in SPHA, and 61% in SPHF. SPHA exhibited a greater decrease than the whey protein isolate (76.2%), which is the most popular protein supplement for athletes to improve muscle functions (Mobley *et al*., 2017). These results agree with clinical studies, which show that high plant protein intake is associated with reduced muscle loss and increased skeletal muscle mass in the elderly (Miki *et al*., 2017; Berrazaga *et al*., 2019). Several studies have reported that oxidative stress and inflammation can influence muscle protein metabolism, thereby affecting muscle atrophy (Huang *et al*., 2017). The increased antioxidant capacity of soy protein (Figure 2), particularly after hydrolysis, could lead to a decrease in myostatin levels.

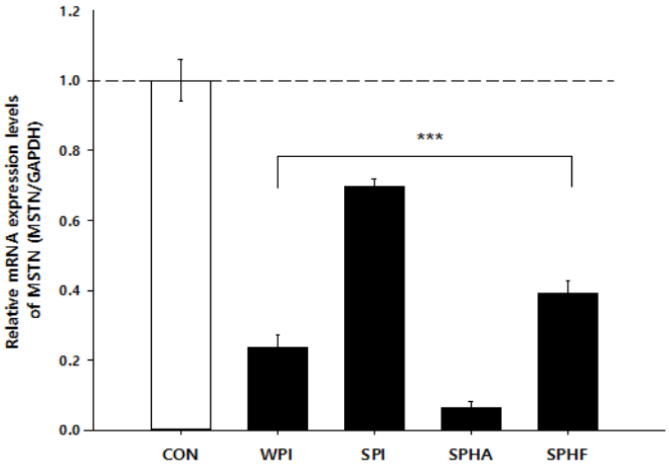

Figure 4. Relative mRNA expression levels of myostatin in soybean protein hydrolysates. WPI: whey protein isolate, SPI: soy protein isolate, SPHA: SPI (> 10 kDa) hydrolysed by Alcalase, SPHF: SPI (> 10 kDa) hydrolysed by Flavourzyme. ***Significant at $p < 0.001$ as compared to CON.

Conclusion

Alcalase exhibited a higher capacity to hydrolyse soy protein isolates than the Flavourzyme. The cell culture results showed the possibility of SPI hydrolysates in facilitating myogenic differentiation, particularly soy protein hydrolysates from Flavourzyme, which had high leucine, BCAA, and essential amino acid contents. SPI hydrolysates induced a decrease in myostatin expression, particularly in soy protein hydrolysates from Alcalase, which had high total phenolic content and antioxidant capacities. The present work demonstrated the possibility of using SPI hydrolysates to prevent muscle loss. However, further studies are needed to confirm the effect of SPI hydrolysates on skeletal muscle atrophy at the mRNA levels of myostatin transcription factors, myostatin downstream genes, and chemical-induced muscle atrophy.

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