Synergetic effect of soluble whey protein hydrolysate and *Panax ginseng* berry extract on muscle atrophy in hindlimb-immobilized C57BL/6 mice

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**ABSTRACT**

**Background:** Sarcopenia, defined as loss of muscle mass and strength with age, becomes a public health concern as the elderly population increases. This study aimed to determine whether the mixture of soluble whey protein hydrolysate (WPH) and *Panax ginseng* berry extract (GBE) has a synergetic effect on sarcopenia and, if so, to identify the relevant mechanisms and optimal mixing ratio.

**Methods:** In the first experiment, C57BL/6 mice were hindlimb immobilized for one-week and then administered WPH 800 mg/kg, GBE 100 mg/kg, WPH 800 mg/kg + GBE 100 mg/kg mixture, and *Fructus Schisandrae* extract (SFE) 200 mg/kg for two weeks. In the second experiment, experimental design was same, but mice were administered three different doses of WPH and GBE mixture (WPH 800 mg/kg + GBE 100 mg/kg, WPH 800 mg/kg + GBE 90 mg/kg, WPH 1000 mg/kg + GBE 75 mg/kg).

**Results:** In the first experiment, we confirmed the synergetic effect of WPH and GBE on muscle mass and identified that GBE was more effective on the protein synthesis side, and WPH tended to be slightly more effective for protein degradation. In the second experiment, among three different ratios, the WPH 800 mg/kg + GBE 100 mg/kg was most effective for muscle mass and strength. The mixtures activated muscle protein synthesis via PI3K/Akt/mTORc1 pathway and inhibited muscle protein degradation via suppressing ubiquitin-proteasome system (UPS) and autophagy-lysosome system (ALS), and these effects were more GBE dose-dependent than WPH.

**Conclusion:** The WPH and GBE mixture having a synergetic effect is a potential agent to prevent sarcopenia.

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1. Introduction

Skeletal muscle is a mechanically and metabolically active organ, consisting of proximately 40% of total body weight [1]. Skeletal muscle atrophy refers to a decrease in muscle mass and strength. It is caused by aging as well as diseases such as cancer cachexia, and chronic inflammation [2]. Especially, age-related muscle atrophy is called sarcopenia [3]. It is reported that human skeletal muscle mass has decreased about 25–30% by age 60–70 [4]. Skeletal muscle plays an essential role in energy production and cellular homeostasis so that decreased muscle mass not only can lower quality of life but also aggravate various metabolic disease for elderly people [5].

Muscle mass is maintained by a balance between muscle protein synthesis and degradation, and it is controlled mainly by the phosphatidylinositol-3-kinase (PI3K)/Akt (protein kinase B) signaling pathway [6]. When this pathway is activated, it up-regulates protein synthesis via phosphorylating mammalian target of rapamycin (mTOR) and down-regulates protein degradation via suppressing ubiquitin-proteasome system (UPS) and autophagy-lysosome system (ALS), and these effects were more GBE dose-dependent than WPH.
eukaryotic initiation factor 4E (eIF4E), the inhibiting assembly of ribosomal subunit and translation. Phosphorylation of 4E-BP1 leads separation itself from eIF4E and processes cap-dependent translation [8]. In terms of protein degradation, there are two kinds of proteolytic systems: ubiquitin-proteasome system (UPS) and autophagy-lysosome system (ALS) [9]. FoxO3a activates those two systems. In the UPS, muscle specific ubiquitin ligase, muscle ring finger-1 (MuRF1), and muscle atrophy F-box protein (Atrogin-1) degrade ubiquitin-conjugated proteins [10]. Bnip3, which selectively removes mitochondria via recruit autophagosome to mitochondria, is involved in ALS [11], p62 serves as a bridge between ALS and UPS by delivering ubiquitinated proteins for autophagic degradation [12]. During muscle atrophy, proteolytic systems are activated, increasing the expression of atrophic factors.

As physical activity is reduced by aging, anabolic resistance of muscle protein to dietary protein intake is developed, and insufficient daily protein intake accelerates sarcopenia [13]. Therefore, it is crucial for elderly people to increase daily protein intake to maintain the protein turnover rate. The recommended daily protein intake for elderly adults is 1.2 g/kg/day [14]. However, Korean elderly people have a minimal amount of protein intake per meal, so more than 70% of the elderly over the age of 60 have insufficient protein intake [15]. Therefore, protein supplements are helpful to meet the amount of daily protein intake for them.

Whey protein is known as a representative source of high-quality protein, separated from the casein in milk or formed as a by-product of cheese making. When it is hydrolyzed, the rate of digestion and absorption increases. In the previous study, we have compared the effect of four types of whey protein hydrolysates on muscle atrophy in the immobilization-induced muscle atrophy mice model and found that soluble whey protein hydrolysate (WPH) was most effective for increasing muscle function and muscle mass [16]. Meanwhile, Panax ginseng berry extract (GBE) is a natural substance that has been actively researched recently and is known for its many effects, such as anti-hyperglycemic [17] and anti-diabetes [18]. Although the anti-sarcopenia effect of GBE is unknown, GBE is expected to be effective because ginseng itself is known for its anti-sarcopenia effect [19,20], and GBE has ginsenoside profiles with higher Re and Rd content than ginseng roots [17] and red ginseng [21]. Therefore, in this study, we aimed to determine whether WPH and GBE have a synergetic effect and identify the relevant mechanisms and optimal mixing ratio.

2. Materials and methods

2.1. Preparation of WPH and GBE

WPH preparation was performed as previously described [16]. Briefly, whey protein concentrate was diluted to 20% with distilled water and adjusted to pH 7 to 7.5 using sodium bicarbonate. Next, 0.2% Alcalase 2.4 L FG (Novozyme, Denmark), 0.2% Protamex (Novozyme, Denmark), and 0.2% Flavourzyme 1000 L (Novozyme, Denmark) were added and incubated, followed by inactivation, filtration, and spray drying. WPH was provided by Neocremar Co.,Ltd. (Seoul, Korea). GBE from which seeds were removed was water extracted under reflux condition for 2 to 5 hours. Then, the extract filtered, followed by evaporation, and spray drying. GBE was provided by Holistic Bio Co., (Seongnam, Korea). Powdered WPH and GBE were dissolved in 0.5% carboxymethylcellulose for oral administration to mice.

2.2. Animal and experimental design

Five-week-old Male C57Bl/6 mice were purchased from Raon Bio (Yongin, Korea) and housed in a standard animal facility maintained at a constant temperature (25 ± 1°C) with a 12:12h light-dark cycle with free access to food and water. There were two animal experiments: One for synergetic effect confirmation and the other for identifying the optimal mixing ratio and the relevant mechanisms. In both experiments, all mice had one week adaptation period, and then hind-limb immobilization (IM) was conducted to induce muscle atrophy, except for the Normal group mice [22]. Two weeks of sample administration proceeded after one week of IM. We showed the experiment design and sample administration groups of each experiment in Fig. 1. All mice were divided into weight-matched groups (n = 7 per group). Fructus Schisandrae extract (SFE), approved for muscle strength improvement effect by the Korea Food & Drug Administration, was used as a positive control. After two weeks of sample administration, mice were sacrificed, and three muscle tissues (quadriceps, gastrocnemius, and soleus) were harvested from the immobilized leg and weighed. The weight of each muscle tissue was divided by the body weight to assess muscle atrophy. Grip strength was measured twice a week during the three weeks experiment using a grip strength meter (Bioseb, Chaville, France). We measured maximum grip strength by placing the mouse on the grid connected to the strength meter and pulling the tail when the mouse grasps the grid. Average grip strength per week is normalized by the animal’s body weight. The protocol of the animal study was approved by the Institutional Animal Care (KHSASP-I9-251) and Use Committee guidelines of Kyung Hee University.

2.3. Histological analysis

Dissected gastrocnemius muscle was fixed in 4% paraformaldehyde and sliced into 5 μm sections. The sections were stained with hematoxylin and eosin (H&E) for 13 hours, and images for cross-sectional area (CSA) were captured using an optical microscope (Olympus, Tokyo, Japan). CSA of myofibers was quantified using Image J software (National Institute of Health, Bethesda, MD, USA).

2.4. Western blot analysis

Extracted muscle tissues were homogenized in liquid nitrogen and lysed using a lysis buffer containing Complete™ Protease Inhibitor Cocktail and PhosSTOP™ (Roche Diagnostics, IN, USA) followed by centrifugation for 15 min at 13,000g at 4°C. The protein concentration was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Each group's same

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**Fig. 1.** Animal experiment design and sample administration groups.
amount of protein was subjected to SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. Next, the membranes were blocked by 5% skim milk for 1 hour and incubated with a specific primary antibody at 4°C overnight. Information on primary antibodies is listed in Table 1. The next day, the membranes were washed by washing buffer and incubated with the corresponding secondary antibodies. The protein bands were visualized by a LAS3000 luminescent image analyzer (Fuji Film, Tokyo, Japan) using the enhanced chemiluminescent (ECL) detection reagent (Thermo Fisher Scientific, Rockford, IL, USA). The visualized protein bands were normalized to the β-actin and quantified using the Image J software (National Institute of Health, MD, USA).

2.5. Real time-PCR (qRT-PCR) analysis

Muscle tissues were homogenized in liquid nitrogen, and total RNA was extracted using an easy-RED™ (iNTRON, Seongnam, Korea), and it was reverse transcribed into cDNA using a PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. qRT-PCR was performed with TB Green™ Premix Ex Taq™ (TaKaRa, Tokyo, Japan) using a Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for RT-PCR: Atrogin-1 forward (5'-AGAAAAAAAGAAGATCAACAACA-3') and reverse (5'-GCTCCCTGTACTTCCTT-3'), Murf1 forward (5'-AGAAGTCAGCTGAGTAACGT-3') and reverse (5'-TAGAGGCTGTCAAACTCTG-3'), Bnip3 forward (5'-TTCCACTACCTCTCTGATGA-3') and reverse (5'-GAAACCGGATTACAGAAAAC-3'), β-actin forward (5'-ATATGCTGGCTGTCTGGTC-3') and reverse (5'-AGATGGCGCTAGGGAGACG-3'). The gene expression levels were normalized to β-actin, and the relative expression level of the target gene was calculated using the 2-ΔΔCT method.

2.6. Statistics

Data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using a one-way ANOVA and Tukey’s test as post hoc analysis. Statistical significance was determined using SPSS version 25 statistical software (Chicago, IL, USA), and was represented as follows: * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to the Normal group. * p < 0.05, ** p < 0.01, and *** p < 0.001 compared to the IM group.

3. Results and discussion

3.1. Synergetic effect of GBE and WPH on muscle atrophy in IM-induced muscle atrophy mice

Mice were subjected to IM to induce muscle atrophy for one week prior to two weeks of sample administration. After sacrifice, we harvested and weighed quadriceps, gastrocnemius, and soleus muscle tissues.

Skeletal muscle is classified as its fiber types; fast-twitch fiber (quadriceps and gastrocnemius) and slow-twitch fiber (soleus). Fast-twitch fiber muscles use anaerobic respiration, and slow-twitch fiber muscles use aerobic respiration, which has a higher concentration of mitochondria and myoglobin than fast-twitch fiber [23]. As a result of muscle mass, two fast-twitch fiber muscles were decreased about 28% each, and slow-twitch fiber muscle was decreased about 35% in the IM group compared to the Normal group (Fig. 2A–C). Consistent with the report that IM changes the characteristic of slow-twitch fiber to fast-twitch fiber [24], the decreasing rate of soleus was higher than quadriceps and gastrocnemius. When comparing muscle mass in the sample administration groups, the WPH800+GBE100 group showed the greatest increase in all three muscles. In fast-twitch fiber muscles, GBE100, WPH800, and SFE200 groups showed similar effects (Fig. 2A–B), but in slow-twitch fiber muscle, the GBE100 group was the second most effective after the WPH800+GBE100 group (Fig. 2C). The more pronounced effect of GBE100 in slow-twitch muscle is consistent with the results of the paper that syringaresinol, one of the constituents of GBE, increases mitochondrial biogenesis [25]. Also, in quadriceps and soleus, the WPH800+GBE100 group had a higher increase rate than the combined increase rate for each group, confirming the synergetic effect of the mixture. The CSA result of gastrocnemius decreased by about 50% in the IM group compared to the Normal group, and the WPH800+GBE100 group recovered the most, identical to the muscle mass results (Fig. 2D). In the CSA distribution chart, the IM groups leaned to the left, the Normal group is widely distributed to the right, and the sample administration groups are concentrated between 1000 μM² and 1500 μM² (Fig. 2E). Among them, the WPH800+GBE100 group is located closer to the Normal group than the WPH or GBE administration group, suggesting that the WPH+GBE mixture is more effective in increasing the size of the muscle fibers.

3.2. Synergetic effect of GBE and WPH on muscle protein synthetic and proteolytic mechanisms

Muscle mass is maintained by a balance between muscle protein synthesis and degradation, and it is controlled by the PI3K/Akt pathway [6]. We measured protein expression of MuRF1, Atrogin-1, S6K1, and 4E-BP1, which are sub-factors of the PI3K/Akt pathway (Fig. 3A–C). In terms of protein synthesis, the ratio of phosphorylation to the total form of S6K1 and 4E-BP1 was increased in the order of WPH800+GBE100 > GBE100 > WPH800 > SFE200. All sample administration groups showed a statistically significant increase, but the most increased in the WPH800+GBE100 group, showing 23% and 31% increase in p-S6K1/t-S6K1 and p-4E-BP1/t-4E-BP1, respectively (Fig. 3A). Since the GBE100 group accounts for

| Antibody     | Host animal | Company             |
|--------------|-------------|---------------------|
| p-P38K (Thr485/199), Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), FoxO3a, p-FoxO3a (Ser253), S6K1, p-S6K1, (Thr389) 4E-BP1, Rabbit | Cell Signalling (MA, USA) |
| p-4E-BP1 (Thr37/46), Bnip3 | Rabbit | Abcam (Cambridge, UK) |
| Atrogin-1, MuRF1 | Mouse | Santa Cruz Biotechnology (CA, USA) |
| p62 | Rabbit | Sigma-Aldrich (St Louis, USA) |
| B-actin | Mouse | GeneTex (CA, USA) |
most of the increase rate of the WPH800+GBE100 group, it is thought that GBE has more effect on the protein synthesis side. In protein degradation, the expression of MuRF1 and Atrogin-1 was significantly increased in the IM group by about 32% and was lowest in the WPH800+GBE100 group. In MuRF1 expression, WPH800 showed a similar decrease rate to the WPH800+GBE100 group, and in Atrogin-1 expression, three groups excepting for the WPH800+GBE100 group showed a similar decrease rate of about 11% (Fig. 3B). Therefore, WPH is judged to be slightly more effective in terms of protein degradation. To sum up, GBE and WPH affect muscle protein synthesis and degradation respectively, resulting in synergetic effects in WPH and GBE mixture.

3.3. Comparison of muscle atrophy effect according to the mixing ratio of GBE and WPH in IM-induced muscle atrophy mice

To find the optimal mixing ratio of GBE and WPH, we compared the effects of the three mixing ratios (WPH800+GBE100, WPH800+GBE90, WPH1000+GBE75). We measured grip strength and muscle mass because muscle atrophy means a decrease in muscle strength and mass [26]. Grip strength was significantly decreased in the IM group compared to the Normal group and significantly increased in the sample administration groups 23%, 20%, and 16% in the order of WPH800+GBE100 > WPH800+GBE90 > WPH1000+GBE75 (Fig. 4A). Quadriceps, a fast-twitch fiber muscle, increased significantly by about 21% in the sample administration groups, and there was no significant difference between groups (Fig. 4B). Another fast-twitch fiber muscle, gastrocnemius, increased GBE dose-dependently, showing the highest increase rate at the WPH800+GBE100 group (Fig. 4C).
Slow-twitch fiber muscle, soleus, was reduced 42% and increased 28% in the WPH800+GBE100 and WPH800+GBE90 groups, 21% in the WPH1000+GBE75 group (Fig. 4D). To sum up the results, the mixture of WPH and GBE significantly increased both muscle strength and muscle mass, and the WPH1000+GBE75 group, which has the highest WPH content, showed a lower increase rate compared to other groups, indicating that GBE plays a crucial role in the synergetic effect of WPH and GBE mixture. Also, CSA of gastrocnemius was enhanced GBE dose-dependently. In the CSA distribution chart, WPH800+GBE90 and WPH1000+GBE75 group showed the 2000~2500 μM² intensive distribution, but WPH800+GBE100 exhibited a wide distribution skewed to the right, showing the tendency most similar to the normal graph (Fig. 4E–G).

3.4. Comparison of protein synthetic and proteolytic mechanisms according to GBE and WPH mixing ratio

In the PI3K/Akt signaling pathway, Akt phosphorylated by PI3K activates mTORC1, the upper factor of S6K1 and 4E-BP1, and inactivates FoxO3a, the upper factor of MuRF1 and Atrogin-1. For muscle protein synthesis, the ratio of phosphorylation to the total form of muscle protein synthesis-related factors was significantly decreased in the IM group and increased in the WPH and GBE administration groups (Fig. 5A). Among three WPH+GBE mixture groups, the WPH800+GBE100 group showed a statistically significant increase in all synthesis-related factors, and the rest of the groups showed the same or lower expression as the WPH800+GBE100 group. In particular, the expression of p-S6K1/t-S6K1 and p-4E-BP1/t-4E-BP1 increased by 25% and 36% in the WPH800+GBE100 respectively, showing a similar increase rate to that of the first experiment.

In terms of expression of protein degradation factor, the expression of p-FoxO3a/t-FoxO3a significantly increased by about 32%, 27% and 11% in the order of WPH800+GBE100, WPH800+GBE90, WPH1000+GBE75, and accordingly, the protein expression of FoxO3a sub-factors, MuRF1, Atrogin1 and Bnip3 were decreased (Fig. 5B). As confirmed in the first experiment, the expressions of Atrogin-1 and MuRF1 of UPS decreased in the WPH+GBE administration groups, and only the WPH800+GBE100 group showed a significant decrease in both protein and mRNA expression (Fig. 5B–C). In addition, the mixture of WPH and GBE was also effective on Bnip3 and p62 of ALS. The protein expression of p62 and Bnip3 decreased significantly in the WPH+GBE100 and WPH800+GBE90 in common (Fig. 5B), mRNA expression of Bnip3 also showed a tendency to decrease, and it was most effective in the WPH800+GBE100 group in the same way as the protein expression (Fig. 5C). Two major proteolytic systems, UPS (ubiquitin-proteasome system) and ALS (autophagy-lysosome system) are closely interlinked to each other to connect with either the proteasome or autophagy [9]. For example, specific ubiquitin linkage is considered as a signal for autophagy degradation [27], so UPS and ALS work compensatory. In this study, we confirmed that a mixture of WPH and GBE effectively inhibited muscle proteolysis, as we verified reductions in both UPS and ALS.

4. Conclusion

This study aimed to determine whether WPH and GBE have a synergetic effect and identify the relevant mechanisms and optimal
mixing ratio. In the first experiment, we confirmed the synergetic effect of WPH and GBE. The mass of skeletal muscle tissues significantly increased the most in the WPH800+GBE100 mixture group compared to the IM group. Also, we found that each WPH and GBE had a greater impact on the degradation side and synthetic side, respectively. In the second experiment, we compared three different doses of WPH and GBE mixture to find out the optimal mixing ratio and confirmed relevant mechanisms in more detail. The WPH and GBE mixture activated muscle protein synthesis via PI3K/Akt/mTORc1 pathway and inhibited muscle protein degradation via suppressing UPS and ALS. This resulted in an increase in the total amount of muscle protein, leading to an increase in muscle mass and strength. Among three different ratios of WPH and GBE groups, the WPH800+GBE100 ratio was most effective for anti-muscular atrophy and related mechanisms, and these effects appear to be more GBE dose-dependent than WPH.

**Declaration of competing interest**

The authors declare no conflict of interest.
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