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Effect Of Insect Protein On Sarcopenia Through Modeling Of Drosophila Melanogaster

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Abstract:
Background*: This study aims to demonstrate the potential of insect protein by identifying insects with high protein content and investigating their effects on muscles using a sarcopenia model of Drosophila. The study examined Drosophila movement and gene expression after insect consumption.*
Materials and Methods*: The analysis of five insect species and chicken protein showed that silkworms have the highest total protein content. Insect protein-based diets were fed to Drosophila for 28 days to develop a sarcopenia model. A comparison of the movement between 3rd instar larvae and 28-day adults revealed that larvae consuming mealworm protein showed longer movement distances and were more active. In adults, consumption of silkworm, cricket, and beetle larvae resulted in larger activity ranges and increased movements. The examination of gene expression related to muscle maintenance in 28-day-old adults showed that Projectin and Titin were expressed after consuming silkworm, cricket, or mealworm protein.*
Results*: These findings demonstrate that the intake of insects with high protein or leucine content can contribute to muscle maintenance. The extent of the effect of different insect consumption varies depending on the growth stages of Drosophila.*
Conclusion: *The findings suggest the potential of insects as an alternative protein source that could be directed at certain age groups of humans.*
Key Word: *Insect protein; Leucine; Sarcopenia; Drosophila melanogester; Ctrax program.*

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I. Introduction

With increased interest in health following the COVID-19 pandemic and people acknowledging protein consumption as an essential nutrient, the global protein market has been experiencing significant growth. By 2033, it is expected to reach $53 billion, representing a 128% increase in market value compared to the previous year[1]. Along with this trend, the edible insect market, appealing to high protein content, has steadily gained attention. Over the past five years, the global edible insect market has grown by an impressive 135%, highlighting the value of insects, which are becoming increasingly commercialized. By 2026, the market is projected to reach $1.5 billion. However, while most people consider beef, pork, and chicken as their primary protein sources, the sustainability of these protein sources is often overlooked.

The traditional methods of meat production lead to serious environmental problems. Greenhouse gas emissions from livestock farming are significantly higher than those from edible insect farming. Per kilogram, cattle emit 35.0 kg, pigs emit 6.95 kg, and chickens emit 5.97 kg of CO_2-equivalent greenhouse gases. In contrast, insects emit only 0.3–3 kg of CO_2-equivalent greenhouse gases per kilogram, making them an environmentally friendly option[2]. Additionally, insect farming is highly efficient. Most edible insects have a short breeding cycle of just 2–4 months, require minimal feed, and can be raised in confined spaces[3]. These advantages make edible insects a viable solution for addressing protein shortages, which is gradually increasing interest in them. In South Korea, the Ministry of Agriculture, Food, and Rural Affairs has been implementing the 3rd Comprehensive Plan for the Development of the Insect and Sericulture Industry, driving consistent growth in the edible insect market through 2025[4].

In South Korea, edible insects are processed and sold in forms such as powders, live insects, and dried products. High-protein foods such as snacks and energy bars made from pupae and silkworms, as well as healthy functional foods containing functional ingredients, are being produced[5]. Most of these products are targeted for use as specialized food ingredients, such as hangover cures and energy supplements[6].

Internationally, in the United States, cricket and mealworm-based hamburger patties and alternative meat products like ham have been produced. Leading U.S. companies, such as Chapul and EXO, have developed energy bars based on technology for extracting Textured Insect Protein (TIP) from insect proteins. In Europe, Proti-Farm is developing products by exploring the breeding methods and food applications of the phoenix worm, an insect

not commonly recognized as edible. Hipromine is manufacturing various products, including feed, protein, and oil, by isolating and refining insect proteins[7,8]. The utilization of insects as a protein source is increasing internationally.

Sarcopenia is a disease posing symptoms of a decline in muscle mass, strength, and physical performance, typically caused by ageing. The most basic approach to preventing sarcopenia is adequate protein intake. Abundant in muscle proteins, Branched-chain amino acids (BCAAs) including valine, leucine, and isoleucine, are important in stimulating muscle growth in the body. Among these three BCAAs, leucine is the most effective in promoting muscle protein synthesis. However, since leucine cannot be naturally synthesized in the body, it must be consumed through food[9]. Therefore, this study aims to identify proteins with high leucine content among insect proteins and demonstrate the applicability of insect proteins. The research involves feeding insect protein to induce sarcopenia in ageing Drosophila melanogaster (fruit flies) and analyzing their movements and genes related to muscles. Through this analysis, the study seeks to verify the effects of insect protein on muscle maintenance and propose its practical applications.

II. Material And Methods

Materials

Animals

The wild-type *Drosophila melanogaster* was used as a model for sarcopenia. Various edible insect species served as protein sources, including dried larvae of *Protaetia brevitarsis* (beetle larvae), *Tenebrio molitor* (mealworm), *Oxya chinensis* (rice grasshopper), *Gryllus bimaculatus* (cricket), and *Bombyx mori* (silkworm). Additionally, powdered chicken breast was used.

Chemicals

Leucine content was measured using the Branched Chain Amino Acid Assay Kit (MET-5056, CellBiolabs). PCR was conducted using an RNA extraction kit (AccuPrep® Universal RNA Extraction Kit, Bioneer) and the One-step Real Time PCR kit (TOPscript RT-PCR Kit, Enzynomics).

Protein isolation

20 g of each of the five selected insect species as protein sources were ground into powder using a mortar and pestle. For each insect sample, including freeze-dried chicken breast, 15 g of the powdered sample was mixed with 60 mL of 2M NaOH solution and dissolved by stirring for one hour. Afterward, 60 mL of distilled water was added, and the solution was further dissolved for 30 minutes. The solution was then filtered through a 100-mesh sieve to remove impurities, and 0.1 M HCl was added to adjust the pH to 7. The mixture was centrifuged at room temperature using a centrifuge (Centrifuge 5415D, Eppendorf), and the supernatant was collected. The extracted supernatant was stored frozen and used as a sample.

Warburg-Christian protein quantification

The protein content of the samples was calculated using the Warburg-Christian method. Ten-fold dilution of samples were made using distilled water and absorbance was recorded at wavelengths of 260 nm and 280 nm using a spectrophotometer (model 7305, JENWAY). Total protein was determined from the absorbance values and the dilution factor using the equation below:

$$\text{mgprotein/mL} = [(1.31*A280)-(0.57*A260)]*\text{dilution factor}$$

Leucine content measurement

Leucine content in each sample was measured using the Branched Chain Amino Acid Assay Kit. The frozen supernatant obtained during protein isolation was melted and transferred into 1 mL tubes. Each sample was centrifuged at 10,000 rpm for 5 minutes. 50 μL of the supernatant was diluted tenfold using 1X phosphate-buffered saline (PBS) and then placed into 24-well plates. To each well, 50 μL of Reaction Mix and 50 μL of 1X Leucine Dehydrogenase Solution were added. The solutions and samples were mixed at room temperature using an orbital shaker. The absorbance of each sample was measured using a spectrophotometer at 450 nm. L-leucine content was determined by using a standard curve prepared with L-leucine Standards, plotting the absorbance against leucine concentration (μM). All measurements were done 3 times and the average was determined.

Preparation of Drosophila media

To evaluate the effects of each insect protein, media containing four types of insect proteins were prepared for the Drosophila (fruit fly) diet. The rice grasshopper, which has a lower leucine protein content compared to other proteins, was excluded during the media preparation process.

The media of the control group was made with mix of corn meal 16 g, glucose 4 g, sucrose 8 g, yeast

3g, agar 1.6g, propanoic acid 0.8 mL, Methyl 4-hydroxybenzonate 1g / 5mL, with 200 mL of distilled water. For the other groups, 2 g of powdered insect was added to the same mixture as the control group.

Sarcopenia modeling

For the control group, no insect protein was added, and three media samples each containing four types of insect proteins (beetle larvae, crickets, silkworms, and mealworms) were prepared. To move the adult flies during transfers, they were temporarily immobilized by being placed at -20˚C for 3 minutes. Two female and two male *Drosophila melanogaster* adults were introduced into each medium for mating. After five days, all adult flies were removed. At the stage when larvae developed into adults, all adult flies were removed again to prevent further mating. Subsequently, five male adults from each medium were separated into new media of the same protein source. This process was repeated at 2-week and 4-week intervals to obtain 5 male adults at three growth stages (1, 2, and 4 weeks), which were used as sarcopenia models.

Locomotion of Drosophila melanogaster

The locomotion ability of *Drosophila* larvae was determined using 3rd instar larvae. A starting line was marked on a square Petri dish, and the starting point of each larva, was indicated with a dot. The larvae were then placed at the starting point, and their final positions were recorded after 5 minutes. The displacement between the initial and final positions was measured. The experiment was repeated five times, and the average displacement was calculated.

For adults, five flies from each growth stage (1, 2, and 4 weeks) were collected and placed in a 60 mm Petri dish. Their movement was recorded for 5 minutes and analyzed using the Ctrax software.

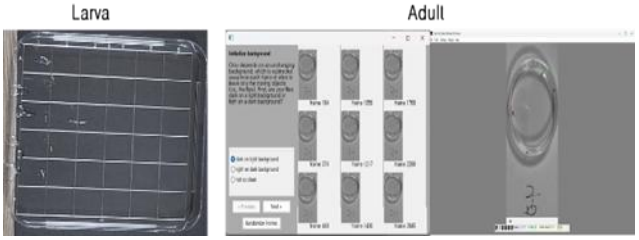

Figure 1. Analysis of larval movements and adult behaviors using the Ctrax program

RNA extraction of Drosophila melanogaster

From each medium, 20 larvae were collected, and for adults, five males from each growth stage were sampled. RNA was extracted using the AccuPrep® Universal RNA Extraction Kit (Bioneer). The collected samples were ground inside 1.5 mL tubes with the addition of 500 μL of distilled water using a pestle. Subsequently, 500 μL of RB Buffer was added, and the mixture was vortexed. The supernatant was transferred to a new 1.5 mL tube following centrifugation, and 200 μL of absolute ethanol was added. The RNA extraction proceeded according to the kit's 'Purification' and 'RNA Clean-Up' rocedure. The sample was transferred to an AccuPrep® Binding Column and centrifuged at maximum speed for 20 seconds. The solution passed through the column was collected and was discarded. 0.7 mL of RWA1 Buffer was added too the column and centrifusged at maximum speed for 20 seconds. Then, the filtered solution was removed and 0.5 mL of RWA2 Buffer was added and centrifuged at maximum speed for 20 seconds. 0.5 ml of RWA2 Buffer was added and centrifuged at same speed for 1 minute to remove all remaining solution. To recover the RNA bound to the AccuPrep® Binding Column, the column was transferred to a new microtube and dried for 1 minute. Then, 0.05 mL of ER Buffer was added, and the sample was centrifuged at 10,000 rpm for 1 minute to isolate the RNA.

Creating PCR primer

To confirm the expression levels of the Titin, Projectin, and Lim1 genes, primers were designed using PrimerBLAST (https://www. ncbi.nlm.nih.gov/tools/primer-blast).

Table. 1 Primer for genes.

Gene	Forward primer	Reverse primer
Glycerol 3 phosphate dehydrogenase	TCGGACTGCGTAGACACTAGA	AGCGCCATCTATGTAAGGATGT
Lim1	CAAAGCATGAGCACCGATAGC	AGTCCGAACTCGTTCACCAT
Projectin	ACACCGAAAGTCGAGGACAC	ATTTGAATTGGCCGGCATCG
Titin	AGCTGTCCAATGTCTGGCTC	CGACGCTGCTCGTAATTTGG

PCR procedure and electrophoresis

PCR cDNA synthesis and PCR amplification for the genes were conducted using the TOPscript RT-PCR Kit (RT410S).

5 μL of TOPscript One-step RT PCR Kit, 3 μL of template (Total RNA), 1 μL of primer, and 10 μL of sterile water, to make a total volume of 20 μL, was prepared for the PCR reaction on ice. PCR amplification was carried out using the MJ Mini 48-Well Personal Thermal Cycler (Bio-Rad) under the conditions shown in Table 2.

Table 2 PCR condition.

Step	Temperature (˚C)	Time
Reverse transciption	50˚C	30 min
Initial denaturation	95˚C	10 min
Denaturation	95˚C	30 sec
Annealing	57-62˚C	40 sec
Elongation	72˚C	1 min
Number of cycles	40 cycle (Denaturation → Anneling → Elongation)	
Final elongation	72˚C	5 min

For electrophoresis, TBE buffer was prepared. To make the 5X TBE buffer, 5.4 g of Tris base, 2.75 g of boric acid, and 20 mL of 0.5M EDTA (pH 8) in 80 mL of distilled water was mixed. Then, 50 mL of 5X TBE buffer was diluted with 450 mL of distilled water to create a 0.5X TBE buffer solution.

To prepare the agarose gel for electrophoresis, 1 g of agarose was added to 100 mL of 0.5X TBE buffer solution in a conical flask. The flask's opening was wrapped with plastic film and heated in a microwave for approximately 5 minutes until fully dissolved. The solution was allowed to cool to room temperature before being poured into a gel tray to solidify. The completed agarose gel was immersed in a solution containing a fluorescent dye for staining.

The electrophoresis apparatus was filled with 0.5X TBE buffer, and the solidified agarose gel was carefully placed into the apparatus, ensuring that the wells faced the negative (-) electrode. Approximately 3 μL of Agarose Gel Loading Buffer (6X) was mixed with the PCR product. A DNA size marker (100 bp) (2 μL) and PCR product (7 μL) were loaded into the wells of the agarose gel. The electrophoresis device was powered at 100 W for 30 minutes. Afterward, the agarose gel was removed and analyzed using a UV visualizer. Photographs of the results were taken. The gel density of the PCR product bands was analyzed using ImageJ software. The relationship was calculated based on band density.

III. Experimental Results

Total protein count

Diets rich in protein are essential in increasing muscle mass and for muscle growth. Protein consumption is known to be able to prevent muscle loss due to aging[10]. Consequently, consumption of proteins provides essential amino acids that induces protein synthesis. Therefore, the purpose of this investigation was to quantify the amount of protein in each insect.

The analysis of protein content showed that all five insects yielded protein concentration more than 10 mg protein/mL. However, the protein content from chicken breast was recorded in an average of less than 8 mg protein/mL; at least 2 mg protein/mL lower than insect's proteins. Bombyx mori larvae (silkworm) contained the highest protein content above 13 mg protein/mL in total protein contents; Protaetia brevitarsis larvae (beetle larvae) also had more than 12 mg protein/mL. Thus, it was confirmed that insect proteins have a higher total protein content compared to livestock proteins such as chicken.

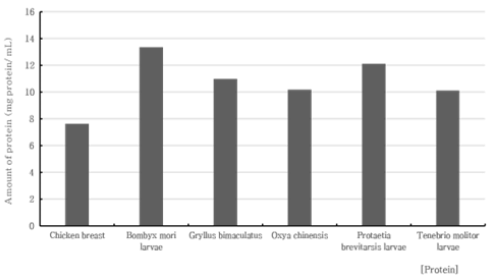

Figure 2. Total protein content analyzed using Warburg -Christian method

Measurement of Leucine content

Previous studies have reported that branched-chain amino acids (BCAAs) are effective in preventing muscle protein breakdown during rest or recovery after exercise, thereby suppressing muscle loss. In this study, the content of BCAAs within proteins was analyzed by measuring L-leucine, L-valine, and L-isoleucine. The measurement was conducted using a colorimetric method, where NADH generated from the reaction of leucine dehydrogenase in the presence of NAD^+ was quantified. The L-leucine content of each sample was determined using a standard curve of the absorbance values of L-leucine standards of known concentrations.

Among the five tested insect species, it was found that L-leucine was present in any three species. Gryllus bimaculatus (cricket) contained a noticeably high L-leucine level, more than 2000 μM. Tenebrio molitor larvae (mealworm) contained less than 500 μM while Protaetia brevitarsis larvae (beetle larvae) contained more than 500 μM. However in Bombyx mori larvae (silkworm), Oxya chinensis (rice grasshopper) and chicken breast, valine, leucine and isoleucine were almost absent. Therefore, it was confirmed that proteins containing leucine as part of the BCAAs were present in crickets, beetle larvae, and mealworms.

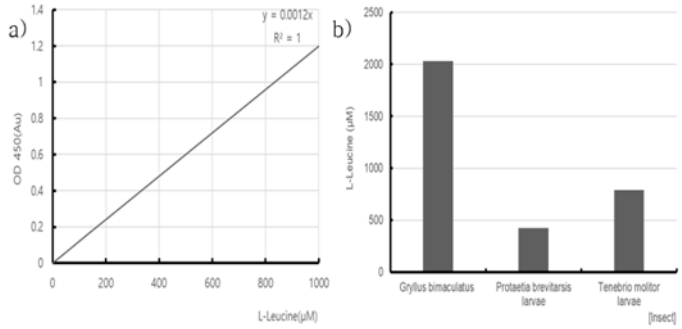

Figure 3. Measurement of Leucine content
Standard curve based on Leucine concentration
Determination of Leucine content in Insect Proteins

Locomotion of Drosophila melanogaster larvae

To investigate the effects of insect proteins on muscle formation and maintenance, media containing cricket, beetle larvae, silkworm, and mealworm proteins were prepared. These were fed to fruit flies as they progressed through the egg, larval, and adult stages. The extent of muscle development in third-instar larvae was assessed by measuring the distance travelled within a square petri dish over 5 minutes.

Muscle development was measured by distance travelled, as greater movement was considered indicative of better muscle development and higher activity levels. Larvae fed Tenebrio molitor (mealworm) proteins exhibited the highest mobility, followed by those fed Protaetia brevitarsis larvae (white-spotted flower chafer). However, larvae fed Bombyx mori larvae (silkworm) proteins travelled approximately half the distance of the control group, making them the only group with less activity than the control.

Larvae fed mealworm proteins travelled more than 2.2 times the distance of the control group, suggesting that mealworm protein consumption promotes muscle development during the larval stage.

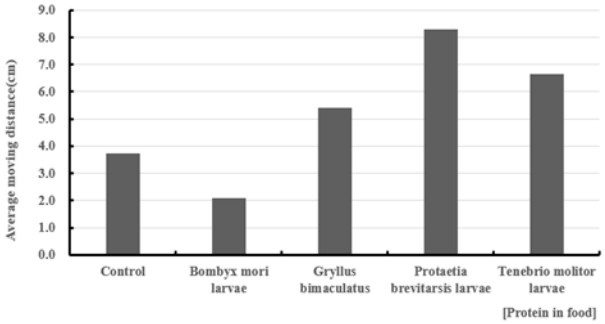

Figure 4. Average distance traveled by third Instar larvae over 5 minutes

Locomotion of Drosophila melanogaster adults

To evaluate the effects of insect proteins in a sarcopenia model, adult fruit flies that consumed insect proteins and underwent 28 days of aging were analyzed. Their movements were recorded for 5 minutes, and the videos were analyzed using the Ctrax program. Location preferences were visualized in red, orange, yellow, and white, with white indicating areas where the flies remained for longer periods.

In the control group, only white and yellow zones were observed, indicating that the flies tended to remain stationary in a single location. In contrast, analysis of the movement of flies fed insect proteins revealed a dominance of red zones, though white zones were also present. Flies fed Bombyx mori larvae (silkworm) proteins showed orange and yellow zones, while flies fed Gryllus bimaculatus (cricket), Protaetia brevitarsis larvae (beetle larvae), and Tenebrio molitor larvae (mealworm) proteins exhibited white and red zones. This indicates that flies consuming insect proteins were more active compared to the control group.

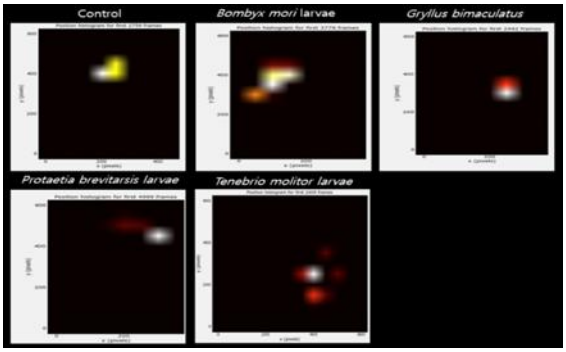

Figure 5. Location distribution of 28-day adult flies analyzed using the Ctrax program

Comparison of gene expression at different growth stages of Drosophila melanogaster

To examine the effects of insect protein consumption on muscle growth and maintenance, gene expression analysis was conducted. RNA was extracted from 3rd instar larvae and 7-day and 28-day adult fruit flies fed insect proteins, and PCR was performed to analyze the expression of the genes Lim1, Projectin, and Titin.

The Lim1 gene plays a role in the early muscle development of fruit flies, particularly influencing embryonic muscle formation. It also contributes to the regeneration of epidermal, visceral, and pharyngeal muscles[11]. In Drosophila melanogaster, the Titin family comprises three genes: Projectin, Kettin, and D-Titin. These genes facilitate the synthesis of the muscle fiber protein Titin, which is essential for elastic muscle movement. This experiment specifically analyzed the expression of Projectin and D-Titin. Projectin impacts muscle contraction and helps prevent muscle damage, while D-Titinis involved in forming multinucleate syncytia, arranging actin-myosin filaments and maintaining the structural stability of muscles[12].

In the 3rd instar larvae, Lim1 expression was observed only in groups fed silkworm, cricket, and beetle larvae proteins. Projectin and Titin were expressed across all groups, including the control, but larvae fed mealworm proteins showed significantly higher expression levels of over 10 times greater than the other groups. This finding confirms that mealworm protein consumption may influence muscle development during the larval stage.

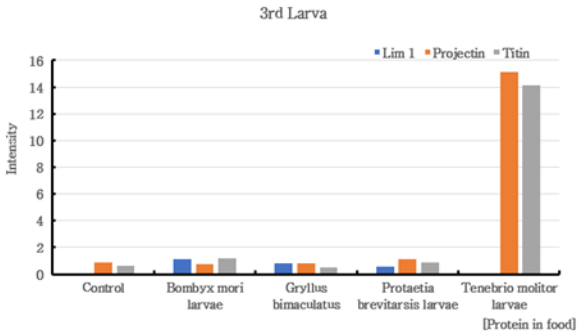

Figure 6. Expression of Lim1, Projectin, and Titin in 3rd Instar larvae

In 7-day adult flies, Lim1 expression was observed regardless of insect protein consumption, but higher expression levels were found in flies that consumed insect proteins. Specifically, flies fed silkworm protein showed approximately 5 times higher gene expression than other groups. Projectin was also expressed across all groups, similar to Lim1, but flies fed insect proteins exhibited higher expression levels, with the following ranking: silkworm > beetle larvae > cricket. Silkworm protein led to the highest gene expression in 7-day adults. For Titin, expression was observed in flies fed silkworm, beetle larvae, and mealworm with the highest expression levels in the following order: silkworm > beetle larvae > mealworm.

As a result, it was confirmed that in 7-day adults, consuming silkworm protein, which has the highest total protein content, promotes the expression of Lim1, Projectin, and Titin, contributing to muscle development, maintenance, and stability.

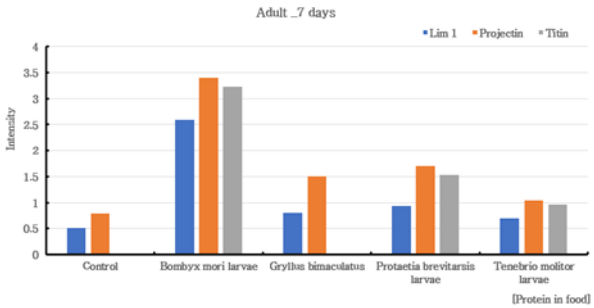

Figure 7. Expression of Lim1, Projectin, and Titin in 7-day adults

In this experiment, 28-day adult flies were used as a sarcopenia model. The average lifespan of fruit flies is around 60 days. However, due to the accelerated growth of fruit flies at temperatures above 30°C, 28-day adults were chosen as a sarcopenia model. Using RNA extracted from the adults, cDNA was synthesized to analyze the expression of Lim1, Projectin, and Titin.

For Lim1, no expression was detected in any group because it is a gene involved in early muscle development. Projectin, which plays a role in preventing muscle damage, was expressed in flies fed silkworm and cricket proteins. Titin, which influences muscle stability, was expressed in flies fed silkworm and mealworm proteins. This suggests that consuming insect proteins is advantageous for muscle maintenance. Silkworm protein demonstrated its benefits through its high total protein content, while cricket and mealworm proteins were distinguished by their leucine content, contributing to differences in gene expression and muscle stability.

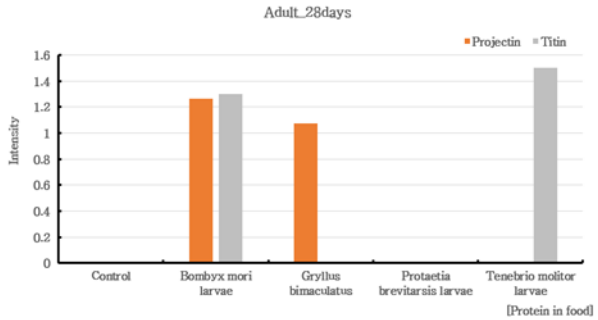

Figure 8. Expression of Lim1, Projectin, and Titin in 28-day adults

IV. Discussion

This study aimed to verify the applicability of edible insect proteins approved in South Korea—Bombyx mori larvae (silkworm), Gryllus bimaculatus (cricket), Oxya chinensis (rice grasshopper), Protaetia brevitarsis larvae (white-spotted flower chafer beetle), and Tenebrio molitor larvae (mealworm)—using a sarcopenia model in fruit flies. The proteins were selected from commercially available products with high consumer preference. The total protein content of these five insect proteins was compared with chicken protein, revealing that all insect proteins had higher protein content. Each insect protein contained more than 10 mg protein/mL, with silkworms exhibiting the highest content at 13.34 mg protein/mL. Leucine was detected in the proteins of crickets, beetle larvae, and mealworms, with crickets having an outstanding content of 2.44 μM, whereas leucine was not detected in chicken protein.

Next, to analyze the effect of consuming insect proteins rich in leucine on muscle enhancement, four proteins—those with high total protein content or containing leucine—were selected to be incorporated into diets. Sarcopenia was induced in Drosophila melanogaster (fruit flies) through various growth stages. Behavioral and gene expression analyses were conducted, with behaviors observed in third instar larvae and 28-day adult flies, and RNA extracted from third instar larvae, 7-day adults, and 28-day adults to compare gene expression. Behavioral analysis showed that third instar larvae fed cricket, beetle larvae, or mealworm proteins exhibited the most movement. Among 28-day adult flies, the control group and those fed mealworm proteins showed little movement, while those fed silkworm, cricket, or beetle larvae proteins displayed significant mobility.

Gene expression analysis revealed that the consumption of insect proteins promoted the expression of

the Lim1 gene in third instar larvae, while mealworm protein also enhanced the expression of Projectin and Titin. In 7-day adult flies, all insect proteins promoted the expression of Lim1, Projectin, and Titin, with silkworm protein showing particularly strong effects. In 28-day adults, a stage where sarcopenia was induced, Projectin and Titin gene expression was observed in groups fed silkworm, cricket, or mealworm proteins but not in the control group. This suggests that leucine-rich proteins such as those in mealworms, crickets, and beetle larvae positively impact muscle development at different stages. Additionally, the high total protein content of silkworms contributed to muscle development and maintenance throughout various stages. Mealworm consumption mainly influenced early muscle development, whereas cricket consumption had more significant effects in later adult stages.

Research into insect protein utilization is ongoing and this study linked insect proteins to diseases like sarcopenia, demonstrating their potential applications. The study scientifically proved that leucine-rich insect proteins suppress sarcopenia and promote muscle development, suggesting their viability as a sustainable protein source for the future. The findings also confirmed that different insect proteins exert varying physiological effects at different growth stages in the fruit fly model, laying a foundation for exploring their potential impacts on human muscle growth. Furthermore, the nutritional value of insect proteins may contribute to various applications, including health supplements and other fields.

This study investigated the effects of insect proteins, particularly leucine, on muscle enhancement, providing evidence of their applicability. The results showed that leucine-rich proteins in mealworms, crickets, and beetle larvae played crucial roles at different growth stages of fruit flies, positively impacting muscle development.

However, the study had limitations. While fruit flies are a suitable model for sarcopenia, physiological differences with humans necessitate further research. Additionally, the study was conducted in summer, creating a high-temperature environment during the breeding and sarcopenia induction phases. Since temperature significantly affects fruit fly growth, it is necessary to confirm whether similar results occur under different temperature conditions. Furthermore, consumer acceptance and allergenic reactions to insect proteins need further examination, particularly the potential immune responses akin to shellfish allergies. Regulatory standards addressing these concerns are essential before widespread adoption.

This study serves as foundational research demonstrating the potential value of leucine-rich insect proteins. Further systematic studies may expand their application across diverse fields.

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