The potential usefulness of the housefly larvae (*Musca domestica*) as materials for the development of multifunctional nutraceuticals

(多機能性食品の開発におけるイエバエ幼虫の素材としての潜在的有用性)

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Contents

Abstract

Many bioactive compounds have been studied for the prevention or treatment of various diseases. Oxidative stress has been confirmed to be related to many chronic diseases, including hypertension and diabetes mellitus. Hypertension is a major chronic adult disease affecting 40% population worldwide, and angiotensin-converting enzyme (ACE, EC 3.4.15.1) plays an important role in elevation in blood pressure. Type 2 diabetes mellitus (DM) is the most common form of diabetes, which account for approximately 90 to 95% of all the diagnosed cases, and dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) is involved in this symptom. Therefore, the development of materials which have antioxidant, ACE-inhibitory (antihypertension), and DPP-IV-inhibitory (antidiabetic) activities become important to the prevention or treatment of these chronic diseases.

Although many chemically synthesized antioxidants, ACE inhibitors, and DPP-IV inhibitors have been developed, it is still unclear whether these chemically synthesized compounds are sufficiently safe for long-term dosing or whether these compounds may have side effects. Therefore, bioactive compounds derived from natural sources are needed. Bioactive compounds are usually derived from plants, aquatic animals, and mammals. Although many types of insects have been used as foods and protein sources, there are few reports on biological activities of insects.

Insects are an abundant resource, that, though not well explored, is expected to be a solution to food security in the future. Among them, the housefly has a high reproductive ability and a short lifecycle; the larvae prefer organic wastes, such as garbage, animal manure, and can convert these wastes into value-added biomass rich in protein, chitin, and fat. Housefly larvae have been used clinically in traditional Chinese medicine for a long time, but little information is available on the bioactive compounds from housefly larvae. Although housefly larvae are considered high-quality protein resource, there are few reports that compare the amino acid composition of housefly larvae with other ideal protein sources. In recent years, several attempts have been reported on the treatment of livestock manure using bioconversion by housefly larvae, and these studies show the potential of housefly larvae for applications with economic and environmental benefits.

In this study, first, the utility of housefly larvae as a protein source was investigated. Second, in order to increase its applicability, the fraction containing protein was extracted and its multifunctional bioactivities were investigated.

In chapter 1, first, the expectation on insects as a natural source was expressed. Second, the housefly larvae, the larval bioconversion system, the utilities of housefly larvae, and the background and objectives of the current study were introduced.

In chapter 2, the utility of housefly larvae was evaluated by amino acid analysis. The housefly larvae contained sufficient amounts of all essential amino acids, and the amino acid composition was comparable to that of fishmeal, beef, chicken, pork, casein, and hen egg. These results showed the potential of housefly larvae as a good protein source.

In chapter 3, we prepared housefly larvae water extract (HLWE) using decoction method and explored the biological activities of the extract for the potential application of the extract as a functional food. HLWE showed significant antioxidant activity (75.4% at 5.00 mg/mL), ACE-inhibitory activity (half-maximal inhibitory concentration [IC_{50}] = 0.430 mg/mL), and DPP-IV-inhibitory activity ([IC_{50}] = 3.52 mg/mL). We found that the low-molecular-weight constituents (< 6 kDa) in HLWE contributed to antioxidant and ACE-inhibitory activities, whereas the high-molecular-weight constituents (> 6 kDa) contributed to DPP-IV inhibition. Our results suggested that housefly larvae may provide a useful source of multifunctional protein.

The results of this study clearly suggest the potential of housefly larvae as a protein resource for feed, food, and functional foods with multifunctional activities with antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activities. Moreover, this is the first time that multifunctional activities such as antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activity were clarified in insects. Our findings provide important insights into the biological activities and applications of housefly larvae.

Keywords: angiotensin-converting enzyme inhibitory activity; antioxidant activity; decoction method; dipeptidyl peptidase-IV-inhibitory activity; housefly larvae water extract; multifunctional activities; multifunctional nutraceuticals

Abbreviations

ACE	Angiotensin-converting enzyme
BCAAs	Branched-chain amino acids
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
BSA	Bovine serum albumin
DM	Diabetes mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase-IV
EAAs	Essential amino acids
FAO	Food and Agriculture Organization of the United Nations
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucose-like peptide-1
HLWE	Housefly larvae water extract
IC_{50}	Half-maximal inhibitory concentration
NEAAs	Nonessential amino acids
RAAS	Renin-angiotensin-aldosterone system
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WHO	World Health Organization
Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
His (H)	Histidine
Ile (I)	Isoleucine
Leu (L)	Leucine
Lys (K)	Lysine
Met (M)	Methionine
Phe (F)	Phenylalanine
Pro (P)	Proline
Ser (S)	Serine

Thr (T)	Threonine
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine

Chapter 1

Introduction

1.1 Background

1.1.1 Expectation on insects

Insects are the largest class within the animal kingdom concerning the species number, which are about one million named species, and account for 80% of all known animal species [1]. Insects can be found in all environments that seem to be very difficult for the living except deep sea and polar regions [1]. Insects do not have an antigen-antibody reaction mechanism as found in vertebrates, but instead, have acquired a strong innate immune mechanism [2]. Insects use this innate immune mechanism to protect themselves.

It is estimated that more than 1,900 species insects have been used as food, which insects are a part of the diets of at least 2 billion people [3]. The most commonly consumed number of insect species by order and the examples of those insects are presented in Fig. 1.1 and Table 1.1, respectively [4]. Insects deliver a host of ecological services and play an important role as pollinators in plant reproduction, in improving soil fertility through waste bioconversion [3]. They also play an important role in natural biocontrol for harmful pest species and provide a variety of valuable products for humans such as honey and silk and medical applications in traditional Chinese medicine. Moreover, insects are an abundant resource that has not been well-explored, which are expected to be a solution to food security in future.

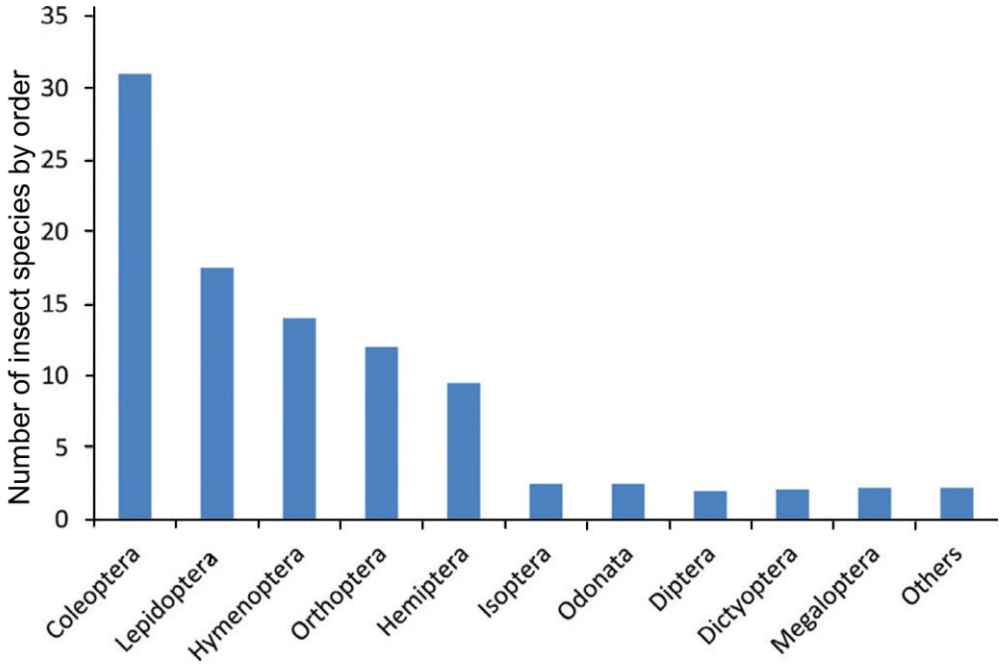

Fig. 1.1 Number of insects consumed worldwide [4].

Table 1.1 Examples of the most consumed edible insects in the world [4].

Order	Species	Location
Lepidoptera	*Anaphe panda* (Boisduval)	DRC, Zambia, Cameroon, Congo, CA Republic, Zimbabwe, Nigeria, Tanzania
	Gynanisa ata Strand	DRC, Zambia, South Africa, Botswana, Burkina Faso
	Anaphe venata Butler	Zambia, Nigeria, Ivory Coast, Sierra Leone, Guinea, Liberia, Guinea Bissau
Orthoptera	*Acanthacris ruficornis* (Fabricius)	DRC, Zambia, South Africa, Botswana, Burkina Faso, Nigeria, Mozambique, Namibia, Ghana, Togo, Chad
	Ruspolia differens (Serville)	DRC, Zambia, South Africa, Cameroon, Congo, CA Republic, Zimbabwe, Burkina Faso, Malawi, Mali
	Zonocerus variegatus (Linnaeus)	DRC, Zambia, South Africa, Cameroon, Zimbabwe, Kenya, Uganda, Tanzania, Malawi
Coleoptera	*Oryctes boas* (Fabricius)	DRC, Cameroon, Congo, CA Republic, Nigeria, Ivory Coast, Sao Tomé, Guinea, Ghana, Liberia
	Rhynchophorus phoenicis (Fabricius)	Thailand, Australia, Nigeria, Ivory Coast, Sierra Leone, Guinea, Liberia, Guinea Bissau DRC, Congo, Botswana
Hymenoptera	*Apis mellifera* (Linnaeus)	Mexico, Cameroon, Congo, CA Republic, Nigeria, Angola, Ivory Coast, Niger, Sao Tomé, Guinea
	Carebara vidua (Smith)	DRC, Zambia, South Africa, Zimbabwe, Botswana, Malawi, Sudan, Kenya, South Sudan
Isoptera	*Macrotermes subhyalinus* (Rambur)	Zambia, Angola, Kenya, Togo, Burundi, Ivory Coast, Canada, the USA
	Macrotermes falciger (Gerstäcker)	Zambia, Zimbabwe, Burkina Faso, Benin, Burundi, Australia, the Netherlands
	Macrotermes natalensis (Haviland)	DRC, Cameroon, Congo, CA Republic, Nigeria, Burundi, South Africa, Zimbabwe, Nigeria, Malawi

Recently, industrial-scale insect farming has been developed by several companies, for example, AgriProtein (South Africa) and EnviroFlight (United States) [3]. AgriProtein uses organic waste to produce protein that will help meet the increasing demand for animal feed. AgriProtein has developed and tested large-scale bioconversion system using housefly larvae. Various types of organic waste including human waste can be used in this system. The target of AgriProtein is a production of 100 tons of larvae per day. The first large factory would require an investment of 8 million dollars; AgriPprotein plan to deploy in Germany, South Africa, the United Kingdom of Great Britain and Northern Ireland, and the United States. EnviroFlight uses the by-product from breweries and ethanol production as a feedstock to rear black soldier fly larvae (*Hermetia illucens*); the fly larvae can be used as a protein source for livestock.

As the consumption of meat increases with the rise in global population, the novel innovation processes shown in Fig. 1.2 and these innovation processes are proposed to satisfy the growing demand for sufficient affordable and sustainable proteins [3]. Furthermore, as insect research progresses, it is believed that insects can make a big contribution to the realization of sustainable agriculture and the solution for the problem of food shortage.

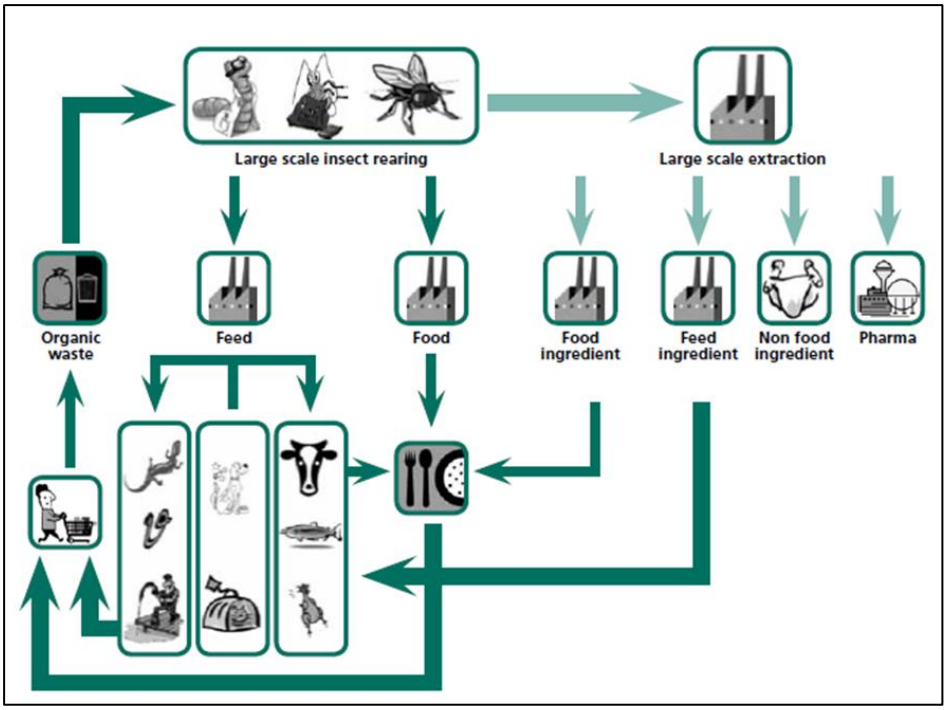

Fig. 1.2 Application of edible insects for designing a circular economy [3].

1.1.2 Housefly larvae (*Musca domestica*) and housefly larvae bioconversion system for livestock manure

1.1.2.1 Housefly larvae

The housefly is a kind of insect that belongs to the housefly family and can be found everywhere in human habitation; which is the most common fly with four growth stages: egg, larva, pupa, and adult (Fig. 1.3) [5]. The housefly has high reproductive ability fast growth, and short lifecycle. Development from egg to adult is fast and fecundity is high [6]. The larval stages typically consist of three stages as follows: (1) first instar is about 20 h-4 days, (2) second instar is about in 24 h to several days, (3) third instar is in 3-9 days. The pupal stage lasts an average of 5 days. The adults have the lifespan of 10-14 days and the females living longer than males. A female housefly can produce up to 1,000 eggs during its lifetime and laying 120-150 eggs per clutch. The larvae are fond of organic wastes, such as garbage, animal manure and can convert these wastes into value-added biomass rich in protein, chitin, and fat [7].

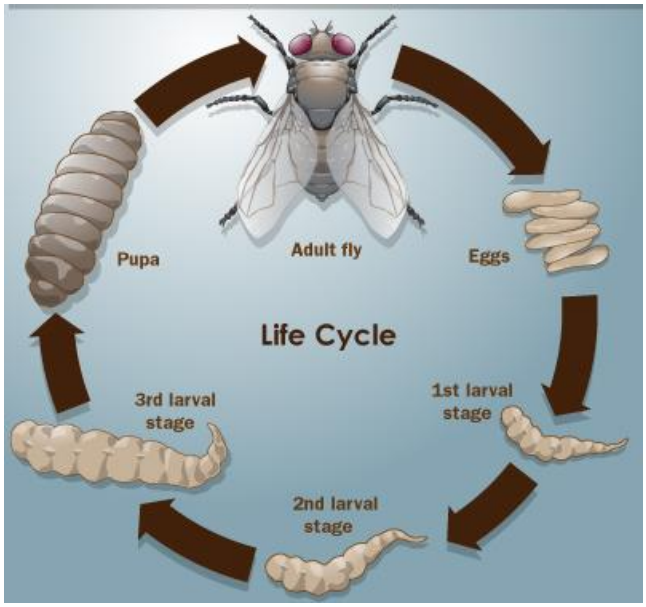

Fig. 1.3 Life cycle of housefly [8].

1.1.2.2 Housefly larvae bioconversion system

As the consumption of meat increases with the rise in global population, livestock waste becomes a serious problem. Livestock manure pollutes the environment in various ways, including air pollution, land degradation, physical impact, and surface and groundwater pollution. Generally, livestock waste can be treated using three methods, i.e., physical processes, chemical processes, and biological processes [9]. Physical processes are a way to treat manure or wastewater by physical forces, including separation of liquid-solids, heating and pressure treatment, drying, incineration, combustion, and gasification. Many chemical materials are needed for the manure-treatment system including many procedures in chemical processes. These conventional livestock manure treatment methods have many problems such as treatment cost, treatment time, instability of compost quality, and so on. Therefore, it is necessary to develop a new method for livestock manure treatment. Housefly larvae prefer spoilage such as livestock manure, food waste and have a short reproductive cycle.

Recently, livestock manure bioconversion system using housefly larvae was developed (Fig. 1.4). Housefly larvae were collected after eclosion approximately 20 h and then transported to larvae bioreactors for bioconversion. The larvae can well grow in bioreactors using raw manure, the bioconversion time differs from 5-7 days due to the season [7]. This system can harvest two products, i.e., housefly larvae and manure residue, which can be used to as animal feed, for research and development of chitosan and antibacterial peptide products, or as an organic fertilizer for agriculture [7].

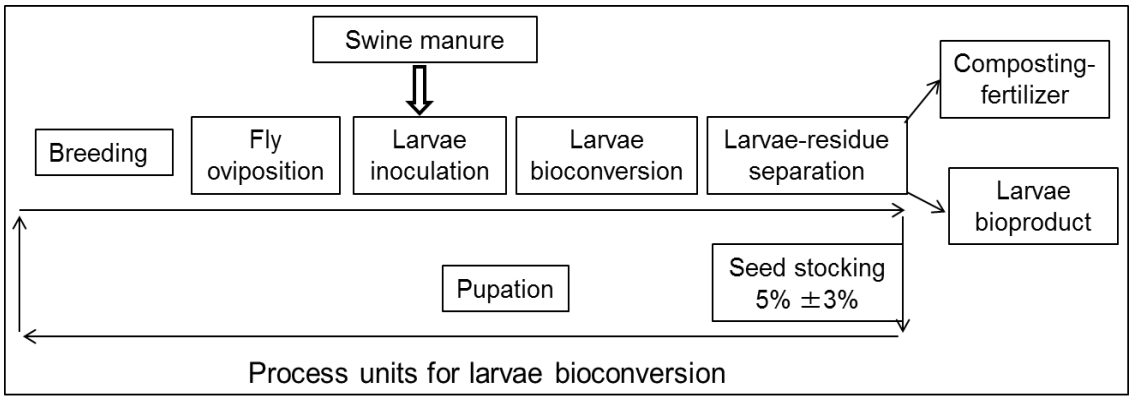

Fig. 1.4 livestock manure conversion system using housefly larvae [7].

In addition, recently, Niu *et al*. produced biodiesel using housefly larvae oil obtained from the bioconversion of food waste by housefly larvae (Fig. 1.5) [10]. It can add more value to waste as compared with conventional waste treatment methods [11]. Therefore, bioconversion of livestock manure or other various wastes by housefly larvae could benefit to applications with economic and environmental benefits.

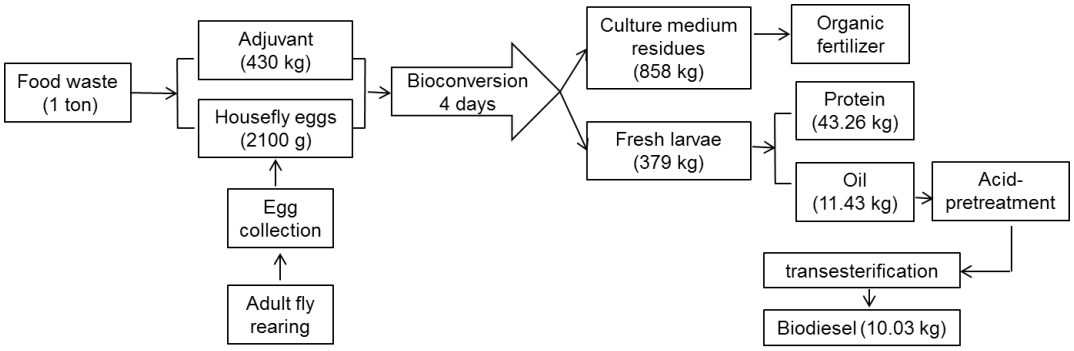

Fig. 1.5. Process and results of the bioconversion of food waste using housefly larvae.

1.1.3 Bioactive compounds derived from natural sources

Recently, researches for obtaining bioactive compounds from natural sources have attracted much attention, because bioactive compounds derived from natural sources are considered to be low toxicity, biodegradability, and renewable [12]. Many bioactive compounds have been isolated and identified from various natural resources including plants, animals, fungi, and microalgae [13]. However, we found only a few reports on bioactive compounds derived from insects.

Bioactive peptides are specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence specific biological activities such as oxidative stress and antihypertensive, and antidiabetic [14]. Bioactive peptides can be naturally occurred or generated by microbial fermentation and enzymatic hydrolysis. They usually have 2-20 amino acid residues, which can be absorbed by the intestine and be transported out intact in the circulatory system to exert physiological effects, or to produce local effects staying in the digestive tract [15]. The first food-derived bioactive peptide was reported by Mellander; he found that milk casein-derived peptides can enhance vitamin D-independent bone mineralization in rachitic infants [16].

1.1.3.1 Antioxidants

Generally, oxidative stress is defined as an excess formation and/or insufficient removal of highly reactive molecules [17]. Oxidative stress can arise when there is an imbalance between reactive oxygen species (ROS) and antioxidant mechanisms [18]. Moreover, oxidative stress has been confirmed to be related to many chronic diseases, such as hypertension, diabetes, cancer and other diseases [17, 19]. Several synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are added to food products to delay lipid peroxidation [20]. However, the synthetic antioxidants are associated with some safety concerns. Therefore, natural antioxidants have gained much attention due to their little side effects.

Recently, bioactive peptides or protein hydrolysates derived from various natural resources have shown potent antioxidant activities. Liu *et al*. identified many antioxidant peptides such as TDK, LDK, and others from the enzymatic hydrolysate of *Mactra veneriformis* by UHPLC-Q-TOF mass spectrometry [21]. Suetsuna *et al*. separated potent antioxidant peptide from the hydrolysate of wheat gluten; the amino acid sequences of these peptides were LQPGQGQQG and AQIPQQ [22]. Zhang *et al*. prepared sweet potato protein hydrolysates (SPPH) using alcalase and identified 5 antioxidant peptides from SPPH [23]. Umayaparvathi *et al*. isolated 7 antioxidant peptides from oyster (*Saccostreacucul-lata*) protein hydrolysate using protease from *B. Cereus* SU12 [24].

1.1.3.2 Angiotensin-converting enzyme inhibitors

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a zinc-dependent dipeptidyl carboxypeptidase and to remove the C-terminal dipeptide from its peptide substrates. ACE is a key component of the renin-angiotensin aldosterone system (RAAS), which plays an important role in regulating blood pressure [25]. ACE catalyzes angiotensin I to potent vasoconstrictor angiotensin II. Thus, ACE inhibitor will result in an overall decrease in blood pressure (Fig. 1.6).

ACE inhibitor is an effective strategy for hypertension treatment. The first ACE inhibitor was found in the venom of snake [26]. Many synthetic ACE inhibitors such as captopril, enalapril have been developed and used to prevent hypertension in clinical. ACE inhibitors are often used to treat myocardial infarction, hypertension, and other cardio-related diseases [27]. Synthetic ACE inhibitors have certain side effects such as a cough, taste disturbances, skin rashes, and high cost [27].

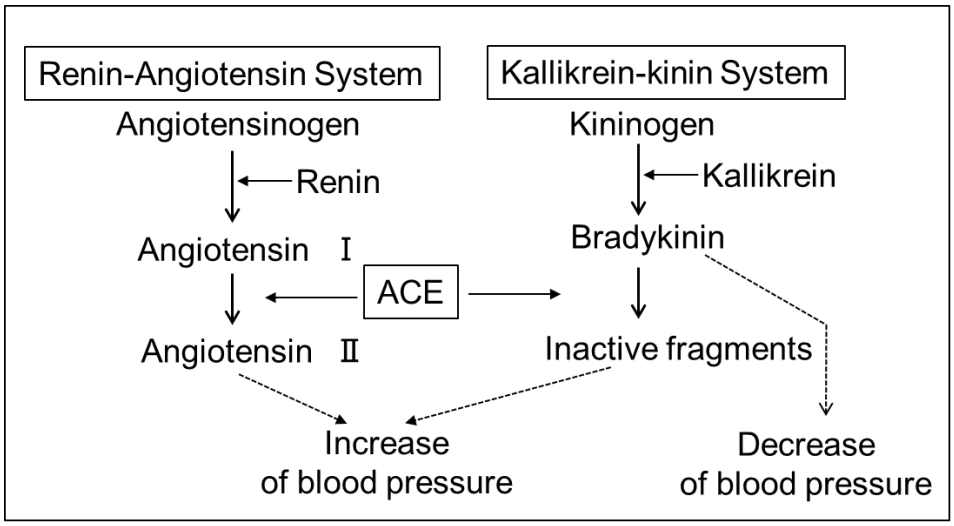

Fig. 1.6 The mechanism of ACE in blood presser regulation [25].

Therefore, it is needed to search non-toxic, safer, innovative and economical ACE inhibitors. Generally, the natural ACE inhibitors are considered to be milder and safer compared with synthetic drugs [27]. Many natural ACE inhibitors have been isolated from food and natural resources. Peptide from Sea cucumber (*Acaudina molpadioidea*) has been shown to have ACE-inhibitory activity [28]. Lee *et al*. prepared tuna frame protein hydrolysates using various enzymes [29]. Qian *et al*. identified a potent ACE-inhibitory peptide from the hydrolysate of bullfrog (*Rana catesbeiana* Shaw) using alcalase [30]. Suetsuna *et al*. identified 5 ACE-inhibitory peptides from the peptic digest from wakame (*Undaria pinnatifida*), and found out their antihypertensive effect in spontaneously hypertensive rats [31].

The report titled "ACE inhibitory activity in enzymatic hydrolysates of insect protein" was published in 2005 [32]. They prepared insect protein hydrolysates of four insects of different orders (*Spodoptera littoralis* (Lepidoptera), *Bombyx mori* (Lepidoptera), *Schistocerca gregaria* (Orthoptera), and *Bombus terrestris* (Hymenoptera)) with gastrointestinal proteases, alcalase, and thermolysin. From then, several literatures on ACE-inhibitory activity from insects were reported. Wang *et al*. prepared hydrolysates of silkworm pupae (*Bombyx mori*) using neutrase, pepsin, acidic protease, flavourzyme, alcalase, and trypsin [33]. Their results suggest that silkworm protein hydrolysates exhibiting ACE-inhibitory activity could be a potential source of ACE inhibitor drugs. Cito *et al*. reviewed ACE-inhibitory activity from insects; except of described above insects, ACE-inhibitory activity was also detected in several other species of insects [34].

1.1.3.3 Dipeptidyl peptidase-IV inhibitors

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) is a serine protease that belongs to the dipeptidyl peptidases (DPPs) [35]. DPP-IV is constitutively expressed on epithelial and endothelial cells of a variety of different tissues, for example, intestine, liver, lung, kidney, and placenta. DPP-IV specifically cleaves X-proline or X-alanine at the N-terminal of polypeptides, transforming them into inactive or even antagonistic species [35].

Incretin hormone is a hormone that stimulates the insulin secretion response to nutrient ingestion in a glucose-dependent manner [36]. The most potent incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 is secreted by intestinal L-cells which stimulates insulin biosynthesis and secretion, reduces glucagon release, delays stomach emptying, reduces appetite, and promote regeneration and differentiation of islet β-cells. GIP is produced by the K-cells of the upper small intestine and is extensively involved in glucose metabolism by enhancing insulin secretion. Both peptides have a short half-life, due to the inactivation by DPP-IV [36]. Therefore, the DPP-IV inhibitor that prevents the inactivation of incretin hormones is seen as an effective method for the management and prevention of type 2 DM (Fig. 1.7).

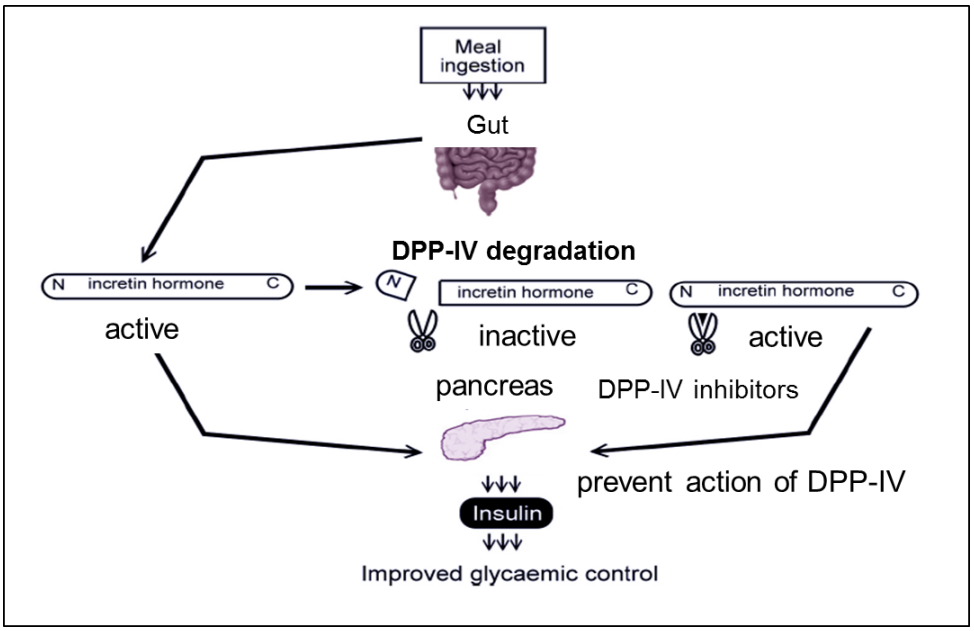

Fig. 1.7 Mechanism of DPP-IV inhibitor in glycaemic control [37].

Recently, DPP-IV has drawn attention as a new treatment option for treatment of Type 2 DM. Type 2 DM is a type of diabetes mellitus, which accounts for 90-95% of the diagnosed cases of DM [38]. DPP-IV inhibitors such as Sitagliptin and Vildagliptin have been developed for type 2 DM treatment [39]. However, these drugs have adverse effects including pancreatitis, angioedema, infective disorders, pancreatic cancer, and thyroid cancer [39]. Therefore, DPP-IV inhibitors that derived from natural sources are expected. Some DPP-IV-inhibitory peptides have been identified from various natural sources. Huang *et al*. isolated 3 DPP-IV-inhibitory peptides from tuna cooking juice hydrolysates by protease XXIII and orientase [40]. Nongonierma *et al*. identified novel DPP-IV-inhibitory peptides from camel milk protein hydrolysate using trypsin [41].

1.2 Utilities of housefly larvae

Pieterse *et al*. performed a nutritional evaluation of housefly larvae and their results suggested that housefly larvae can be regarded as a good-quality protein source that suitable for animal feeding [42]. In addition, Hussein *et al*. conducted nutrition assessment on housefly larvae grown using cattle manure. As a result, the larvae have been shown to be potentially attractive alternatives for use as protein feed ingredients for livestock and aquaculture [43].

Several bioactive compounds from housefly larvae have been reported. Ai *et al*. prepared chitosan from housefly larvae which exhibited antioxidant and antitumor activities [44], and Cao *et al*. purified a new lectin that displays antitumor activity against human breast cancer cells using affinity chromatography and HPLC [45]. Hou *et al*. prepared an extract from housefly larvae; the extract exhibited antibacterial and antitumor activities [46].

Zhang *et al*. prepared housefly larvae hydrolysates using alcalase and neutral proteinase, these hydrolysates exhibited antioxidant activity [47]. Holman Mark *et al*. isolated a diuretic myokinin neuropeptide from housefly larvae [48]. Wang *et al*. extracted a protein-enriched fraction from housefly larvae using buffer treatment, which showed anti-influenza activity [49]. Ito *et al*. isolated insect lysozyme from housefly larvae and investigated its digestive function [50]. Lu *et al*. prepared a protein-enriched fraction from housefly larvae and the fraction exhibited anti-hepatitis B virus activity [51]. Recently, Guo *et al*. identified a novel antimicrobial protein AMP17 from housefly larvae, which exhibits potent antifungal activity against *Candida albicans* [52].

However, there is no report on ACE-inhibitory and DPP-IV-inhibitory activities from housefly larvae. Moreover, there is no report on DPP-IV inhibition from insects.

1.3 Objectives

Insects contain abundant and high-quality protein, fat, vitamins, and minerals; many of them (edible insects) have been used in the human diet in most parts of the world [3]. Housefly is a type of insect, and so far nutrition assessment has been done on housefly larvae, but most of these studies focused on application to livestock feed. There are few studies on the potentiality of the housefly larvae as food or functional foods, which have limited its application in this regard.

Oxidative stress is caused by a large variety of free radicals, which would damage DNA, membranes lipids, and proteins [17]. Moreover, oxidative stress has been confirmed to be related to many chronic diseases, such as hypertension, diabetes, cancer and other diseases [16, 18]. Bioactive compounds are usually derived from plant, aquatic animals, and mammalian animals [12]. However, reports on bioactive compounds from insects are extremely scarce compared to other natural resources.

Housefly larvae have been used in traditional Chinese medicine for a long time, however, little information is available on the bioactive compounds from housefly larvae. Recently, extracts/fractions from housefly larvae have been shown to possess antioxidant, antitumor, antibacterial, and other activities [44-46]. Moreover, to date, those extracts or fractions from housefly larvae were mainly prepared by using buffer treatment, enzymatic hydrolysis. However, these treatment methods are inconvenient and high-cost, which may limit the use of housefly larvae in large-scale. Therefore, it is necessary to develop a convenient and low-cost method to well use housefly larvae for bioactive compounds. In addition, although various biological activities from housefly larvae have been reported, there is no report on ACE-inhibitory and DPP-IV-inhibitory activities from housefly larvae.

As described above, the specific objectives of in current study are as follows:

(1) To evaluate the amino acid composition of housefly larvae to clarify the possibility of housefly larvae as materials for functional foods.

(2) To prepare bioactive compounds showing multifunctional activities such as antioxidant activity, ACE-inhibitory activity, and DPP-IV-inhibitory activity from housefly larvae by a simple method. Housefly larvae water extract (HLWE) was prepared by using decoction method that is a convenient, safe and low-cost method for extraction of bioactive compounds in traditional Chinese medicine.

Chapter 2

Evaluation of usefulness of housefly larvae based on amino acid analysis

Abstract

In this chapter, the utility of housefly larvae was evaluated by amino acid analysis. The housefly larvae contained all the essential amino acids and satisfactory for the amino acid requirements for humans specified by Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO). The amino acid composition of housefly larvae was comparable to ideal protein resources such as fishmeal, beef, chicken, pork, casein, and hen egg.

2.1 Introduction

Fishmeal is a high-quality protein source which mainly used as feed; it can be made from almost any type of seafood [1]. However, the price of fishmeal is rising due to an increase of utilization of fishmeal. Therefore, it is needed to search a substitute of fishmeal for livestock producers. Several studies have reported the nutritional value of housefly larvae and have shown that housefly larvae may be a substitute for fishmeal [2, 3]. Pieterse *et al*. performed a nutritional evaluation of housefly larvae and their results suggested that housefly larvae can be regarded as a good-quality protein source that suitable for animal feeding [2]. Hussein *et al*. conducted nutrition assessment on housefly larvae grown using cattle manure and showed the potential of housefly larvae as a protein feed ingredients for livestock and aquaculture [3]. However, these reports only compared the amino acid composition of one type of fishmeal, and it seems that there has not been any report on comparison with the amino acid composition of several types of fishmeal at the same time. The amino composition of fishmeal is depended on the type of fish used to manufacture fishmeal. Therefore, it should be compared with multiple fishmeal to well evaluate the usefulness of housefly larvae.

Most of the nutritional assessments of housefly larvae have been aimed at evaluating the applicability to livestock feed. However, there are very few reports on the potentiality of the housefly larvae as food or functional foods. In this chapter, the utility of housefly larvae was evaluated as follows. (1) The amino acid composition of housefly larvae was compared with commercially available fishmeal. (2) The potential of housefly larvae as food or functional foods was investigated by comparing the amino acid composition of housefly larvae with beef, chicken, pork, casein, and hen egg, which are considered as ideal protein resources.

2.2 Materials

Dried housefly larvae reared with swine manure and 5 types of fishmeal (A, B, C, D, E) were kindly provided by E's Inc. (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.3 Methods

2.3.1 Amino acid analysis of housefly larvae and fish meal

Dried housefly larvae were smashed using a mixer grinder (Iwatani IFM-800DG), and 2 mg of the powder was hydrolyzed using 2 mL of 6 M HCl for 24 h at 110°C in vacuum tubes sealed under a nitrogen atmosphere. After hydrolysis, the HCl was removed *in vacuo* and the powder was dissolved in 0.02 M HCl. To determine the tryptophan (Trp) contents of housefly larvae, 4 M methane sulfonic acid solution was used to hydrolyze the powder under the same conditions (housefly larvae : methane sulfonic acid = 1 mg : 1 mL). After hydrolysis, 4 M sodium hydroxide solution was used to neutralize the hydrolysis solution to pH 2–3. After neutralization, the hydrolysate solution was filtered to remove the insoluble matter. All fishmeal were also hydrolyzed under the same conditions as the housefly larvae powder. Amino acid analysis was performed using an automatic analyzer (L-8800; Hitachi, Tokyo, Japan). The experiments were performed in duplicate, and the results are presented as means.

2.3.2 Comparison of amino acid composition between housefly larvae and fishmeal

To well evaluate the potential of housefly larvae as feed, the comparison of amino acid composition between housefly larvae and 5 types of fishmeal were performed. The amino acid composition of these protein resources and housefly larvae were compared by radar chart analysis. In order to capture the difference intuitively, the content of each amino acid containing in fishmeal A, B, C, D, and E was considered as a standard (100%) and the relative content of each amino acid in housefly larvae compared with each fishmeal was calculated.

In addition, the comparison between housefly larvae and 5 types of fishmeal in the contents of BCAAs (Ile, Leu, Val), Lys, and the sum of Lys, Met, Thr, and Trp were also performed due to these specific amino acids are important to animals.

2.3.3 Comparison of amino acid composition between housefly larvae and beef, chicken, pork, casein, and hen egg

To evaluate the potential of housefly larvae as food, the comparison of amino acid composition between housefly larvae and other ideal protein sources including beef, chicken, pork, casein, and egg were performed. Similar to the Section 2.3.2, the amino acid composition of these protein resources and housefly larvae were also compared by radar chart analysis to capture the difference intuitively.

2.3.4 Calculation of the degree of similarity between housefly larvae and other resource in amino acids

In order to clearly grasp the difference in amino acid composition between housefly larvae and other protein resources, we adopted the modified Canberra distance reported by Wang *et al*. [4] to calculate the degree of similarity in amino acids between the housefly larvae and beef, chicken, pork, casein, and eggs. Canberra distance is always used in cluster analysis for data mining. The value of the degree of similarity can reflect how the amino acid composition of housefly larvae is similar to that of those protein resources. The larger value of the degree of similarity nearer to 1 means the higher nutritional value of housefly larvae as a protein resource, which can comparable to those protein resources. The similarity degree was calculated using the following formula:

$$\text{Canberra distance: } d(a_k, \mu_k) = \sum_i \frac{|a_k - \mu_k|}{|a_k + \mu_k|}$$

$$\text{Degree of similarity: } \mu(a_k, \mu_k) = 1\text{-}0.09\sum_i \frac{|a_k - \mu_k|}{|a_k + \mu_k|}$$

(a_k: the content of i^{th} amino acids containing in protein sources, μ_k: the content of i^{th} amino acids containing in housefly larvae)

2.4 Results

2.4.1 Amino acid composition of housefly larvae

The amino acid composition of housefly larvae reared with swine manure is shown in Table 2.1 and Fig. 2.1. The housefly larvae had all essential amino acids (EAAs); the top six most abundant amino acids in housefly larvae were as follows: Glx (14.2%; the sum of Glu and Gln), Asx (10.4%; the sum of Asp and Asn), Lys (8.1%), Val (8.0%), Tyr (7.7%), and Phe (7.4%). In the total amino acids of housefly larvae, hydrophobic, aromatic, and acidic amino acids accounted for 35.6%, 15.5%, and 24.6%, respectively. The contents of Ile, Leu, and Val, i.e., branched-chain amino acids (BCAAs), accounted for 18.4% of the total amino acids of housefly larvae. Our results were similar to the amino acid composition of housefly larvae reared with different methods (e.g., cattle manure) reported by Hussein *et al*. [3], as shown in Table 2.1. The contents of Lys and BCAAs in our study (8.1% and 18.4%, respectively) were higher than those in the previous study (Lys, 7.1%; BCAAs, 14.0%). In contrast, the contents of Met (7.2%) and Trp (1.3%) in the previous study were higher than those in our study (1.7% and 0.4%, respectively).

2.4.2 Amino acid composition of housefly larvae and fishmeal

The comparison of amino acid composition between housefly larvae and fishmeal is shown in Table 2.2 and Fig. 2.2-2.9. The most abundant amino acids were Glx and Asx in fishmeal A, D, and E, which are same to that of housefly larvae; whereas the most abundant amino acids containing in fishmeal B and C were Gly and Glx (or Alx). The content of Gly contained in housefly larvae (4.2%) was lower compared to all types of fishmeal. The sum of Lys, Met, Thr, and Trp (these amino acids are limiting amino acids in animal feed) contained in fishmeal A, B, C, D, and E were 12.1, 12.7, 13.6, 16.4, and 14.4%, respectively, which is lower than that of housefly larvae (14.8%) except for fishmeal D (16.4%).

The contents of BCAAs accounted for 18.8, 15.0, 17.3, 18.3, and 18.5% of the total amino acids in fishmeal A, B, C, D, and E, respectively; it accounted for 18.4% in that of housefly larvae, which is comparable to all 5 types of fishmeal. In addition, the contents of Lys in fishmeal A, B, C, D, and E were 5.8, 5.5, 6.8, 8.3, and 6.6%, respectively. Housefly larvae contained more Lys (8.1%) compared to all other fishmeal, except for fishmeal D (8.3%). Housefly larvae contained higher essential amino acids compared with all fishmeal. The ratio of EAAs to NEAAs in fishmeal A, B, C, D, and E were 0.6, 0.5, 0.6, 0.7, and 0.6, respectively, which was somewhat lower than that of housefly larvae (0.8).

Table 2.1 Amino acid composition of housefly larvae (% of total amino acids).

Amino acid	Housefly larvae	
	Current study	Hussein *et al*. [a] [3]
Essential amino acids		
Ile	3.8	3.3
Leu	6.6	6.0
Lys	8.1	7.1
Met	1.7	7.2
Phe	7.4	6.9
Thr	4.6	4.5
Trp	0.4	1.3
His	3.2	2.9
Val	8.0	4.7
Nonessential amino acids		
Arg	5.8	5.8
Ala	5.3	5.3
Asx*	10.4	9.4
Cys	NA	1.4
Glx*	14.2	15.2
Gly	4.2	4.0
Pro	4.1	4.5
Ser	4.4	4.2
Tyr	7.7	6.3
EAAs[b]	44.0	43.8
NEAAs[c]	56.0	56.2
EAAs/NEAAs	0.8	0.8
BCAAs[d]	18.4	14.0

NA: not analyzed.

* Asx: Asp + Asn, Glx: Glu + Gln

[a] In order to unify the significant figures in the data, data conversion and rounding were performed.

[b] EAAs (E): essential amino acids

[c] NEAAs (N): nonessential amino acids

[d] BCAAs: branched-chain amino acids (Ile, Leu, Val)

Table 2.2 Amino acid composition of housefly larvae and fishmeal from different manufacturers (% of total amino acids).

Amino acid	Housefly larvae	Fishmeal				
		A	B	C	D	E
Essential amino acids						
Ile	3.8	3.9	3.2	4.0	4.6	4.6
Leu	6.6	8.2	6.3	7.6	8.2	8.1
Lys	8.1	5.8	5.5	6.8	8.3	6.6
Met	1.7	1.5	2.3	1.6	2.5	2.8
Phe	7.4	4.9	3.9	4.2	4.4	4.7
Thr	4.6	4.3	4.9	4.6	4.8	4.4
Trp	0.4	0.5	0	0.6	0.8	0.6
His	3.2	2.8	2.8	2.4	2.3	2.0
Val	8.0	6.7	5.5	5.7	5.5	5.8
Nonessential amino acids						
Arg	5.8	7.4	6.7	7.2	6.6	7.2
Ala	5.3	7.0	8.0	8.1	7.4	5.8
Asx*	10.4	9.4	7.7	9.5	10.2	9.5
Cys	NA	NA	NA	NA	NA	NA
Glx*	14.2	15.3	12.0	14.4	14.9	19.0
Gly	4.2	8.2	15.8	10.2	7.6	5.9
Pro	4.1	6.2	8.1	6.7	5.1	6.1
Ser	4.4	5.7	4.2	4.8	4.4	4.7
Tyr	7.7	2.8	3.2	2.2	3.5	3.7
EAAs[a]	44.0	38.4	34.4	37.5	41.0	40.0
NEAAs[b]	56.0	61.6	65.6	62.5	59.0	60.0
EAAs/NEAAs	0.8	0.6	0.5	0.6	0.7	0.6
BCAAs[c]	18.4	18.8	15.0	17.3	18.3	18.5

NA: not analyzed.

* Asx: Asp + Asn, Glx: Glu + Gln

[a] EAAs (E): essential amino acids

[b] NEAAs (N): nonessential amino acids

[c] BCAAs: branched-chain amino acids (Ile, Leu, Val)

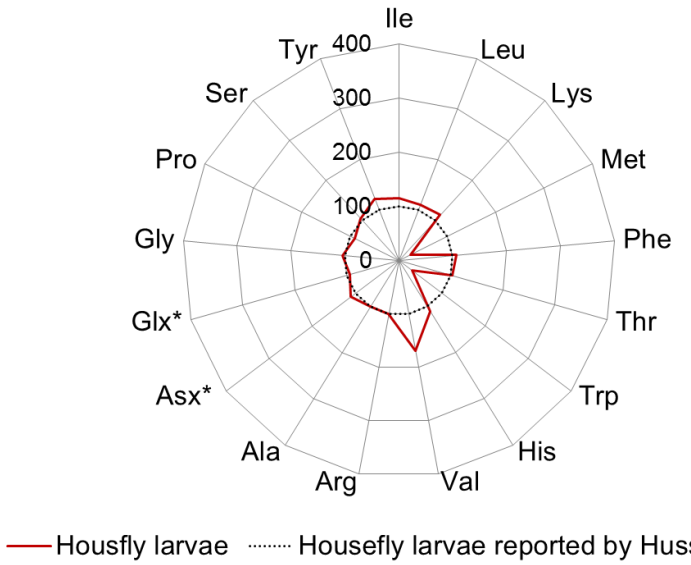

Fig. 2.1 Radar chart analysis of the amino acid composition (except Cys) between housefly larvae in the current study and the housefly larvae reported by Hussein *et al*. [3]. The circle represents the amino acid composition of housefly larvae reported by Hussein *et al*, which was regarded as a standard (100%). The amino acid composition of housefly larvae in the current study was expressed as relative contents of each amino acid compared with that reported by Hussein *et al*. Asx: Asp + Asn, Glx: Glu + Gln.

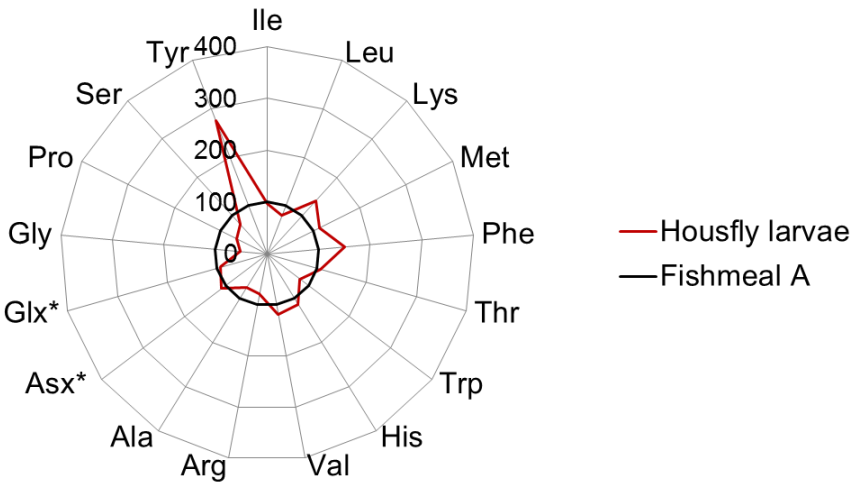

Fig. 2.2 Radar chart analysis of the amino acid composition (except Cys) between housefly larvae and fishmeal A. The circle represents the amino acid composition of fishmeal A, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with fishmeal A. Asx: Asp + Asn, Glx: Glu + Gln.

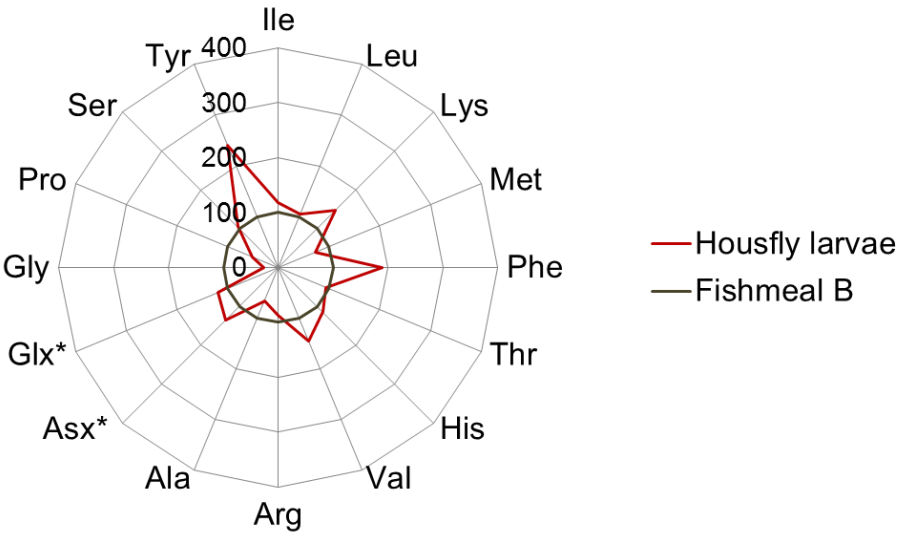

Fig. 2.3 Radar chart analysis of the amino acid composition (except Cys and Trp) between housefly larvae and fishmeal B. The circle represents the amino acid composition of fishmeal B, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with fishmeal B. Asx: Asp + Asn, Glx: Glu + Gln.

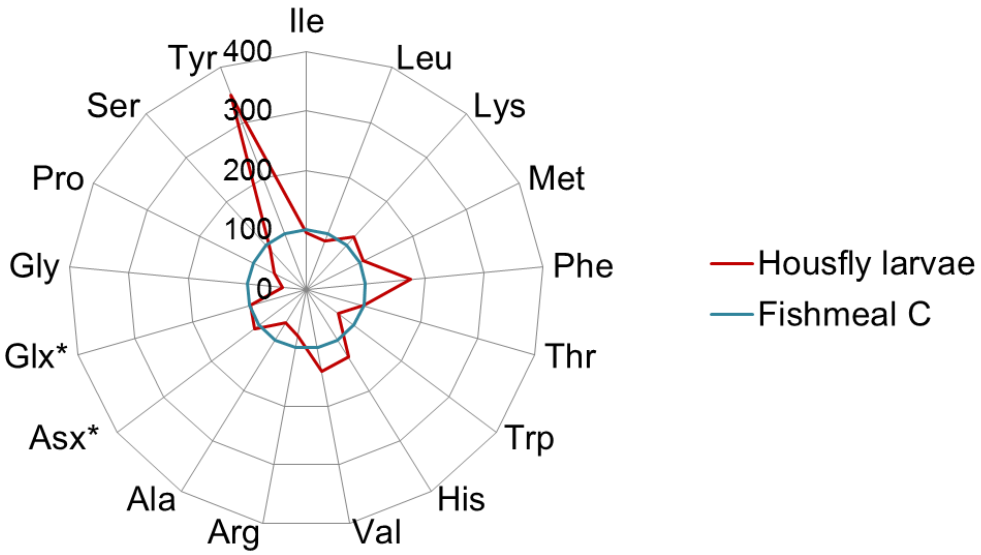

Fig. 2.4 Radar chart analysis of the amino acid composition (except Cys) between housefly larvae and fishmeal C. The circle represents the amino acid composition of fishmeal C, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with fishmeal C. Asx: Asp + Asn, Glx: Glu + Gln.

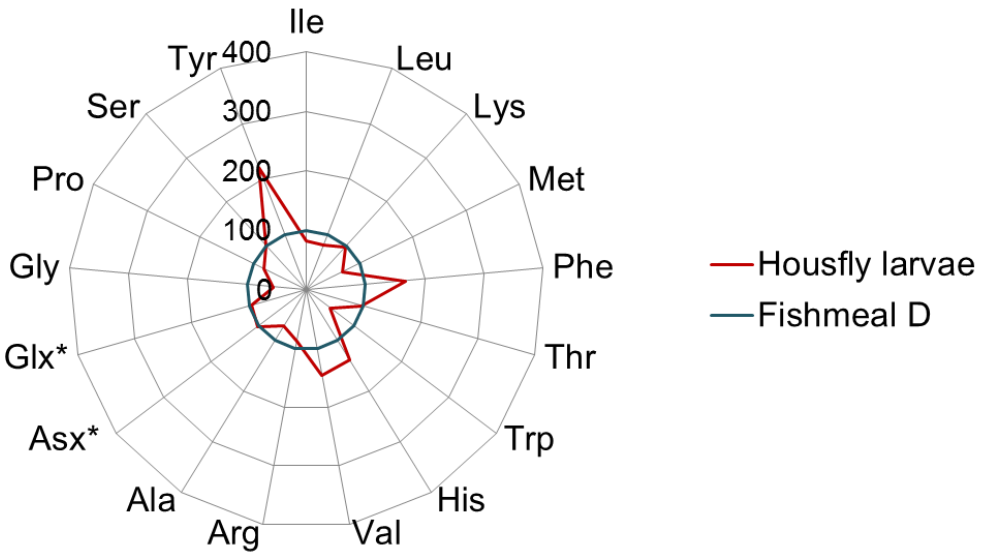

Fig. 2.5 Radar chart analysis of the amino acid composition (except Cys) between housefly larvae and fishmeal D. The circle represents the amino acid composition of fishmeal D, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with fishmeal D. Asx: Asp + Asn, Glx: Glu + Gln.

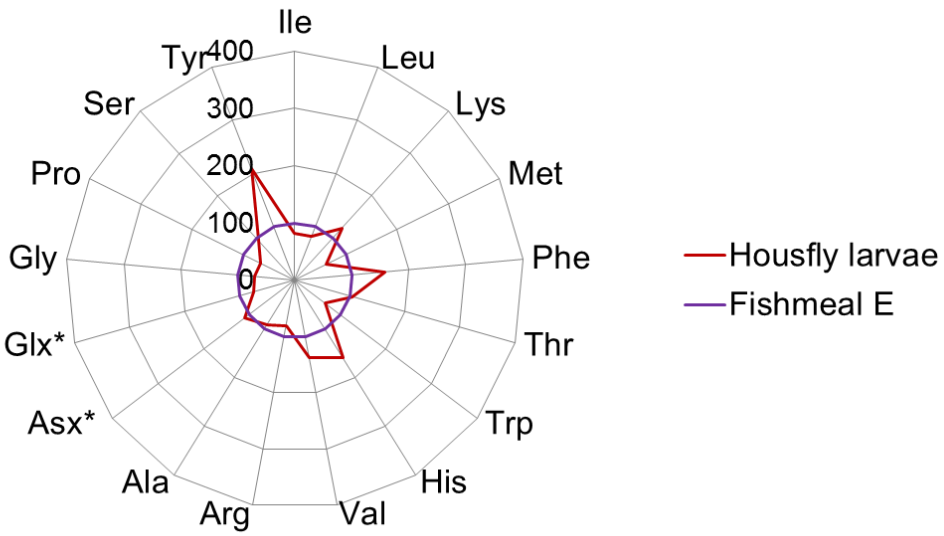

Fig. 2.6 Radar chart analysis of the amino acid composition (except Cys) between housefly larvae and fishmeal E. The circle represents the amino acid composition of fishmeal E, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with fishmeal E. Asx: Asp + Asn, Glx: Glu + Gln.

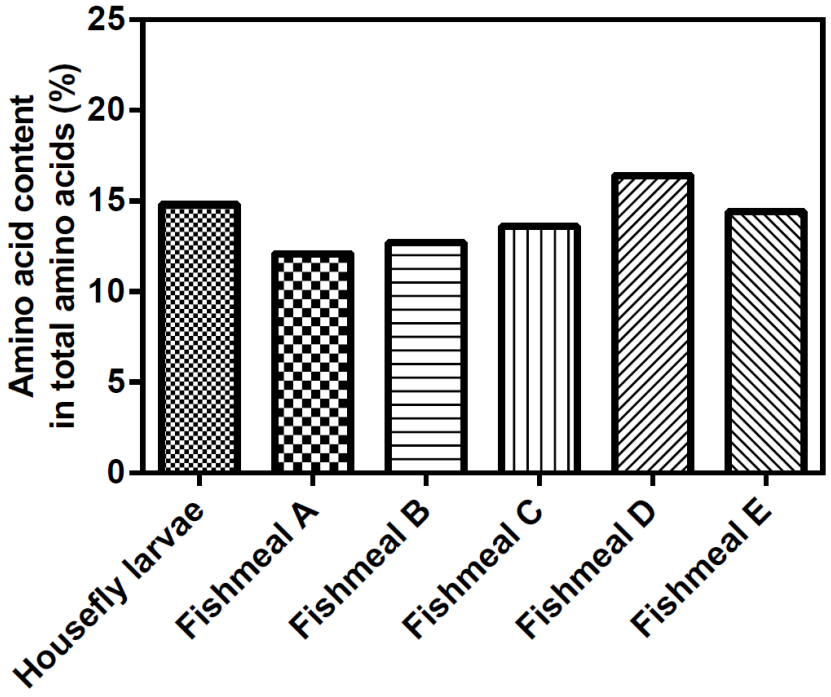

Fig. 2.7 Comparison of the total contents of Lys, Met, Thr, and Trp of housefly larvae with 5 types of fishmeal.

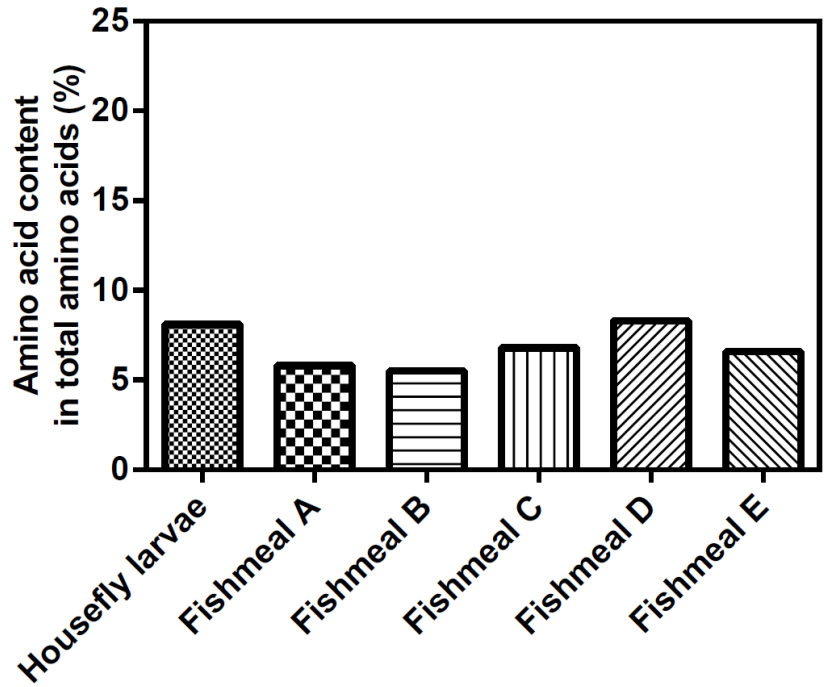

Fig. 2.8 Comparison of the content of Lys of housefly larvae with 5 types of fishmeal.

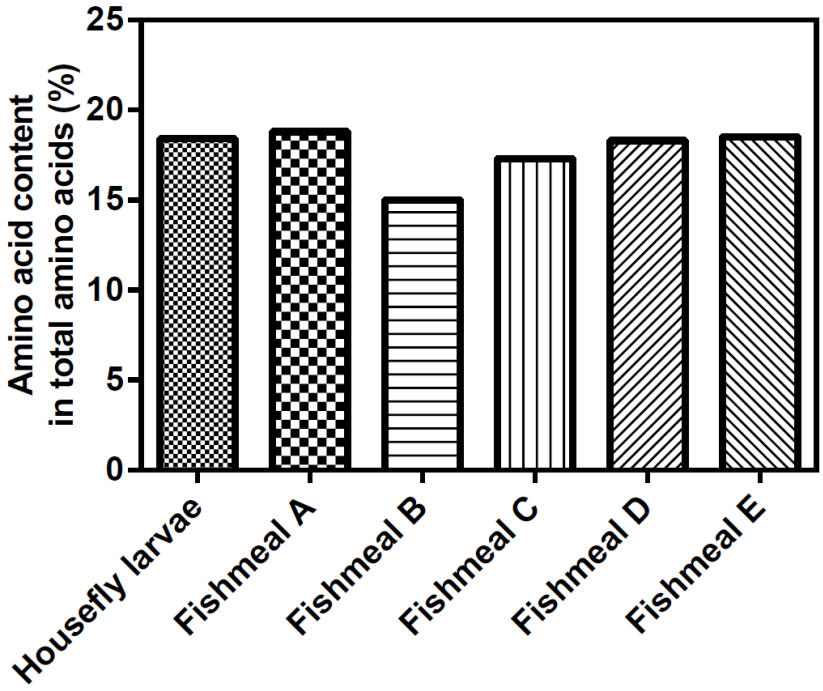

Fig. 2.9 Comparison of the total contents of Ile, Leu, and Val between housefly larvae and 5 types of fishmeal.

2.4.3 Amino acid composition of housefly larvae and other protein sources

We compared the amino acid composition of housefly larvae with other protein sources to confirm the potential of housefly larvae as food and functional foods (Table 2.3, Fig. 2.10-2.16). The BCAA content in total amino acids of housefly larvae is 18.4%, which is comparable to that of beef (18.5%), chicken (19.5%), and pork (18.4%). The content of Lys in total amino acids of housefly larvae is 8.1%, which is higher than that of egg (6.8%) and casein (7.5%), and comparable to that of beef (9.2%), chicken (8.7%) and pork (8.4%), which is described in Table 2.3. In addition, the amino acid compositions of housefly larvae and beef, chicken, pork, casein, and hen egg protein, which are known to be good nutrient sources, were compared by radar chart analysis (Fig. 2.10-2.16). The amino acid composition of housefly larvae was similar to that of beef, chicken, and pork, except for Tyr, Val, Trp, and Phe; it was similar to that of casein, except for Tyr, Pro, Trp, and Phe; it was similar to that of hen egg, except for Ser, Tyr, Trp, and Phe. Furthermore, housefly larvae provided satisfactory nutrient contents according to the amino acid requirements for humans specified by FAO/WHO, i.e., the content of EAAs was 44% that greater than 40%, and the ratio of essential to nonessential amino acids (EAAs/NEAAs) was 0.8 which greater than 0.6 [6].

Table 2.3 Amino acid composition of housefly larvae and other proteins (% of total amino acids).

Amino acid	Housefly larvae	Beef[a] [5]	Chicken[a] [5]	Pork[a] [5]	Casein[a] [5]	Egg[a] [5]
Essential amino acids						
Ile	3.8	5.0	5.9	5.3	5.0	6.1
Leu	6.6	8.4	8.1	7.8	8.7	8.5
Lys	8.1	9.2	8.7	8.4	7.5	6.8
Met	1.7	2.8	2.8	2.8	2.6	3.3
Phe	7.4	4.5	4.4	4.3	4.8	5.6
Thr	4.6	4.7	4.4	5.1	4.3	5.0
Trp	0.4	1.2	1.1	1.4	1.5	1.4
His	3.2	3.5	2.9	3.4	2.7	2.4
Val	8.0	5.2	5.6	5.4	6.2	6.6
Nonessential amino acids						
Arg	5.8	6.5	6.1	6.6	3.4	5.9
Ala	5.3	6.0	3.7	5.7	2.8	5.7
Asx*	10.4	9.3	10.1	9.2	6.6	9.3
Cys	NA	1.3	1.4	1.2	0.3	2.4
Glx*	14.2	15.7	16.5	14.9	20.3	12.3
Gly	4.2	5.0	5.8	5.9	1.8	3.2
Pro	4.1	3.9	4.6	4.7	10.6	4.0
Ser	4.4	4.2	4.3	4.3	5.6	7.4
Tyr	7.7	3.7	3.7	3.7	5.4	4.0
EAAs[b]	44.0	44.4	43.8	43.8	43.2	45.6
NEAAs[c]	56.0	55.6	56.2	56.2	56.8	54.4
EAAs/NEAAs	0.8	0.8	0.8	0.8	0.8	0.8
BCAAs[d]	18.4	18.5	19.5	18.4	19.9	21.3

NA: not analyzed.

* Asx: Asp + Asn, Glx: Glu + Gln

[a] In order to unify the significant figures in the data, data conversion and rounding were performed.

[b] EAAs (E): essential amino acids

[c] NEAAs (N): nonessential amino acids

[d] BCAAs: branched-chain amino acids (Ile, Leu, Val)

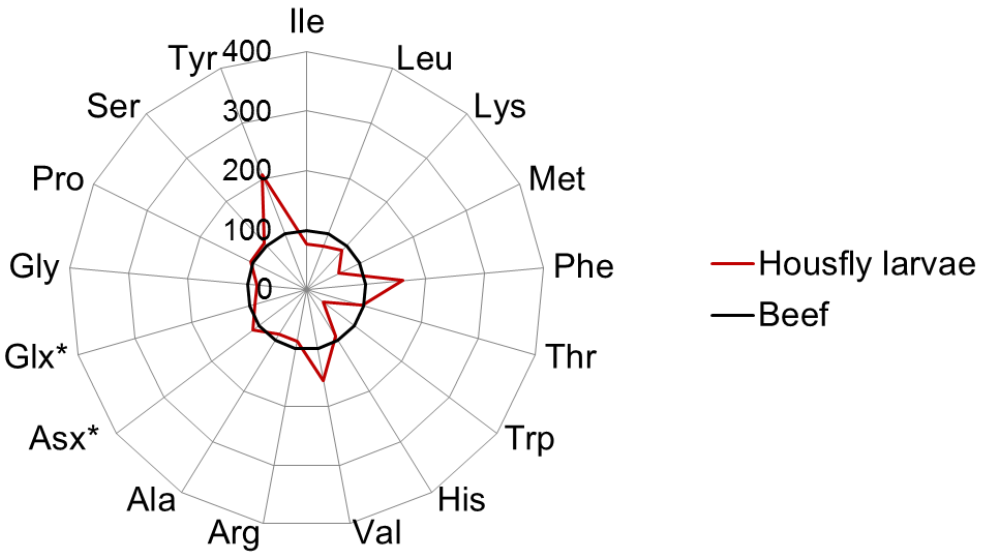

Fig. 2.10 Radar chart analysis of the amino acid composition of housefly larvae and beef. The circle represents the amino acid composition of beef, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with beef. Asx: Asp + Asn, Glx: Glu + Gln.

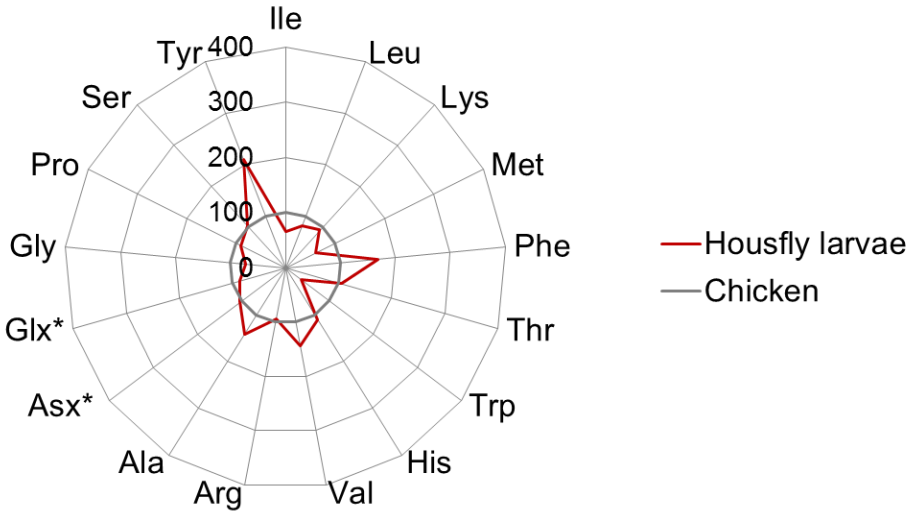

Fig. 2.11 Radar chart analysis of the amino acid composition of housefly larvae and chicken. The circle represents the amino acid composition of chicken, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with chicken. Asx: Asp + Asn, Glx: Glu + Gln.

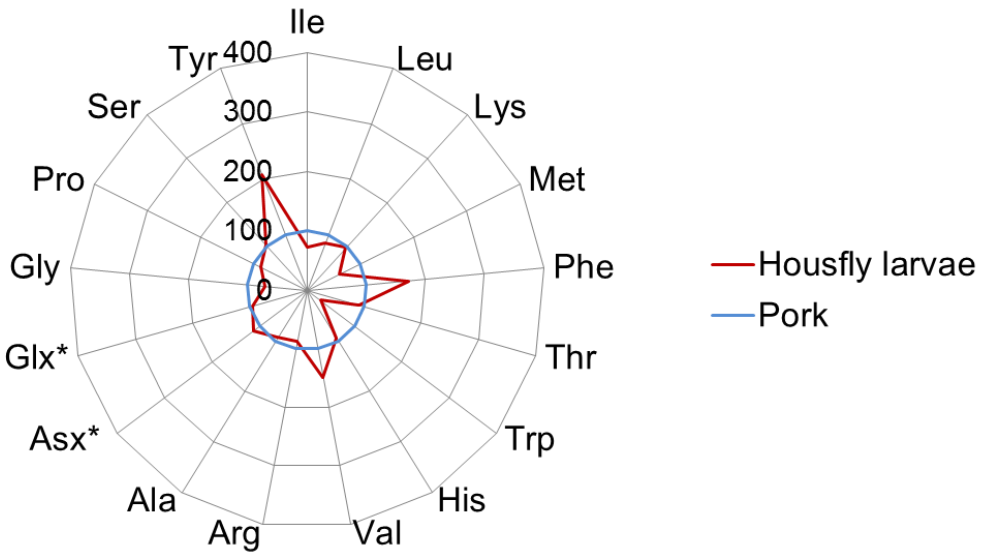

Fig. 2.12 Radar chart analysis of the amino acid composition of housefly larvae and pork. The circle represents the amino acid composition of pork, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with pork. Asx: Asp + Asn, Glx: Glu + Gln.

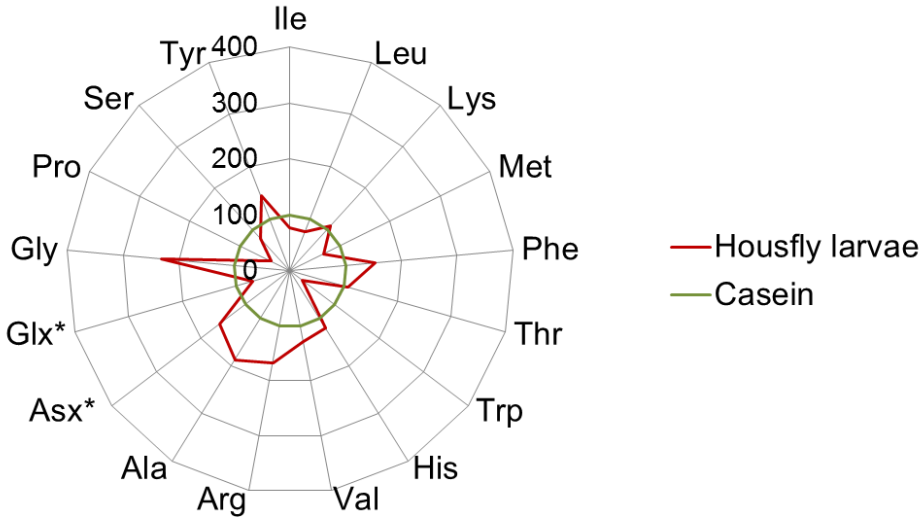

Fig. 2.13 Radar chart analysis of the amino acid composition of housefly larvae and casein. The circle represents the amino acid composition of casein, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with casein. Asx: Asp + Asn, Glx: Glu + Gln.

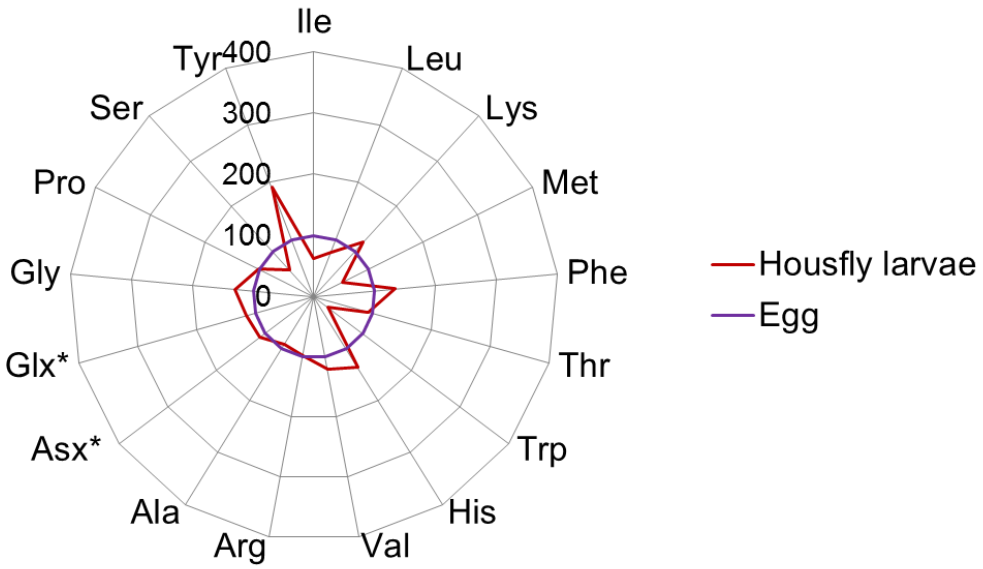

Fig. 2.14 Radar chart analysis of the amino acid composition of housefly larvae and hen eggs. The circle represents the amino acid composition of egg, which was regarded as a standard (100%). The amino acid composition of housefly larvae was expressed as relative contents compared with egg. Asx: Asp + Asn, Glx: Glu + Gln.

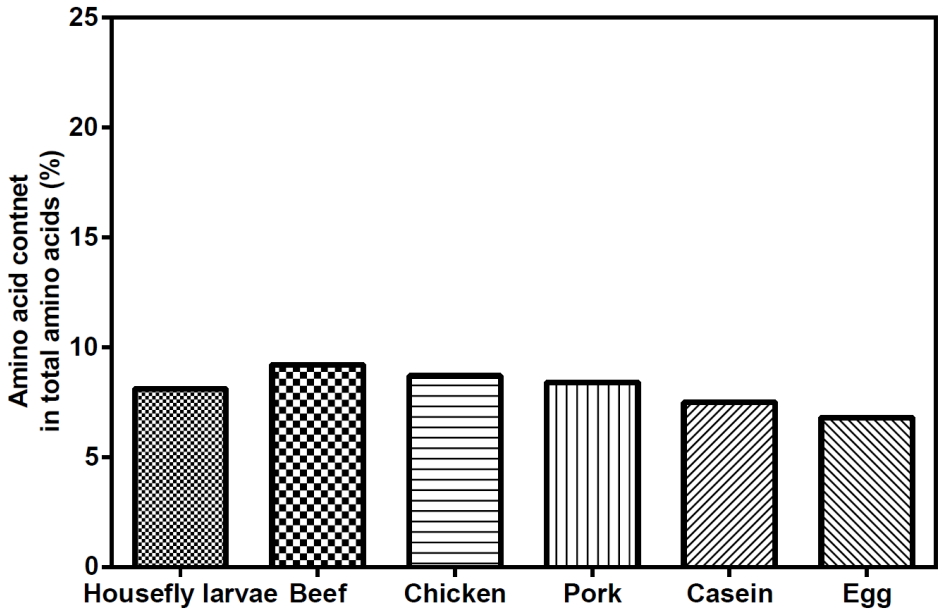

Fig. 2.15 Comparison of Lys of housefly larvae with beef, chicken, pork, casein, and egg.

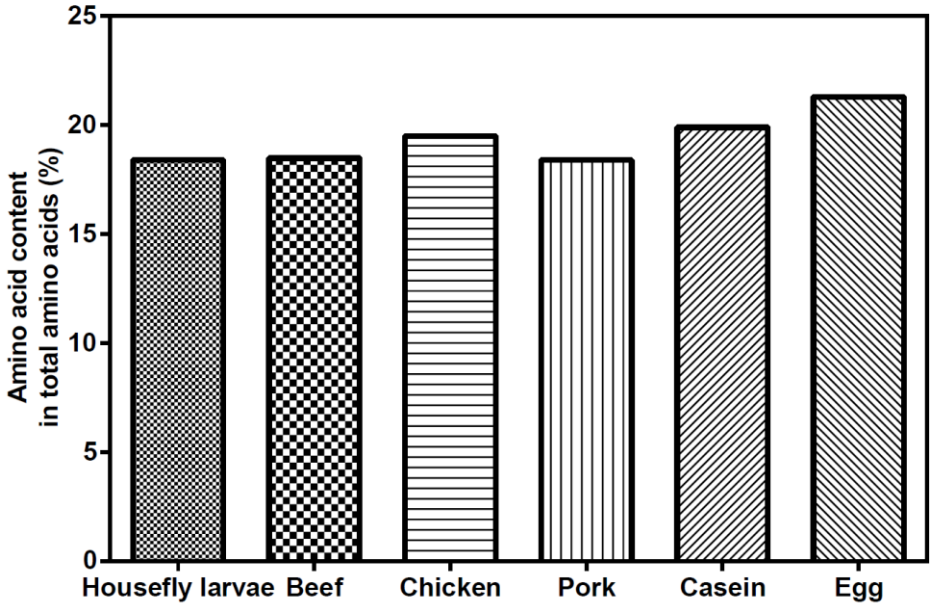

Fig. 2.16 Comparison of the total contents of Ile, Leu, and Val between housefly larvae and beef, chicken, pork, casein, and egg.

2.4.4 The degree of similarity between housefly larvae and other resources in amino acids

In the previous section, the amino acid composition of several protein resources and housefly larvae was compared by radar chart analysis. In this section, we calculated the degree of similarity in entire and essential amino acids between housefly larvae and other protein sources in order to quantify how the amino acid composition of housefly larvae are close to other protein sources.

As shown in Table 2.4-2.5, the degree of similarity of housefly larvae and 5 types of fishmeal in entire amino acids are close to 0.8 except that when compared with fishmeal B (0.65); the degree of similarity in essential amino acids are bigger than 0.82 which is close to 1. The degree of similarity between housefly larvae and other protein sources are same as the degree of similarity when compared with fishmeal. These results suggest that the amino acid composition of housefly larvae can be comparable to that of fishmeal, beef, chicken, pork, casein, and egg.

Table 2.4 The degree of similarity between housefly larvae and 5 types of fishmeal.

Fishmeal	The degree of similarity	
	in entire amino acids	in essential amino acids
A	0.79	0.92
B	0.65	0.82
C	0.76	0.91
D	0.78	0.88
E	0.78	0.88

Table 2.5 The degree of similarity between housefly larvae and other protein sources.

	The degree of similarity	
	in entire amino acids	in essential amino acids
Beef	0.79	0.86
Chicken	0.78	0.86
Pork	0.79	0.86
Casein	0.67	0.86
Egg	0.76	0.84

2.5 Discussion

Insects have been used as human food in many parts of the world. Since insects have a well-balanced nutrient profile, the study on the use of insects as food is becoming an attractive and new area [7-9]. Belluco *et al*. highlighted that insects are a good protein resource and they are safe for human consumption [10]. In this chapter, the usefulness of housefly larvae as feed, food, and functional foods was evaluated by amino acid analysis.

The amino acid composition of the housefly larvae reared with swine manure in the current study was compared with that of the same species reared with cattle manure recently reported by Hussein *et al*. [3]. They showed similar amino acid ratio, and the contents of Lys, BCAAs (Ile, Leu, and Val), Met, and Trp were somewhat different. The differences in amino acid compositions despite using the same species may be attributable to differences in their growing conditions, such as harvest time and breeding substrate. Moreover, Hussein *et al*. investigated the nutrient components of housefly larvae including fatty acid and mineral composition in detail, and they remarked the usefulness of this species as protein feed ingredients for livestock and aquaculture. Thus,

our findings, combined with this previous study, suggested that housefly larvae might be a promising candidate source of useful nutrients. Our radar chart analysis showed that the amino acid composition of housefly larvae was similar to that of fishmeal and other protein sources.

The BCAA content in total amino acids of housefly larvae is 18.4%, which is comparable to that of beef (18.5%), chicken (19.5%), and pork (18.4%). The content of Lys in total amino acids of housefly larvae is 8.1%, which is higher than that of egg (6.8%) and casein (7.5%), and comparable to that of beef (9.2%), chicken (8.7%) and pork (8.4%), BCAAs may increase growth hormone circulation, which may be related to anabolic mechanisms causing muscle growth [11]. In addition, elevated plasma BCAAs levels may be related to insulin resistance and the incidence of type 2 diabetes [12]. Lys is a limiting amino acid in swine and poultry feed; consequently, supplementation with Lys or Lys-rich substances is required for the growth of these animals.

Moreover, housefly larvae contained all EAAs and satisfied the amino acid requirements for humans specified by the FAO/WHO. Therefore, these results suggested that housefly larvae could be a high-quality protein resource that could be used to develop amino acid supplements or additives.

Recently, the new concept of functional amino acids (FAAs) was proposed by Wu [13]. FAAs are amino acids that can participate in and regulate key metabolic pathways to improve the health, survival, growth, development, lactation, and reproduction of organisms [13]. Deficiencies in FAAs (either EAAs or NEAAs) impair not only protein synthesis but also whole-body homeostasis [13]. Housefly larvae were found to contain all EAAs and NEAAs, and the amino acid balance was similar to that of hen egg protein and other food proteins, which are typically considered an ideal protein resource. Therefore, our findings suggested that housefly larvae could have applications as a good source of protein.

In the total amino acids of housefly larvae, 35.6% of the hydrophobic amino acids, 15.5% of the aromatic amino acids and 24.6% of the acidic amino acids (Glx + Asx) were included, respectively. According to previous reports related to antioxidant peptides, ACE-inhibitory and DPP-IV-inhibitory peptides, amino acids such as Trp, Tyr, Lys, His, Met, Glu, and Asp may significantly contribute to the antioxidant activity of peptides [14]; hydrophobic amino acids such as Trp, Tyr, Phe, and Pro are important for ACE-inhibitory and DPP-IV-inhibitory activity of peptides [15]. Since housefly larvae contained abundant these amino acids and having well-balanced amino acids, these results suggest that antioxidant, ACE-inhibitory, and DPP-IV-inhibitory peptides could also be present in housefly larvae.

The life cycle of insects is very short compared to other animals such as pig and cattle [16]. Moreover, insects have a higher feed conversion efficiency compared to other conventional livestock and have less production of greenhouse gases, water consumption and land requirement than pig and cattle [17, 18]. Several studies have shown that insects have a higher efficiency of matter assimilation than livestock; the amount of plant nutrients needed to produce 1 kg of meat is over 10-times that needed to produce the same amount of insect zoomass [19]. Hence the rearing of insects for production of insect-based food causes much less strain on ecosystem services than livestock-based food [19], which may contribute to overcoming food and feed security problems in the future.

2.6 Conclusion

In this chapter, the amino acid composition of housefly larvae as a protein resource was evaluated. The housefly larvae contained all the essential amino acids and satisfactory for the amino acid requirements for humans specified by Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO). In addition, the amino acid composition of housefly larvae was comparable to fishmeal and those ideal protein sources including beef, chicken, pork, casein, and egg. Our results indicate the potential of housefly larvae as a protein source that can be used to feed, food, and agents for functional foods.

Chapter 3

Preparation of housefly larvae water extract (HLWE) with multifunctional activities

Abstract

In this chapter, it was investigated the usefulness of housefly larvae as multifunctional nutraceuticals. First, we prepared housefly larvae water extract (HLWE) using decoction method. Second, it was explored the biological activities of the extracts. HLWE showed significant antioxidant activity (75.4% at 5.00 mg/mL), ACE-inhibitory activity (half-maximal inhibitory concentration [IC_{50}] = 0.430 mg/mL), and DPP-IV-inhibitory activity ([IC_{50}] = 3.52 mg/mL). The low-molecular-weight constituents (< 6 kDa) in HLWE contributed to antioxidant and ACE-inhibitory activities, whereas the high-molecular-weight constituents (> 6 kDa) contributed to DPP-IV inhibition.

This is the first report of ACE-inhibitory and DPP-IV-inhibitory activities in housefly larvae, and these findings provide important insights into the biological activities of housefly larvae. Moreover, our results suggested that housefly larvae may be useful source of peptides for developing multifunctional nutraceuticals that could prevent oxidative stress, hypertension, and type 2 diabetes.

3.1 Introduction

Housefly larvae have been an important resource in traditional Chinese medicine for the treating malnutritional stagnation, decubital necrosis, ecthyma, and lip boil since the 14th century [1] and are thought to represent a high-quality protein source that could be used as a livestock feed [2]. Recently, some reports on housefly larvae extracts or peptide fractions have shown that they possess useful bioactivities, including antioxidant, antibacterial, and antitumor activities [1, 3-5]. However, it is unclear whether these bioactive compounds possess other multifunctional activities, and insects possessing multifunctional activities, such as antioxidant activity, ACE-inhibitory activity, and DPP-IV-inhibitory activity have not been reported.

The purpose of this study is to investigate whether multifunctional bioactive compounds with antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activities present in housefly larvae. First, we prepared housefly larvae water extract (HLWE) using decoction method. Second, we investigated the biological activities of HLWE, including antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activities to explore the usefulness of housefly larvae as materials for the development of multifunctional nutraceuticals.

3.2 Materials

Dried housefly larvae were kindly provided by E's Inc. (Tokyo, Japan). 2,2-Diphenyl-1-picrylhydrazyl (DPPH, D4313) and fluorescent substrate H-(2)Abz-Acp(6)-Ala-Phe(4-NO_2)-Leu-OH (N09830) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), respectively. ACE from rabbit lungs (EC 3.4.15.1, A6778, ≥ 2 U/mg protein), porcine DPP-IV (EC 3.4.14.5, D7052, ≥ 10 U/mg protein), and Gly-Pro-*p*NA (G0513) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Centricon Plus-70 centrifugal filter units were purchased from Merck Millipore Co. (Tokyo, Japan). Quick-CBB PLUS and all other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3.3 Methods

3.3.1 Preparation of HLWE

Housefly larvae are an important resource in traditional Chinese medicine. In generally, water is often used to extract the active ingredients from herbs in traditional Chinese medicine. Hence, we used water to extract the bioactive components from housefly larvae in this study. We prepared housefly larvae water extract using decoction method, which is one of the common methods of processing medicinal herbs for the treatment of diseases using Chinese medicine [6]. The decoction method (Fig. 3.1) was performed as follows:

First, dried housefly larvae powder (0.5 g) and deionized water (25 mL) were added to an earthen pot, and the housefly larvae were immersed for 30 min. The samples were then simmered for 10 min (heat was reduced after the water began to boil), after which the heat was turned off, and the supernatant was removed. An equal volume of deionized water was added to the pot, and the same procedure was repeated. All supernatants were combined and centrifuged at 4,900 × *g* for 10 min. The supernatants were freeze-dried, and the HLWE was obtained. The HLWE was used for further studies. The extraction yield of HLWE was calculated using the following equation:

$$\text{Extraction yield of HLWE (\%)} = W/M \times 100$$

where W is the weight of obtained HLWE, M is the weight of dried housefly larvae powder used in the extraction.

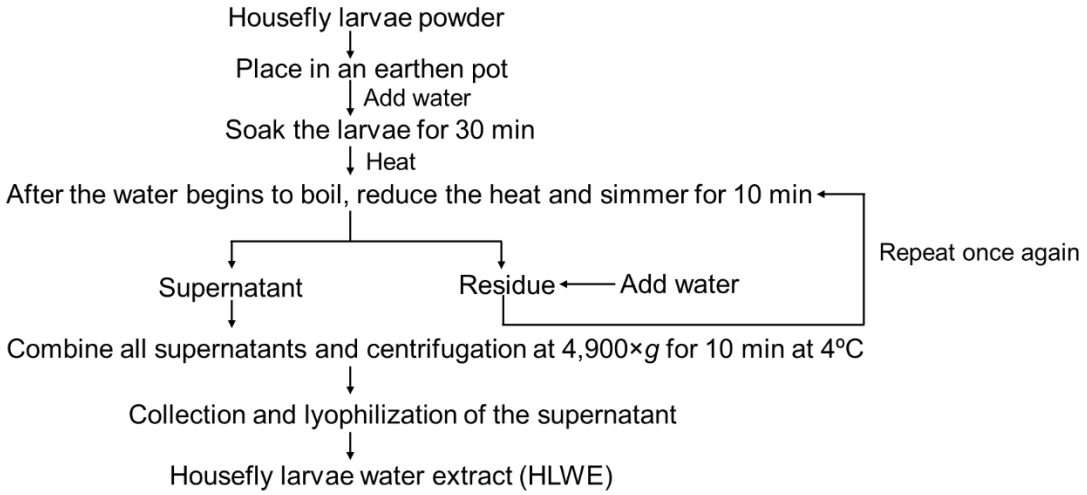

Fig. 3.1 Preparation procedures for housefly larvae water extract (HLWE).

3.3.2 Molecular distribution of HLWE by SDS-PAGE

To determine the molecular weight distribution of the HLWE, we carried out SDS-PAGE using 15% resolving and 5% stacking gels. The gels were then stained with Quick-CBB PLUS.

3.3.3 Fractionation of HLWE by ultrafiltration

For fractionation, the HLWE was prepared (5 g of housefly larvae powder was used) according to the procedures described in Section 3.3.1, and the extract was separated by ultrafiltration using a Centricon Plus-70 centrifugal filter device. HLWEs were divided into two fractions by molecular weight, and the fractions were designated HLWE-L (the low-molecular-weight fraction) and HLWE-H (the high-molecular-weight fraction). All fractions were collected and lyophilized for further experiments.

3.3.4 Confirmation of fractionation of HLWE by tricine-SDS-PAGE

To confirm whether the HLWE was fractioned successfully, the HLWE, HLWE-H, and HLWE-L were characterized by tricine-SDS-PAGE modified by Helen J *et al*. [7]. The gels were then stained with Quick-CBB PLUS.

3.3.5 Gel filtration chromatography

The molecular weight distributions of HLWE, HLWE-H, and HLWE-L were determined by gel filtration chromatography on a 1220 Infinity LC System (Agilent

Technologies, Santa Clara, CA, USA) equipped with a TSK gel G2000 SW_{XL} column (7.8 × 300 mm, 5 μm; Tosoh Co., Tokyo, Japan) with 50 mM phosphate buffer containing 0.3 M NaCl (pH 6.9). The flow rate was 0.2 mL/min, and absorbance was measured at 220 nm. A molecular weight calibration curve was prepared using molecular weight markers (1,350–670,000 Da; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3.3.6 Amino acid analysis of HLWE and its fraction

HLWE and its fractions were hydrolyzed using 6 M HCl for 24 h at 110°C in vacuum tubes sealed under a nitrogen atmosphere. After hydrolysis, the HCl was removed *in vacuo* and the powder was redissolved in 0.02 M HCl. Amino acid analyses were performed using an automatic analyzer (L-8900; Hitachi, Tokyo, Japan). The amount of each amino acid was calculated based on the peak area in comparison with that of the standard. The experiments were performed in duplicate, and the results are presented as means.

3.3.7 Biological activity assays of the HLWE and its fractions

3.3.7.1 Measurement of antioxidant activity

To determine the antioxidant activity of the HLWE, we performed the DPPH radical scavenging assay according to the method described by Dey *et al*. [8], with some modifications. DPPH was dissolved in 99.5% ethanol solution (0.15 mM). HLWE samples were dissolved in distilled water at final concentrations of 1.25, 2.50, and 5.00 mg/mL. Bovine serum albumin (BSA) and vitamin C were used as negative and positive controls, respectively, with concentrations of 1.25, 2.50, and 5.00 mg/mL. Briefly, the test samples (120 μL) were pipetted onto 96-well microplates, and DPPH solution (120 μL) was then added. The microplates were incubated at 25°C for 30 min, and the absorbance of the DPPH radical was measured at 517 nm with a microplate reader (Immuno-Mini NJ-2300; Nalge Nunc International Co., USA). Lower absorbance indicated higher free radical scavenging activity. The DPPH radical scavenging activity was determined using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = (Ac - [As - Asb]) / Ac \times 100$$

where Ac is the absorbance of the control (DPPH solution without test sample), As is the absorbance of the test sample plus DPPH solution, and Asb is the absorbance of the test sample plus ethanol without DPPH solution.

3.3.7.2 ACE inhibition assay

ACE-inhibitory activity was measured by the fluorescence method, as described by Ando *et al*. [9-11]. This method is based on the ability of ACE to hydrolyze the fluorescent substrate H-(2)Abz-Acp(6)-Ala-Phe(4-NO_2)-Leu-OH. Briefly, HLWE solution (1.00 mL) was mixed with Tris-HCl plus NaCl buffer solution (0.22 M, pH 7.4, 1.55 mL). Then, substrate solution (79.8 μM, 300 μL) was added and pre-incubated for 3 min at 37°C. The reaction was initiated by adding ACE solution (50.0 mU/mL, 150 μL), and the solution was incubated at 37°C for 15 min. Test samples were dissolved at final concentrations of 0.75, 1.50, and 3.00 mg/mL. BSA and tripeptide IPP were used as a negative and positive control, respectively. The fluorescence was measured using a spectrofluorimeter (JASCO FP-6500; JASCO Co., Tokyo, Japan) with excitation and emission wavelengths of 340 and 415 nm, respectively. ACE inhibition (%) was calculated as follows:

$$\text{ACE inhibition (\%)} = (C - T) / C \times 100$$

where C is the fluorescence intensity in the absence of the HLWE, and T is the fluorescence intensity in presence of the HLWE.

3.3.7.3 DPP-IV inhibition assay

DPP-IV-inhibitory activity was determined using a colorimetric method with Gly-Pro-*p*-nitroanilide (Gly-Pro-*p*NA) as a substrate. The DPP-IV inhibitory assay was performed as previously described [12] with some modifications. Briefly, the reaction system was composed of enzyme, substrate, and test samples in Tris-HCl buffer (0.1 M, pH 8.0). Test samples (125 μL) and substrate solution (0.8 mM, 125 μL) were mixed and incubated at 37°C for 10 min. The reaction was initiated by adding the DPP-IV solution (5.00 mU/mL, 250 μL), and the solution was incubated at 37°C for 60 min. The reaction was terminated by adding 3% acetic acid (0.5 mL), and the absorbance of the released *p*NA was measured using a UV-spectrophotometer (JASCO Ubest V-560; JASCO Co., Tokyo, Japan) at 380 nm [13]. Samples were dissolved in buffer with final concentrations of 1.25, 2.50, and 5.00 mg/mL. BSA and IPP were used as negative and positive controls, respectively. The DPP-IV inhibition (%) was calculated as follows:

$$\text{DPP-IV inhibition (\%)} = (Ac - [As - Asb]) / Ac \times 100$$

where Ac is the absorbance without inhibitor, As is the absorbance in the presence of inhibitor, and Asb is the absorbance of the sample without DPP-IV solution.

3.3.8 Statistical analysis

All experiments were performed in triplicate. The significance of the differences between HLWE and BSA was evaluated by Student's *t*-tests ($P < 0.05$). All data are expressed as means ± standard deviations (SDs). In addition, the significance of the differences in some data was evaluated by analysis of variance test ($p < 0.05$) (ANOVA).

3.4 Results

3.4.1 Preparation of HLWE and molecular distribution of HLWE by SDS-PAGE

We prepared housefly larvae water extracts using decoction method, and examined its molecular weight distribution by SDS-PAGE. The extraction yield of the HLWE was 29.2% ± 2.43% (in the scale of 0.5 g) and 41.6% (in the scale of 5 g), respectively. The molecular weight distribution of protein contained in the HLWE was estimated by SDS-PAGE, as shown in Fig. 3.2. Proteins with a wide range of molecular weights were observed in the HLWE, and many proteins had molecular weights of less than 25 kDa. Some clear bands around 37, 50, 75, 150, and above 250 kDa were also observed.

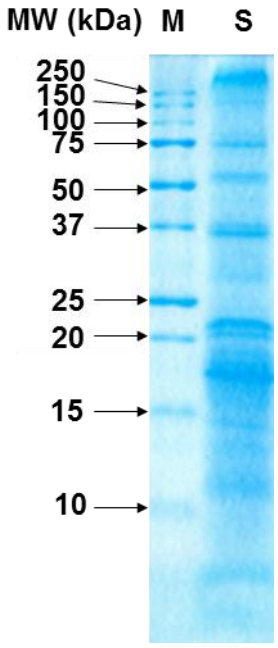

Fig. 3.2 SDS-PAGE of HLWE. Lane M showed the molecular weight of marker (10-250 kDa), and Lane S showed the profiles of HLWE.

3.4.2 Fractionation of HLWE and confirmation of fractionation by tricine-SDS-PAGE

HLWE was fractionated into two fractions, the low-molecular-weight fraction (HLWE-L) and high-molecular-weight fraction (HLWE-H) by ultrafiltration. The recovery rate of HLWE-L and HLWE-H were 41.6% and 34.8%, respectively. The profiles of fractionation of HLWE by ultrafiltration were confirmed by tricine-SDS-PAGE, as depicted in Fig. 3.3. It was confirmed that HLWE was fractionated around at 10 kDa. In order to confirm the molecular weight distribution of each fraction in more detail, gel filtration chromatography was performed. The details are described in Section 3.4.3.

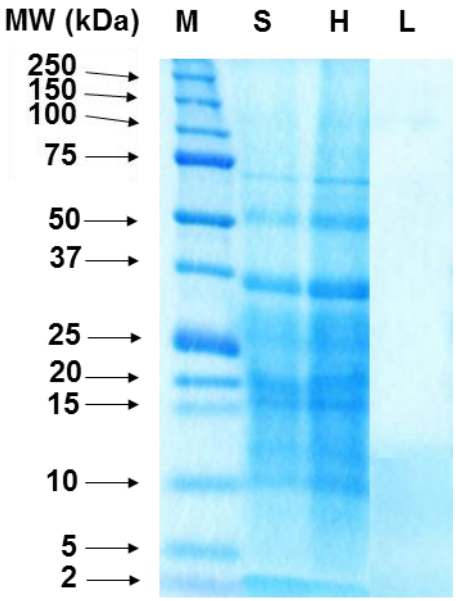

Fig. 3.3 Tricine-SDS-PAGE of HLWE and its fractions by ultrafiltration. Lane M showed the molecular weight of marker (2-250 kDa), and Lane S, Lane H, and L showed the profiles of HLWE, HLWE-H, and HLWE-L, respectively.

3.4.3 Determination of molecular distribution of HLWE and its fractions by gel filtration chromatography

As shown in Fig. 3.4, in order to confirm the molecular weight distribution of HLWE and its fractions in detail, gel filtration chromatography was performed. Similar to the results of tricine-SDS-PAGE described in Section 3.4.2, it was shown that the HLWE was efficiently fractionated by ultrafiltration. The constituents of HLWE were

distributed over a broad range, and many peaks were observed between 0.4 and 70 kDa. Several peaks around 2.5, 3.5, 23, and 34 kDa were quantified, and HLWE was further fractionated around at 6 kDa, which was established as the boundary between low-molecular-weight (0.4–6 kDa) and high-molecular-weight (6–70 kDa) HLWEs. The two fractions, HLWE-L (< 6 kDa) and HLWE-H (> 6 kDa) were obtained.

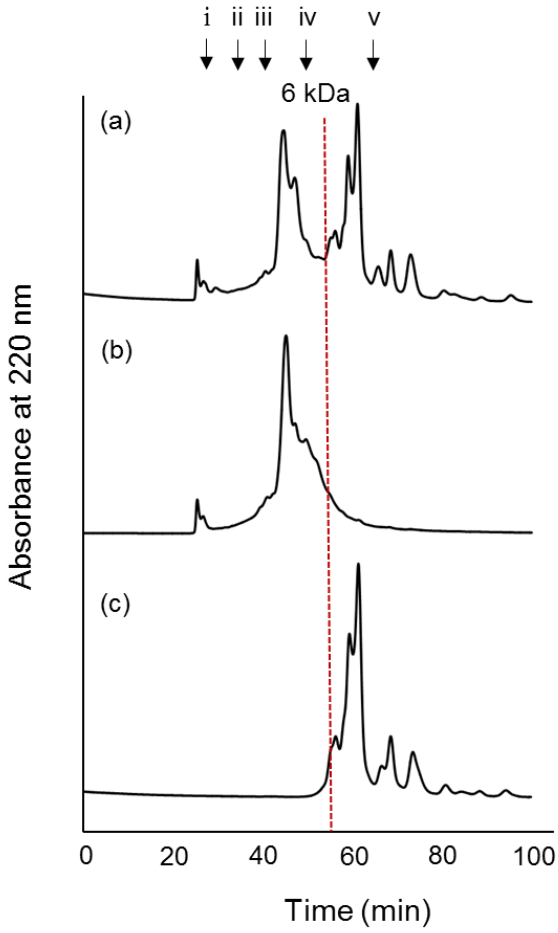

Fig. 3.4 Chromatograms profile of HLWE and its fractions derived from gel filtration chromatography. Chromatograms for (a) HLWE, (b) HLWE-H, and (c) HLWE-L. Elution times of the molecular mass standards: (i) thyroglobulin (670,000 Da), (ii) γ-globulin (158,000 Da), (iii) ovalbumin (44,000 Da), (iv) myoglobin (17,000 Da), and (v) vitamin B_{12} (1,350 Da) are indicated with arrows. The vertical line in the figure indicates 6 kDa.

3.4.4 Amino acid composition of HLWE and its fractions

The amino acid compositions of HLWE, HLWE-L, and HLWE-H are shown in Table 3.1. The overall constituent amino acids of these fractions were similar. Hydrophobic amino acids were found at similar percentages in HLWE-L (28.3%) and

HLWE-H (30.0%). The contents of aromatic and acidic amino acids in HLWE-L (14.3% and 44.7%, respectively) were greater than those in HLWE-H (7.5% and 31.3%, respectively), whereas the percentage of basic amino acids in HLWE-L (12.8%) was lower than that in HLWE-H (18.4%).

Table 3.1 Amino acid composition of HLWE and the fractions of HLWE by ultrafiltration (% of total amino acids).

Amino acid	HLWE	HLWE-L	HLWE-H
Essential amino acids			
Ile	2.5	1.5	3.3
Leu	4.2	2.3	5.5
Lys	6.9	3.6	9.3
Met	0.7	0.7	0.9
Phe	6.4	9.4	4.2
Thr	3.7	2.0	5.0
Trp	NA	NA	NA
His	3.9	1.5	5.6
Val	4.6	2.3	6.2
Nonessential amino acids			
Arg	5.3	7.7	3.5
Ala	5.7	4.8	6.4
Asx*	9.7	5.9	12.3
Cys	NA	NA	NA
Glx*	27.2	38.8	19.0
Gly	5.2	4.2	5.7
Pro	5.9	8.0	4.4
Ser	4.3	2.5	5.5
Tyr	3.8	4.9	3.3
Aromatic amino acids (AAAs)	10.2	14.3	7.5
Hydrophobic amino acids (HAAs)	29.3	28.3	30.0
Acidic amino acids	36.9	44.7	31.3
Basic amino acids	16.1	12.8	18.4

NA: not analyzed.

* Asx: Asp + Asn, Glx: Glu + Gln

3.4.5 Biological activities

3.4.5.1 Antioxidant activity of HLWE and its fraction

Analysis of DPPH radical scavenging activity is one of the most commonly used methods for antioxidant activity measurement. The HLWEs showed concentration-dependent antioxidant activities of 48.3% (1.25 mg/mL), 59.7% (2.50 mg/mL), and 75.4% (5.00 mg/mL), as depicted in Fig. 3.4. BSA was used as a negative control. Notably, the HLWE showed significantly higher DPPH radical scavenging activity than BSA at all assay concentrations (Fig. 3.5). HLWE-L showed higher antioxidant activity than HLWE-H at all assay concentrations (Table 3.2, Fig. 3.6); HLWE-L showed relative antioxidant activities of 91.0% (1.25 mg/mL), 78.9% (2.50 mg/mL), and 65.3% (5.00 mg/mL) using HLWE as a standard (100%), and HLWE-H showed relative antioxidant activities of 35.1% (1.25 mg/mL), 36.1% (2.50 mg/mL), and 36.5% (5.00 mg/mL), respectively.

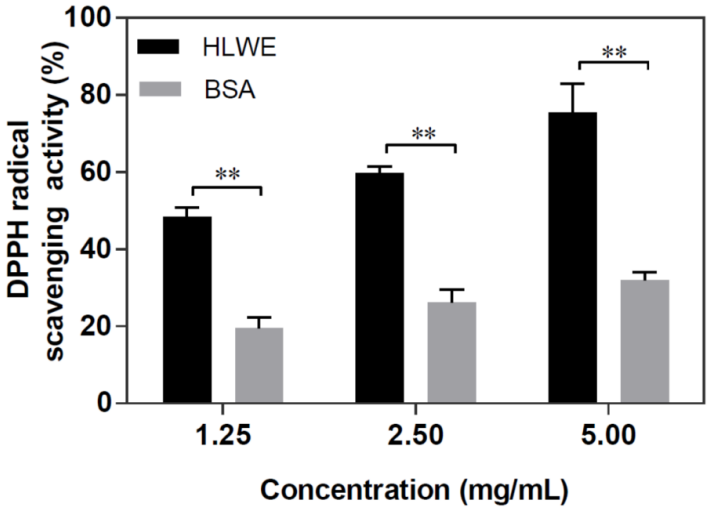

Fig. 3.5 Antioxidant activities of the HLWE and BSA. Concentrations: 1.25, 2.50, and 5.00 mg/mL. BSA was used as a negative control. Values are expressed as means ± SDs. $^{**}P < 0.01$.

Table 3.2 Relative antioxidant activity of the fractions of HLWE by ultrafiltration.

Fraction	Concentration (mg/mL)		
	1.25	2.50	5.00
	Relative activity (%)		
Original HLWE	100	100	100
HLWE-L	91.0 ± 5.3	78.9 ± 3.7	65.3 ± 1.8
HLWE-H	35.1 ± 4.7	36.1 ± 5.0	36.5 ± 3.7

The activity of the fractions of HLWE after ultrafiltration is shown as activity relative to HLWE.

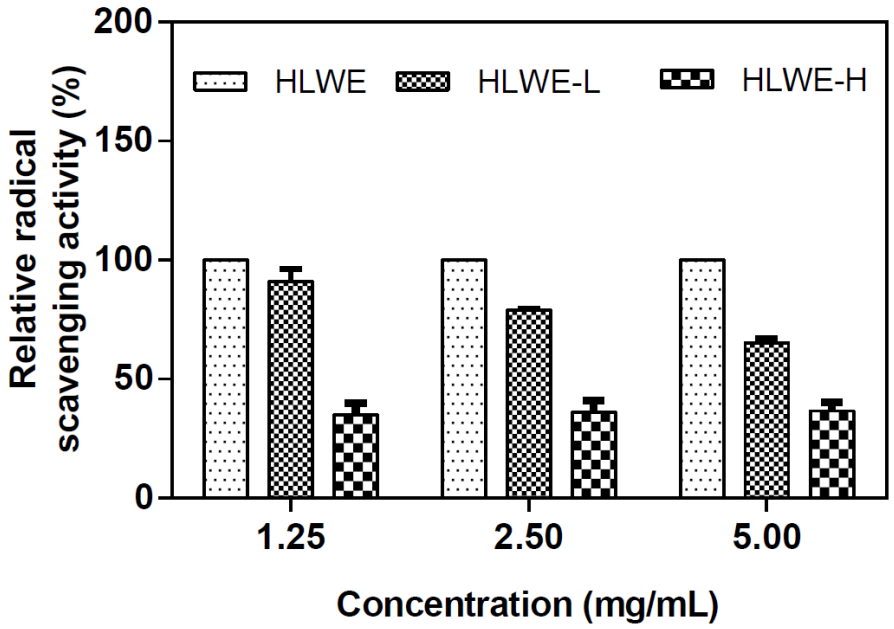

Fig. 3.6 Relative antioxidant activities of HLWE, HLWE-L, and HLWE-H. Concentrations: 1.25, 2.50, and 5.00 mg/mL. The antioxidant activity of HLWE was used as a standard (100%). The antioxidant activities of HLWE-L and HLWE-H are expressed as relative activity compared with HLWE.

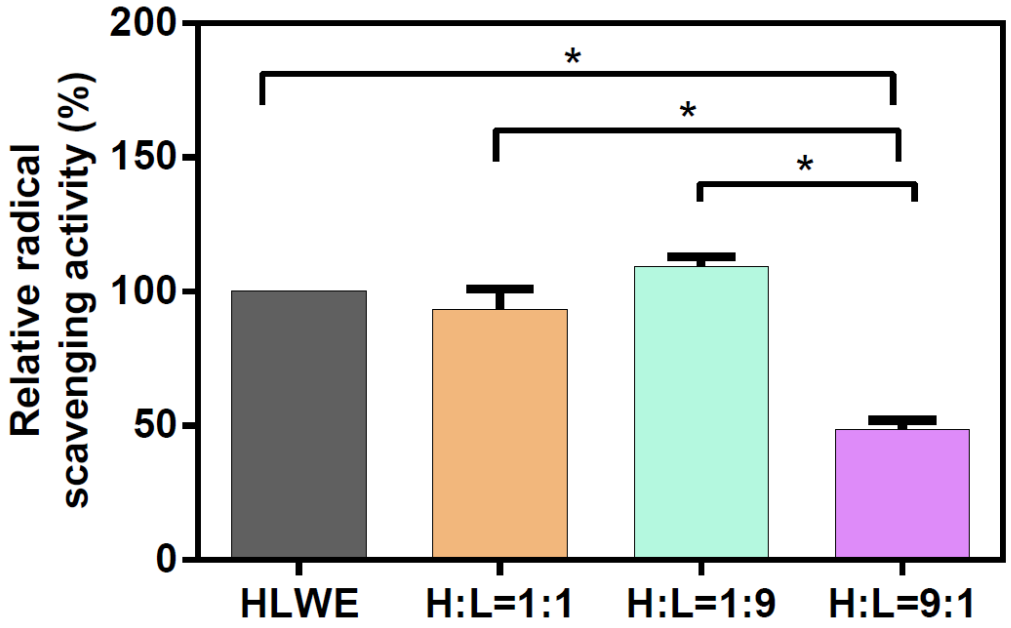

Fig. 3.7 Relative antioxidant activities of the mixtures of HLWE-H and HLWE-L at the different ratio (1:1, 1:9, and 9:1) (w/w) at concentrations of 1.25 mg/mL. H and L are the abbreviations of HLWE-H and HLWE-L, respectively. The antioxidant activity of HLWE was used as a standard (100%). The antioxidant activities of HLWE-L and HLWE-H at different ratios are expressed as relative activity compared with HLWE. Values are expressed as means ± SDs. *$P < 0.05$.

As depicted in Fig. 3.6, HLWE-L showed higher antioxidant activity than HLWE-H (Fig. 3.6). However, both fractions of HLWE-H and HLWE-L showed lower activity compared with original HLWE. In order to confirm why HLWE-L and HLWE-H exhibited lower antioxidant activity compared with HLWE, the antioxidant measurements of a mixture of HLWE-H and HLWE-L at three different ratios (1:1, 1:9, and 9:1) were performed. The results are shown in Fig 3.7. As the ratio of HLWE-L increased, the antioxidant activity of the mixture increased. In addition, it was confirmed that as the ratio of HLWE-L was increased (50% → 90%), the antioxidant activity of the mixtures gradually increased to the level of the antioxidant activity exhibited by the original HLWE. From these results, it was suggested that HLWE-L mainly contribute to the antioxidant activity of HLWE, and the synergistic effect of the HLWE-H and HLWE-L may also affect the activity of HLWE.

3.4.5.2 ACE-inhibitory activity assay

The ACE-inhibitory activity of the HLWE was determined at different concentrations (Fig. 3.8). HLWE showed concentration-dependent ACE-inhibitory activities of 52.7% (0.75 mg/mL), 68.9% (1.50 mg/mL), and 87.2% (3.00 mg/mL), similar to the antioxidant activities. The half-maximal inhibitory concentration (IC_{50}) value with regard to ACE-inhibitory activity was 0.430 mg/mL. BSA was used as a negative control. HLWE showed significantly higher ACE-inhibitory activity than BSA at all concentrations. In particular, ACE inhibition by the HLWE was about 9 times greater than that of BSA at a concentration of 0.75 mg/mL. These findings suggested that the HLWE may be a useful foodstuff with moderate ACE-inhibitory activity.

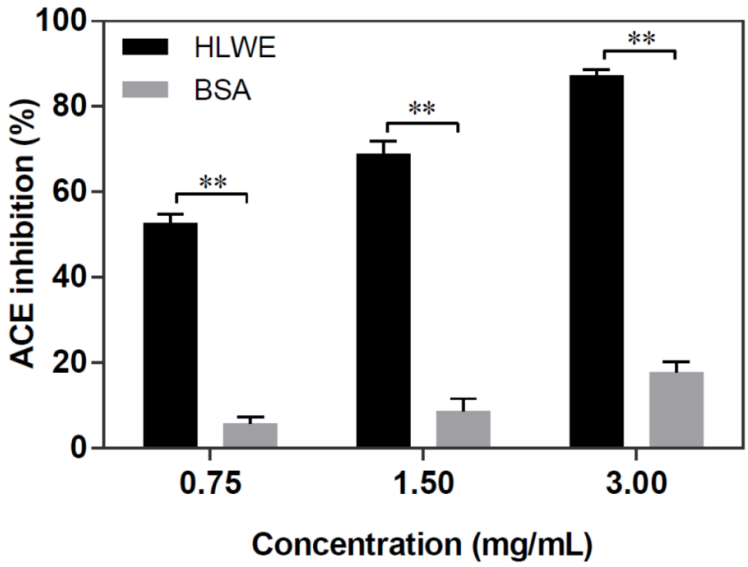

Fig. 3.8 ACE-inhibitory activities of the HLWE and BSA. Concentrations of 0.75, 1.50, and 3.00 mg/mL. BSA was used as a negative control. Values are expressed as means ± SDs. $^{**}P < 0.01$.

Table 3.3 Relative ACE inhibition of the fractions of HLWE by ultrafiltration.

Fraction	Concentration (mg/mL)		
	0.75	1.50	3.00
	Relative inhibition (%)		
Original HLWE	100	100	100
HLWE-L	142 ± 9.0	120 ± 0.6	112 ± 0.3
HLWE-H	15.7 ± 3.6	28.8 ± 1.2	78.7 ± 1.5

The activity of the fractions of HLWE after ultrafiltration is shown as activity relative to HLWE.

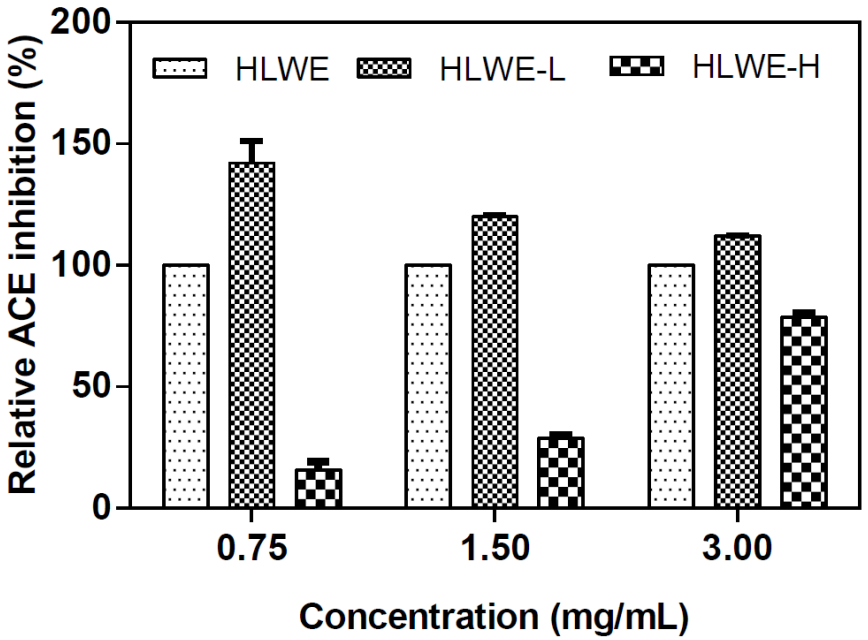

Fig. 3.9 Relative ACE-inhibitory activities of HLWE, HLWE-L, and HLWE-H at different concentrations. Concentrations: 0.75, 1.50, and 3.00 mg/mL. The ACE inhibition of HLWE was used as a standard (100%). The ACE inhibition of HLWE-L and HLWE-H are expressed as relative activity compared with HLWE.

Table 3.3 and Fig. 3.9 show the relative ACE-inhibitory activity of the two fractions (HLWE-L and HLWE-H) compared with that of the HLWE. These fractions showed strikingly different inhibitory activities. In particular, HLWE-L exhibited potent ACE-inhibitory activity compared with HLWE-H; specifically, HLWE-L showed ACE-inhibitory activities about 1.4- and 9-fold stronger than those of HLWE and HLWE-H, respectively, at a concentration of 0.75 mg/mL (Table 3.3).

3.4.5.3 DPP-IV-inhibitory activity assay

The HLWE exhibited concentration-dependent DPP-IV-inhibitory activity (23.1% at 1.25 mg/mL, 40.0% at 2.50 mg/mL, and 66.3% at 5.00 mg/mL; Fig. 3.10). The IC_{50} value was 3.52 mg/mL. In this study, BSA and IPP were used as negative and positive controls, respectively. The HLWE showed significantly higher DPP-IV inhibitory activity than BSA at all concentrations (Fig. 3.10). IPP is a DPP-IV-inhibitory peptide [14], and the HLWE showed strong DPP-IV-inhibitory activity (66.3%) compared with that of IPP (73.3%) at the same concentration (5.00 mg/mL; data not shown). In contrast to the other two biological activity assays, in DPP-IV-inhibitory assays, HLWE-L did not show stronger activity than HLWE-H (Table 3.4, Fig. 3.11). HLWE-L only showed 69.6% relative activity compared with HLWE at a concentration of 1.25 mg/mL, whereas, HLWE-H exhibited higher relative activity compared with HLWE at all concentrations; 136% (1.25 mg/mL), 125% (2.50 mg/mL), and 131% (5.00 mg/mL).

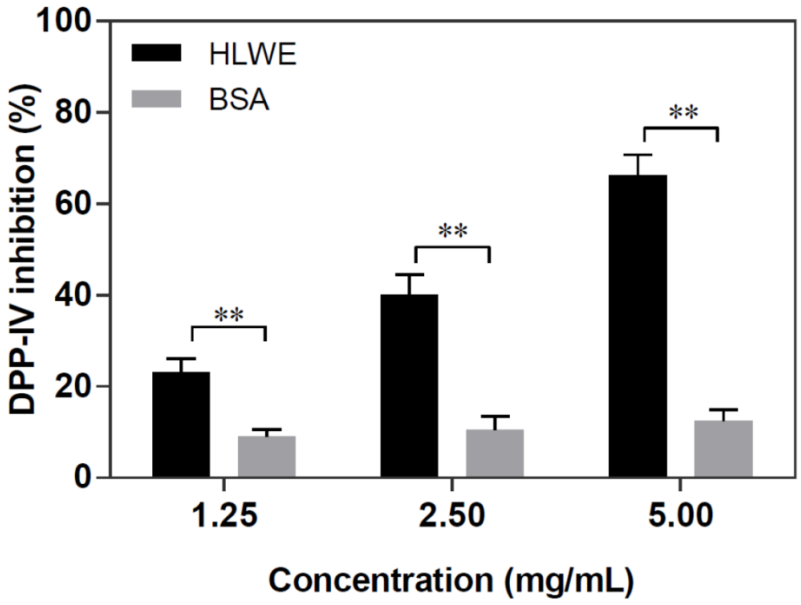

Fig. 3.10 DPP-IV-inhibitory activity of the HLWE (1.25, 2.50, and 5.00 mg/mL). BSA was used as a reference. Values are expressed as means ± SDs. $^{**}P < 0.01$.

Table 3.4 Relative DPP-IV inhibition of the fractions of HLWE by ultrafiltration.

Fraction	Concentration (mg/mL)		
	1.25	2.50	5.00
	Relative inhibition (%)		
Original HLWE	100	100	100
HLWE-L	69.6 ± 2.9	45.5 ± 4.8	35.6 ± 4.1
HLWE-H	136 ± 2.9	125 ± 9.7	131 ± 3.9

The activity of the fractions of HLWE after ultrafiltration is shown as activity relative to HLWE.

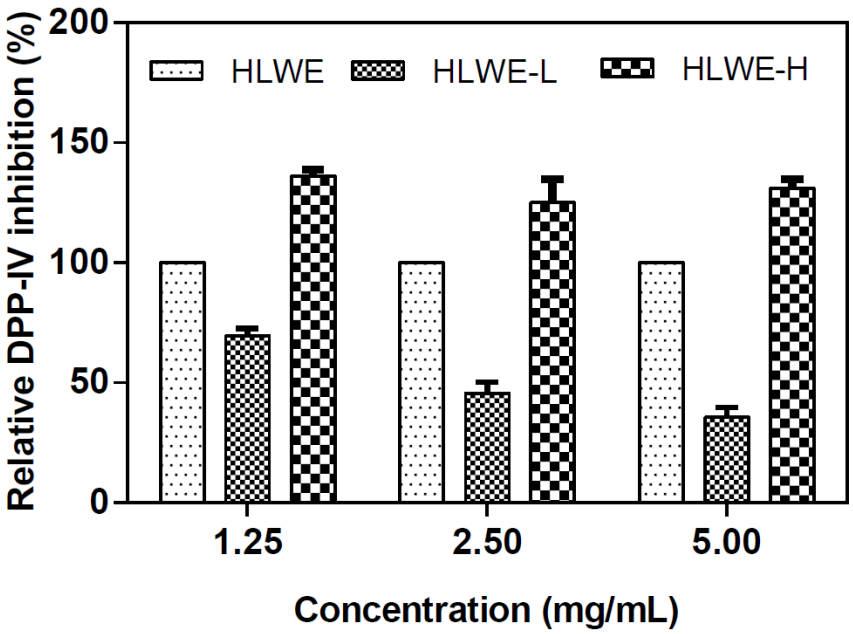

Fig. 3.11 Relative DPP-IV-inhibitory activities of HLWE, HLWE-L, and HLWE-H at concentrations of 1.25, 2.50, and 5.00 mg/mL. The DPP-IV inhibition of HLWE was used as a standard (100%). The DPP-IV inhibition of HLWE-L and HLWE-H are expressed as relative activity compared with HLWE.

3.5 Discussion

Insects have not been well explored or exploited compared with other types of organisms [15]. In 2013, the FAO published a report entitled, “Edible insects: future prospects for food and feed security” [16]. According to this report, more than 1,900 species of insects, including housefly larvae, have been used as foods, and these edible insects could be a solution for food and feed security. Hence, it is necessary to evaluate the usefulness of insects based on scientific investigation.

Houseflies are a common type of fly found worldwide. In this study, we prepared housefly larvae water extract using decoction method to evaluate the applicability of housefly larvae extracts. The decoction method is an extraction method for active ingredients from herbs in traditional Chinese medicine. The decoction method has been used for thousands of years and is considered a convenient and safe method because water is used as a solvent. Liu *et al*. prepared a water extract (WE) of *Holotrichia parallela* Motschulsky (large black chafer, a beetle of the Scarabaeidae subfamily) with a microwave-accelerated reaction system and measured the extract yield of the WE (25.03% ± 1.85%) [17]; their results were similar to our results (29.2% ± 2.4%, in the scale of 0.5 g) in the current study. In addition, Chu *et al*. prepared housefly larvae protein-enriched extracts (PE) by homogenization treatment, and bands with molecular weights of 14–20 kDa were confirmed [18]. Moreover, bands of 14–25 kDa could be linked to housefly larvae lysozyme and a thermal stable antimicrobial protein with a molecular weight of approximately 16 kDa [19, 20]. We detected even more protein bands in SDS-PAGE of the HLWE compared with that of the PE. These results suggested that the decoction method, which is frequently used in traditional Chinese medicine, could be an ideal, safe, convenient method for HLWE preparation.

The HLWE showed 48.3% DPPH radical scavenging activity at a concentration of 1.25 mg/mL, which was comparable to that reported by Zhang *et al*. In their report, the housefly larvae hydrolysates prepared using alcalase and neutral proteinase exhibited 36.5% and 39.6% DPPH radical scavenging activities, respectively, at a concentration of 1.00 mg/mL [3]. Whey and egg white protein hydrolysates prepared by enzymes exhibited 62.8–73.1% and 73.1% DPPH radical scavenging activity, respectively, at a concentration of 10 mg/mL, respectively [21, 22]. Thus, based on these results, the HLWE could be a strong antioxidant. Generally, smaller peptides showed relatively higher antioxidant activity [23]. HLWE-L showed higher antioxidant activity than HLWE-H at all assay concentrations, which could be attributed to the smaller constituents in HLWE-L. However, HLWE-L did not display higher antioxidant activity compared with HLWE. This result was consistent with the results reported by Zhou *et al*., who

prepared abalone viscera hydrolysates (AVHs) using five proteases and fractionated each hydrolysate; the low-molecular-weight fraction of AVH did not show stronger antioxidant activity [23]. In this study, HLWE-L exhibited stronger antioxidant activity compared with HLWE-H at all assay concentrations. This may be due to the presence of peptides that are rich in hydrophobic and aromatic amino acids in the HLWE-L. According to a report by Chi *et al*., hydrophobic amino acids and aromatic amino acids are important for the antioxidant activity of peptides [24]. Our results suggested that antioxidant activity was associated not only with the molecular weights of peptides and proteins but also with the amino acid composition and amino acid sequence of peptides/proteins.

The IC_{50} value of the HLWE for ACE-inhibitory activity was 0.430 mg/mL. This value was lower than that of the Tris/HCl buffer extracts from *Bombus terrestris* and *Schistocerca gregaria* (two insect species), which exhibited ACE-inhibitory activities with IC_{50} values of 3.935 ± 0.014 and 3.109 ± 0.546 mg/mL, respectively [25]. The hot water extract from the muscle of golden freshwater clams exhibited ACE-inhibitory activity with an IC_{50} value of 1.95 mg/mL [26]. Notably, the ACE-inhibitory activity of the HLWE was higher than those reported previously. Our results suggested that the HLWE could be applied as an ACE-inhibitory agent in the development of functional foods and nutraceuticals. HLWE-L showed about 9-fold higher ACE inhibition than HLWE-H at a concentration of 0.75 mg/mL. This result may be attributed to the low-molecular-weight peptides contained in HLWE-L. According to several reports on ACE inhibition, it has been reported that low-molecular-weight-peptides made a greater contribution to ACE-inhibitory activities than high-molecular-weight-peptides [22, 26]. In addition, according to a report by He *et al*., hydrophobic amino acids and aromatic amino acids are important for the ACE-inhibitory activities of peptides [27]. HLWE-L contained a higher proportion of hydrophobic and aromatic amino acids (42.6%) than did HLWE-H (37.5%). Therefore, the low molecular size and amino acid composition of HLWE-L may synergistically contribute to ACE-inhibitory activity.

The HLWE exhibited high DPP-IV-inhibitory activity at 5.00 mg/mL, comparable with IPP at same concentration (5.00 mg/mL). The DPP-IV-inhibitory activity reported in our study was higher than that reported for brewer's spent grain protein-enriched isolate, which exhibits DPP-IV-inhibitory activity of less than 30% at 3.50 mg/mL [28]. The extract from salmon skin showed DPP-IV-inhibitory activity of about 10% at a concentration of 5.00 mg/mL [29]. Our results were comparable to those of these previous reports. Generally, smaller peptides show more potent DPP-IV inhibition than larger peptides [29]. However, in this study, HLWE-H showed higher DPP-IV inhibition

than HLWE-L. Gallego *et al*. reported that the DPP-IV-inhibitory activity of the < 1–3 kDa peptide fraction was higher than that of the smaller peptide fraction (< 1 kDa), which derived from Spanish dry-cured ham [30]. This report and our study suggested that the amino acid sequence and constituents are also important in addition to the molecular weight of peptides for mediating DPP-IV-inhibitory activity. According to a report by Xia *et al*., Ile, Leu, Phe, Val, Ala, Gly, and Pro are involved in determining the DPP-IV-inhibitory activities of peptides [31]. HLWE-H contains more those amino acids (total content: 35.7%) than HLWE-L was (total content: 32.5%). This may contribute to stronger DPP-IV inhibition by HLWE-H than by HLWE-L.

The HLWE exerted potent ACE-inhibitory activity, remarkable antioxidant effects, and DPP-IV-inhibitory activity. There are previous reports that ACE-inhibitory peptides possess antioxidant or DPP-IV-inhibitory activities simultaneously [32, 33]. In this study, it was elucidated for the first time that housefly larvae possess ACE-inhibitory and other two activities simultaneously. Moreover, the fractions of HLWE by ultrafiltration exhibited significantly different antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activities when compared with the original HLWE; we assumed that these multifunctional activities of HLWE were due to the presence of peptides. As revealed by Rao *et al*., proteins with abundant hydrophobic amino acid contents and positively charged amino acids may stimulate the generation of multifunctional peptides [34]. The amino acid analysis for housefly larvae (chapter 2) and HLWE showed that both materials contain hydrophobic and positively charged amino acids abundantly, supporting the finding that the HLWE exhibited multifunctional activities. Generally, antioxidant, ACE-inhibitory, and DPP-IV-inhibitory peptides can be generated by enzymatic hydrolysis [33, 34]. In this study, however, we prepared HLWE with multifunctional activities using a simple extraction method without enzymatic hydrolysis.

In chapter 2, housefly larvae were found to contain all EAAs and NEAAs, and the amino acid balance was similar to that of beef, chicken, pork, casein, and hen egg, which are typically considered ideal protein resources. Therefore, the results in chapter 2 combined with the results in this chapter suggested that housefly larvae could have applications as a good source of protein and multifunctional peptides possessing relevant biological activities. Further studies of the identification of constituents of the HLWE possessing various bioactivities are needed in order to determine why the HLWE exhibits these multifunctional activities.

3.6 Conclusion

In this chapter, the biological activity assays of the HLWE prepared using the decoction method were performed to investigate the usefulness of housefly larvae as a multifunctional nutrient agents. The current study indicated that housefly larvae may contain many antioxidant, ACE-inhibitory, and DPP-IV-inhibitory proteins, facilitating the development of foods and drugs in the future. Moreover, the decoction method could be an effective method to extract bioactive components from housefly larvae.

This is the first report of ACE-inhibitory and DPP-IV-inhibitory activities from housefly larvae. In addition, this is the first time that multifunctional activities such as antioxidant activity, ACE-inhibitory activity, and DPP-IV-inhibitory activity are confirmed in insects. Our findings provide important insights into the biological activities and applications of housefly larvae. Moreover, further researches described below will lead to progress of beneficial use of housefly larvae.

(1) Identification of multifunctional peptides contained in HLWE.
(2) Analysis of the structure-activity correlation of multifunctional peptides by molecular docking method.
(3) The design of more potent peptides exhibiting stronger activities according to the molecular docking results, which may contribute to drug design in future.
(4) To confirm whether HLWE exhibit other beneficial effects on therapeutic benefit.
(5) The conduction of toxicity test on HLWE for applying HLWE to functional foods *in vitro* and *in vivo*.

Chapter 4

Summary

The main objective of this research is to discover the potential usefulness of the housefly larvae as multifunctional nutraceutical agents. We first investigated its utility as feed and food, based on its amino acid composition. Second, we prepared a housefly larvae water extract (HLWE) using the decoction method i.e., a method used to extract active ingredients from herbs in traditional Chinese medicine. Subsequently, we investigated the multifunctional activities of this extract to explore the potential of housefly larvae as functional food agents.

In chapter 2, the utility of housefly larvae was evaluated by amino acid analysis. The amino acid composition of housefly larvae was compared with fishmeal, beef, chicken, pork, casein, and egg using the radar chart. In addition, in order to confirm the manner in which the housefly larvae were similar to protein sources such as fishmeal, beef, chicken, pork, casein, and egg, the degree of similarity in amino acid composition was calculated. This was done using the Canberra distance, a method used in cluster analysis. Here, the housefly larvae were found to contain sufficient amounts of all the essential amino acids. In addition, the amino acid composition was comparable to that of fishmeal, beef, chicken, pork, casein, and egg.

In chapter 3, in order to increase its applicability, the usefulness of the larvae as materials for the development of multifunctional foods was investigated. The housefly larvae water extract (HLWE) was prepared using the decoction method. This process has been used for centuries in traditional Chinese medicine. Subsequently, the biological activities of HLWE were explored. As a result, remarkable multifunctional activities, such as antioxidant activity, ACE-inhibitory activity, and DPP-IV-inhibitory activity were confirmed. Our results suggest the potential of housefly larvae as agents of antioxidant, ACE-inhibitory, and DPP-IV-inhibitory peptides for functional foods. Therefore, the usefulness of the housefly larvae was demonstrated as materials for the development of multifunctional nutraceuticals.

In chapter 4, we summarized the results of the current dissertation. In addition, the contributions and the special points of the current research were also described.

In this study, the potential utility of housefly larvae as a protein source was clearly suggested. The larvae have the potential to be used as feed, food, and agents for the development of multifunctional nutraceuticals with antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activities. The findings of this dissertation provide important insights into the biological activities and applications of the housefly larvae. Moreover, the current results will lead to the progress of the beneficial use of other insects. The contributions and the special points of this research are described as follows.

The contributions are as follows:

(1) ACE-inhibitory and DPP-IV-inhibitory activity were discovered from housefly larvae for the first time.
(2) It is the first time that multifunctional activities such as antioxidant, ACE-inhibitory, and DPP-IV-inhibitory activity were clarified in insects.
(3) The potential utility of the housefly larvae as materials for the development of multifunctional nutraceuticals was confirmed.
(4) The results indicated the potential of the housefly larvae as a protein source, which can be used as feed, food, and agents for the development of antioxidants, ACE inhibitors, and DPP-IV inhibitors.
(5) It was suggested that decoction method could be an effective method to extract bioactive compounds from the housefly larvae.
(6) This research provides a basis for further research on bioactive compounds from the housefly larvae.

The special points are as follows:

(1) For comparing the amino acid composition between the housefly larvae and fishmeal, beef, and other protein sources, the Canberra distance, a method to determine the distance, commonly used in the cluster analysis, was adopted.
(2) The decoction method, which has been used in traditional Chinese medicine, was incorporated into the extraction of bioactive compounds from the housefly larvae.

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Chapter 3

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