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Antioxidant Properties of Peptides Extracted from *Tenebrio molitor* Larvae

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The goal of this study was to identify new bioactive peptides in extracts derived from *Tenebrio molitor* (*T. molitor*) larvae for the development of functional foods. After extraction from freeze-dried *T. molitor* larvae with various solvents on time course, the extracts showed the highest 2,2,1-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity at 5 and 10 hr per total protein and solid contents, respectively. When the water extract was fractionated, a high methanol concentration led to a reduced level of high-molecular-weight proteins in the centrifugal supernatant, whereas increased DPPH activity in the supernatants suggests low-molecular-weight peptides may mediate antioxidant activity in the supernatant. Most of the organic solvent partitions, excluding butanol, showed similar activities in the water phases, and the organic solvent partition fraction exhibited a 28~44% decrease in activity following heat treatment, implying that some components in the fraction become unstable in the presence of heat. The addition of proteinase K to the water extract increased DPPH activity by 10~20%, suggesting that peptides, when released from total proteins, partially increase antioxidant activity. Therefore, we suggest that the antioxidants in *T. molitor* larval extracts make them a potential source of functional animal food.

Key words : Antioxidant, methanol fractionation, organic solvent extraction, partition, *T. molitor*

Introduction

More than a million species of insects have been reported, and millions more remain to be discovered. They constitute the largest number of species next only to microscopic life on earth and are the most abundant species in sheer diversity [3]. Since insects have evolved quite differently from vertebrates, plants and microbes, they may synthesize specific compounds with a unique structure as an environmental adaptation [20]. Extraction of a physiologically active substance from insects with pharmacological activity can be used to develop therapeutic drugs [16]. In addition, insects have been used widely in folk medicine in many areas to treat diseases or facilitate recovery from illness [14]. However, since research into substances with pharmacological activity from insects is limited to specific Asian countries, further systematic investigations are needed to discover biologically active compounds [14].

Tenebrio molitor, mealworm, belongs to Tenebrionidae. *Tenebrio molitor* larvae have traditionally been used in Asian countries including Korea to treat liver diseases, including liver cancer [18]. *Tenebrio molitor* is widely distributed worldwide and is known as a nocturnal insect that thrives on cereals. Since the development of large-scale breeding and supply systems, mealworm has been produced industrially as well as used as a food source in China and Netherlands [25]. Edible varieties of mealworm have been approved by Korea Food & Drug Administration (KFDA) since 2016 owing to their high protein content and nutritional value.

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The previous Studies for *T. molitor* larvae have reported bioactive materials such as anti-microbial peptides (AMPs), including tenecin 1, 2, 3 and 4 [6]. In addition to tenecin, several AMPs including pacifastin-related serine proteases have been identified via proteome analysis [26]. AMPs exhibit not only antibacterial action, but also anti-cancer effects [10, 15]. Functional analysis revealed the key role of crustacean pacifastin in immune response [5]. Additionally, pacifastin-like peptides in insects exhibit multiple regulatory functions associated with immunity, reproduction, and phase transition [5]. Recently, research on *T. molitor* larvae has been done on protein analysis and various component changes according to food sources, which the interest in the functional components of has been increasing in terms of future food resource development [9, 17]

In this study, bioactive substances in *Tenebrio molitor* larvae were investigated by separating fractions with antioxidant activity via organic solvent extraction, methanol fractionation, and variable organic solvent partition, followed by analysis of antioxidant activity related to peptides.

Materials and Methods

Measurement of DPPH activity

T. molitor larvae were purchased from an insect farm in South Korea (Insect Nala co. LTD). The larvae were freeze-dried, and stored at -70℃ or directly used for the study.

The DPPH-radical scavenging activity was measured using a modified method from a previous study [4]. Briefly, the reaction mixture was prepared with equal volumes of DPPH solution (D9132, Sigma, USA) and extract (0~1 mg/ml), mixed well, and then reacted for 20 min at room temperature. The solution was measured at a wavelength of 525 nm with a microplate reader (Multiscan GO, Thermo Scientific Co. Ltd., USA). Ascorbic acid was used as a positive control. Free radical-scavenging activity was determined by the difference in absorbance ratio between the control and sample, and calculated as follows: DPPH radical scavenging capacity (%) = [1 − (AS − AS0)/ A0] × 100%, where A0 is the absorbance of the negative control group without sample, AS0 represents the absorbance of the sample solution, and AS refers to the absorbance of the treated group with sample. IC50 value (mg/ml) indicates peptide or total solid (mg) to show the activity of 50% of DPPH radical scavenging capacity in ml reaction solution.

Assays of protein and total solid contents, and SDS-PAGE

Protein content was examined using the Bio-Rad protein assay kit (Bio-Rad, USA), according to Bradford method. Bovine serum albumin (BSA) was used as a protein standard to evaluate the unknown protein content. An aliquot of sample solution containing the total solid content was placed on a drying dish, and dried for 12 hr or more at 105℃. When the dried solid showed constant weight, the total solid content was measured.

SDS-PAGE was performed by mixing each sample with an SDS-sample loading buffer [21], and then boiled for 5 min. The extracted peptides were separated by SDS-PAGE, and the separated bands were visualized by Coomassie staining.

Assay of antibacterial activity

Antibacterial activity was evaluated using the diffusion method with 8 mm discs [2]. The applied extract was adjusted to a concentration of 20 mg/ml. Bacteria from the Korean Collection for Type Cultures (KCTC) were distributed and used as follows: Gram-negative bacteria including pathogenic *Escherichia coli* (ATCC11775), *Salmonella typhimurium* (ATCC14028), and *Vibrio parahaemolyticus* (ATCC 17802D-5); Gram-positive bacteria including *Bacillus cereus* (ATCC 1178IFO), *Clostridium perfringens* (ATCC13124), *Listeria monocytogenes* (ATCC 19114), and *Staphylococcus aureus* (ATCC112692); and the yeast *Candida albicans* (00432 ATCC 1023 IFO 1594). The allocated bacteria were subcultured in a nutrient broth (Difco, USA). The subcultured bacteria were smeared on nutrient agar and then positioned on an 8 mm disc with 0.2, 1, 2 and 4 mg/ml of each extract, followed by incubation for 24 hr at 37℃. After incubation, the antibacterial activity was evaluated based on the size of the clear zone around the disc.

Extraction depending on time with organic solvent

Organic solvent extraction was performed using pure water, methanol and ethanol. One part of the dried mealworm for extraction was mixed with 9 parts of the organic solvents. The mixed samples were homogenized for 5 min at 12,000 rpm using a homogenizer (T25basic, IKA Malaysia), and then reacted for 0, 5, 10, and 24 hr at 4℃. Insoluble precipitate from the reacted samples was separated by centrifugation at 3,000 × g for 20 min at 4℃. The supernatant was filtered with Whatman filter paper No. 2, and used to analyze the protein and total solid content, as well as DPPH and anti-

bacterial activity.

Methanol fractionation

The water extract was adjusted into final methanol concentrates of 0, 30, 50 and 70%, and incubated for more than 1 hr at 4℃. The reacted solutions were centrifuged at 3,000 × g for 20 min at 4℃, and then separated into methanol-soluble supernatant and insoluble precipitate. The supernatant was concentrated using a vacuum evaporator (RW-0252G 4000/G1, Heidolph, Germany), and powdered by freeze-drying (PVTFD10R, Ilsinbiobase, Korea). The powder was appropriately diluted to analyze the protein content, total solids, and DPPH activity.

Partition by organic solvent

The supernatant from 70% methanol fractionation was mixed with equal volumes of butanol, chloroform, diethyl ether, and hexane, and then incubated for more than 1 hr at 4℃. The reacted solutions were centrifuged at 3,000× g for 20 min at 4℃, and then extracted into water and organic solvent phases. The recovered phases were concentrated by a vacuum evaporator (RW-0252G 4000/G1, Heidolph, Germany) and then powdered by freeze-drying (PVTFD10R, Ilsinbiobase, Korea). The powder was appropriately diluted to analyze the protein content, total solids, and DPPH activity.

Analyses of thermal stability and peptide bioactive substances

For heat stability, the samples were reacted by the water phases obtained from the partition of organic solvent for 0, 10, 20 and 30 min at 80℃, and cooled to room temperature under ice water. The cooled sample was analyzed for DPPH activity.

To analyze the bioactive peptides, the water extract of *T. molitor* was reacted for 0, 0.5, 1, 2, and 10 hr at 37℃ with 40 mg/ml proteinase K, and then deactivated for 10 min at 80℃. The solution was analyzed for DPPH and antibacterial activities.

Statistical analysis

When significant differences were detected, the mean values were separated by differences in probability. The results are presented as least square means with standard deviations. Duncan's multiple range tests (MRT) were used to verify significant differences ($p<0.05$) between sample types. All the analyses were performed using the SAS statistical software package [version 9.1, SAS Inst., Inc., USA], and differences were considered significant at $p<0.05$.

Results and Discussion

Evaluation of antioxidant activity depending on extraction time

Although antibacterial compounds derived from *T. molitor* larvae were partially analyzed in previous studies [1, 11, 16], most antioxidants and other bioactive substances from *T. molitor* remains to be discovered. Therefore, in this study, the antioxidative activity was initially evaluated in water, methanol and ethanol extracts of *T. molitor* larvae. The results showed that the water extract had the highest DPPH activity, whereas the ethanol extract showed the least activity (Fig. 1A, Fig. 1C). Protein analysis showed that the water extract exhibited 3.3~3.9 and 0.8~1.7-fold higher DPPH activity than the ethanol and methanol extracts, respectively. The water and ethanol extracts showed the strongest activities at 0~5 hr extraction times (Fig. 1A, Fig. 1B, Fig. 1C). The methanol extract exhibited the strongest activity in the sample incubated for 0 hr at 4℃, whereas the others showed similar activity. Since the *T. molitor* larvae exhibited superior antioxidant activities when the extracts were treated with solvents briefly, it is suggested that the substances in free state in the cell are easily extracted.

In terms of total solid content, DPPH activity of the water extract was 4.8~9.0 and 2.4~3.2-fold higher than that of ethanol and methanol extracts, respectively. The differences in activity between water and ethanol or methanol extracts depending on total solid content were 1.1~5.1 and 1.7-fold higher than those based on protein, respectively. In addition, the water extract in total solid showed the highest activity at 10 hr, whereas the methanol and ethanol extracts exhibited the highest activities at 0 and 5 hr, respectively. Since the DPPH activities based on protein and total solid contents showed slight differences according to extraction time, it is suggested that extraction solvent and time for the activity varied depending on the final extraction of bioactive substances. However, in this study, since we were interested in the extraction of physiologically active peptides, the water extract at 0~5 hr was employed in the next experiment. All extraction methods from *T. monitor* showed antioxidant activity, but the most effective extraction method was found in ethanol and water mixture [8]. From these results, antioxidants are estimated to be slightly more water-soluble than fat-soluble, and it seems to be a pattern consistent with the results of this study.

Although antibacterial activity of all the extracts was ex-

A

Extraction time (hr)			0	5	10	24
IC50 (mg/ml)	protein	Water	0.25^{Cb}	0.23^{Cc}	0.34^{Bb}	0.48^{Ac}
		methanol	0.32^{Bb}	0.38^{Ab}	0.38^{Ab}	0.37^{Ab}
		Ethanol	0.98^{Ba}	1.06^{Ba}	1.62^{Aa}	1.58^{Aa}
	Total solid	Water	4.89^{ABb}	5.75^{Ac}	3.74^{Cc}	4.45^{BCc}
		methanol	12.10^{Cb}	14.83^{Ab}	12.75^{Bb}	14.59^{Ab}
		Ethanol	35.10^{Aa}	27.39^{Ba}	33.56^{ABa}	38.76^{Aa}

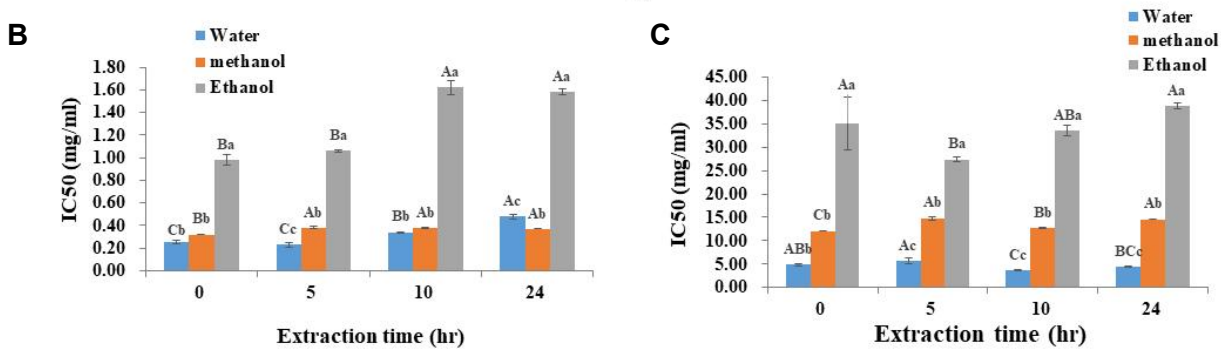


Fig. 1. DPPH activities depending on solvent extraction time. (A) IC50 value of DPPH activity at each extract time. (B) Analysis of activity at protein level. (C) Analysis of activity at total solid level. X- and Y-axes of A and B represent extraction time and DPPH IC50 value, respectively. This result was carried out by three replications. $^{a-c}$Valuesare significantly different in the same column from each other. $^{A-C}$Valuesare significantly different in the same row from each other.

amined by disc method, the activity was not detected in all samples (data not shown). It is assumed that the activity was not detected owing to the low concentration of the substances with antibacterial activity.

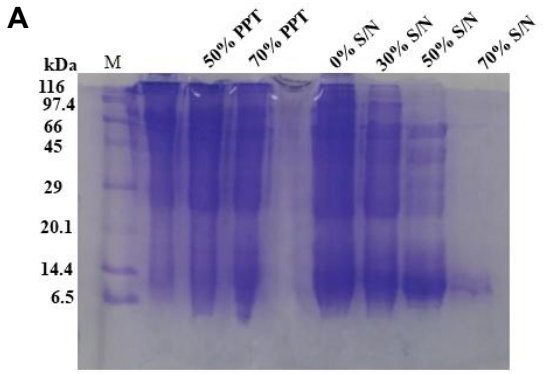
Evaluation of antioxidant activity according to methanol fractionation

To analyze the fractionated proteins depending on methanol concentration, high-molecular-weight proteins were transferred into the pellet precipitated via centrifugation in proportion to methanol concentration (Fig. 2A). However, DPPH activities in each supernatant fraction were increased in proportion to methanol concentration (Fig. 2B, Fig. 2C). Low or absent levels were detected in the precipitated pellets of methanol fractionation in this study (data not shown). Most of the bioactive peptides were composed of 2~20 amino acids, and their activities were analyzed depending on amino acid composition and sequence [9, 22]. Based on the study results, the high-molecular-weight peptides or proteins in the supernatants were removed due to increased methanol concentration. Since the antioxidant activity was increased by removing high molecular weight peptides, the relative antioxidant activity was improved by the decrease in high-molecular-weight materials.

Although the activity based on total solids was similar to that of proteins from 0 to 50% methanol concentration, the activity was sharply reduced at 70% methanol concentration (Fig. 2D). Therefore, it is suggested that the extracted substances showed varying content and composition depending on methanol concentration.

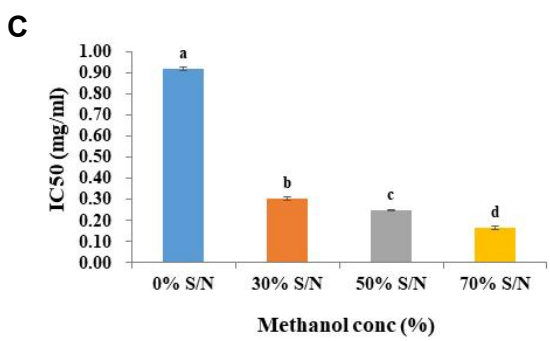
Evaluation of antioxidant activity according to organic solvent partition

To compare the antioxidant activity according to the polarity of organic solvent, we performed partition of each organic solvent. SDS-PAGE of peptides obtained from partition was detected by only low molecular weight. Especially, the butanol phase of butanol/water partition was transferred by a few amounts of low molecular weight (Fig. 3A). However, peptides and solids dissolved in the organic solvent phases were hardly observed in the other organic solvent phases such as chloroform, diethyl ether and hexane partitions (Fig. 3A and data not shown). It is assumed that the removed high-molecular-weight peptides were located in the interphase of each phase and removed during the phase separation process after centrifugation. Furthermore, when compared with IC50 values of 70% methanol fractionation and organic solvent partition, since the water phases of organic solvent partition were 10-fold lower than that of 70% methanol fractionation, the decrease in antioxidant activity is due to the interphase peptides removed to maintain partially hydrophobic properties.



B

Methanol concentration (%)		IC50 (mg/ml)
protein	0% S/N	0.92[a]
	30% S/N	0.30[b]
	50% S/N	0.25[c]
	70% S/N	0.17[d]
Total solid	0% S/N	24.75[a]
	30% S/N	8.52[c]
	50% S/N	5.73[d]
	70% S/N	10.98[b]



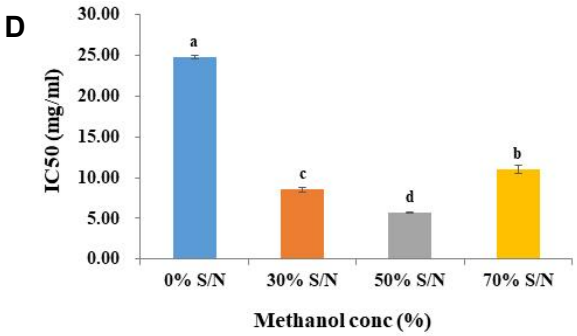
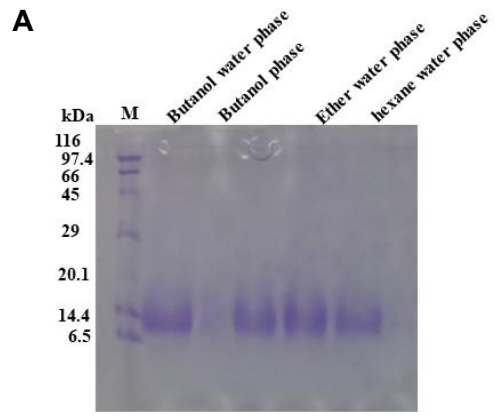
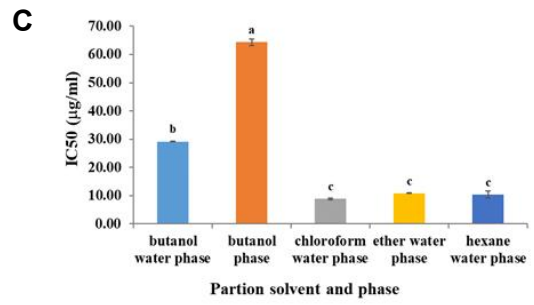


Fig. 2. Result of methanol fractionation. (A) Result of SDS-PAGE; (B) IC50 values of DPPH at each extraction time. (C) Analysis of activity at protein level. (D) Analysis of activity at total solid level. The water extracts were fractioned after adjusting each methanol concentration. X- and Y-axes of C and D represent methanol concentrations and DPPH IC50 values, respectively. M, molecular marker. Methanol conc; methanol concentrate, S/N; supernatant, PPT: precipitate pellet.



B

Solvent type			IC50 (μg/ml)
Protein	butanol	water phase	29.16[b]
		butanol phase	64.33[a]
	Chloroform water phase		8.91[c]
	ether water phase		10.86[c]
	hexane water phase		10.40[c]
Solvent type			**IC50 (mg/ml)**
Total solid	butanol	water phase	5.64[a]
		butanol phase	4.20[b]
	chloroform water phase		1.62[c]
	ether water phase		1.86[c]
	hexane water phase		1.76[c]



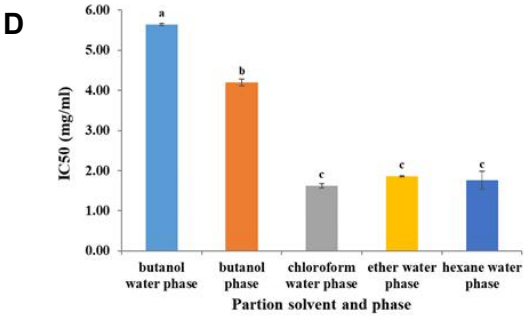


Fig. 3. Results of organic solvent partition. (A) Results of SDS-PAGE; (B) IC50 values of DPPH in each organic solvent fraction. (C) Analysis of activity at protein level. (D) Analysis of activity at total solid level. X- and Y-axes of C and D indicate organic solvent type and DPPH IC50 values, respectively. M, molecular marker.

The hydrophobic peptides prevent a oxidized product due to access to hydrophobic radical species and polyunsaturated fatty acids (PUFAs) [7, 19, 24]. Therefore, the removed interphase peptides may maintain partial hydrophilic property and play a critical role for antioxidation.

The results of protein and total solids suggest that the water phases from chloroform, diethyl ether and hexane partitions exhibit similar activities, except for butanol with relative polarity. The water and butanol phases of butanol partition showed contrasting protein and total solid content (Fig. 3B, Fig. 3C, Fig. 3D). The phenomenon is attributed to differences in the solubility of each material. The results suggest that when nonpolar organic solvent is employed for organic partition, the substances maintain higher antioxidant activity by transferring into the water phase. In addition, since most of the active substances are displayed in the water phase based on the results of phase separation, except for interphase peptides, it is assumed that their molecules exhibit polarity. Otherwise, due to the partial antioxidant activity in butanol phase, various substances exhibit antioxidant activity to maintain a difference in the degree of polarity.

Evaluation of peptide antioxidant activity

To analyze thermal stability of the extracted substances, DPPH activity of the water phase of partition was examined after treatment for the indicated time at 80℃ (Fig. 4A, Fig. 4B). When compared with untreated extract, the activities based on proteins and total solids were decreased to 44% and 32%, respectively. Especially, heat treatment for 30 min was initially reduced for DPPH activity in both protein and total solid content. However, the activity was partially increased at the sample treated for 30 min more than those of the samples treated for 10 and 20 min. Substance modifications or structural differences owing to heat treatment might alter antioxidant activity, which induces a partial increase in activity.

Proteinase K was introduced for evaluation of changes in antioxidant activity following treatment with protease. The degradation of peptides was clearly observed depending on the treated times and concentration of proteinase K (Fig. 5A). The antioxidant activity showed a tendency to increase to 12~23% depending on proteolysis (Fig. 5B, Fig. 5C, Fig. 5D). Furthermore, since the activity based on total solids showed a pattern similar to protein level activity, the differential activity may depend on proteolysis via proteinase K. It was confirmed that the antioxidant effect was increased when the *T. molitor* larval powder was decomposed into various proteases [13]. From these results, it is clear that the hydrolysate of mealworm proteins is involved in the antioxidant effect, and it was consistent with the results of this study.

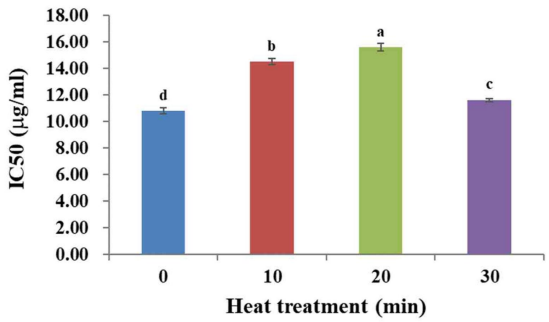
Based on this result, we suggest that the conversion of peptides from higher to lower molecular weight may improve the antioxidant activity depending on the products of protein degradation. Most bioactive peptides consist of 2~20 amino acids with a molecular weight less than 6 kDa [12, 23]. Therefore, the increase in antioxidant activity following proteinase K treatment may be attributed to low-molecular-weight peptides owing to proteolysis.

In summary, the highest

The highest antioxidant activities were detected in the water extract, and in 70% (based on protein) and 50% (based

A

time (min)	0	10	20	30
Protein IC50 (μg/ml)	10.81^{d}	14.53^{b}	15.59^{a}	11.63^{c}
decrease rate (%)	0^{a}	-34.43^{b}	-42.89^{b}	9.00^{a}



B

time (min)	0	10	20	30
TS IC50 (mg/ml)	1.97^{b}	2.53^{a}	2.61^{a}	2.11^{ab}
decrease rate (%)	0^{a}	-34.35^{b}	-45.57^{d}	-9.09^{b}

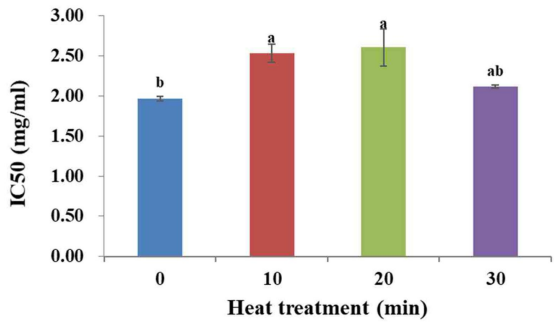
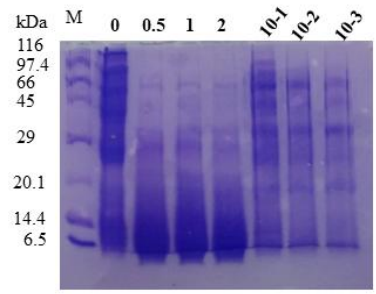


Fig. 4. Change in DPPH activity according to heat treatment. (A) Analysis of activity at protein level. (B) Analysis of activity at total solid level. X- and Y-axes of A and B indicate heat-treated time at 80℃ and DPPH IC50 values, respectively.

A

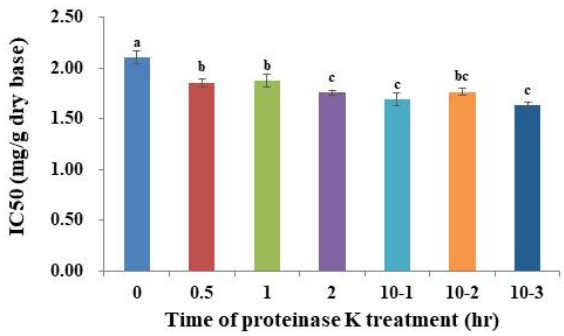


B

time (min)	0	0.5	1	2	10-1	10-2	10-3
Protein IC50 (mg/ml)	2.11^{a}	1.86^{b}	1.88^{b}	1.76^{bc}	1.69^{c}	1.76^{bc}	1.63^{c}
increase rate (%)	0^{d}	11.70^{c}	10.93^{c}	16.31^{bc}	19.91^{ab}	16.17^{bc}	22.49^{a}

time (min)	0	0.5	1	2	10-1	10-2	10-3
TSIC50 (mg/ml)	8.78^{a}	7.76^{b}	7.84^{b}	7.35^{bc}	7.05^{c}	7.37^{bc}	6.82^{c}
increase rate (%)	0^{d}	11.55^{c}	10.75^{c}	16.15^{bc}	19.77^{ab}	16.04^{bc}	22.33^{a}

C



D

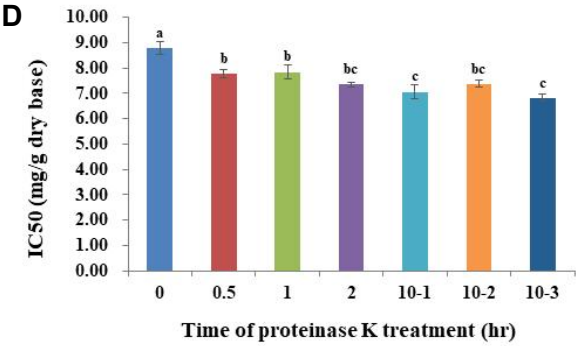


Fig. 5. Change in DPPH activity according to proteinase K treatment. (A) Results of SDS-PAGE; (B) IC50 values of DPPH at each treated time. (C) Analysis of activity at protein level. (D) Analysis of activity at total solid level. Each sample was treated for the indicated time at 37℃ with 40 mg/ml Proteinase K. Samples 10-1, 10-2, and 10-3 were treated for 10 hr at 37℃ with 40, 80, and 200 mg/ml Proteinase K, respectively. M, molecular marker.

on total solid) extracts derived from methanol fractionation. However, organic solvent partition yielded similar activity, except for butanol. In addition, the antioxidant activity was associated with the low molecular weight of *T. moritos*. We confirmed that a part of the antioxidant activity is originated from peptides. Therefore, we suggest that peptides extracted from *T. moritos* manifest effective bioactive properties following treatment with a suitable protease.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 갈색거저리 유충에서 추출한 펩타이드의 항산화 특성

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본 연구는 기능성 식품의 개발을 위해 갈색거저리 유충 유래 추출물에서 새로운 생리활성 펩타이드를 확인하는 것이다. 동결건조된 *T. molitor* 유충에서 다양한 용매로 시간에 따라 추출하였다. *T. molitor* 유충 추출 결과 물 추출물에서 5시간과 10시간에서 총 단백질과 고형분 함량당 각각 가장 높은 2,2,1-diphenyl-1-picrylhydrazyl (DPPH) 활성을 보였다. 물 추출물을 메탄올 농도에 따라 분획하였을 때, 높은 메탄올 농도는 상등액 중에 고분자량 단백질의 수준을 감소시키는 반면, 상등액의 DPPH 활성 수준을 증가시켜 저분자량 펩타이드가 항산화 활성을 매개하는 것으로 나타났다. 유기용매 분배에서 부탄올을 제외한 유기용매 분배에서 물층에서 유사한 활성을 보였다. 유기용매분획물을 열처리 후 활성이 28~44% 감소하여 열에 대한 불안정한 성분이 존재하는 것으로 추정되었다. 물 추출물에 프로테아제 K를 첨가하면 DPPH 활성이 10~20% 증가하여 전체 단백질에서 방출되는 펩타이드가 항산화 활성을 부분적으로 증가시켰음을 알 수 있다. 그러므로, *T. molitor* 유충 추출물은 항산화제로써 기능을 수행할 수 있는 물질이 존재하는 것으로 제의할 수 있다.