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Purification and identification of novel antioxidant peptides derived from *Bombyx mori* pupae hydrolysates

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ABSTRACT

The biological importance of antioxidant peptides was the focus of new natural sources of food preservatives. *Bombyx mori* pupae are considered a valuable by-product of the silk-reeling industry due to their high-quality protein content. This study aimed to purify and identify the antioxidant peptides obtained from enzymatically hydrolyzed *B. mori* pupae, which could be used as new sources of natural food preservatives. Among the prepared hydrolysates, pepsin hydrolysate with the highest antioxidant activities was purified sequentially using ultrafiltration and reversed-phase high-performance liquid chromatography (RP-HPLC). The DPPH radical scavenging and ferrous ion chelating activity were used to evaluate antioxidant activity. Fractions with high activity were further analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS). Three peptides were identified as Glu-Asn-Ile-Ile-Leu-Phe-Arg (ENIILFR), Leu-Asn-Lys-Asp-Leu-Met-Arg (LNKDLMR), and Met-Leu-Ile-Ile-Ile-Met-Arg (MLIIIMR), respectively. All three novel identified peptides exhibited significantly stronger antioxidant capacity than synthetic antioxidants used in the food industry, including butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT). ENIILFR showed the best antioxidant activity. These findings indicate that the three peptides have potential applications as natural antioxidants in the food industry.

1. Introduction

Antioxidants are substances that can slow down or prevent the spoilage of food caused by oxidation, thus extending the lifespan of products. A broad range of antioxidants is available from both natural and synthetic sources, which are commonly used in food. Several synthetic antioxidants are used in the chemical and food industry including butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) [1]. Many people are concerned about the safety of food additives. Large amounts of BHT may cause skin, eye, and respiratory irritation in humans [2]. BHA may cause endocrine disruption, leading to weight gain and delayed sexual maturation [3].

There has been a growing interest in finding natural sources of bioactive peptides that can be used for food preservation while being safe to consume [4,5]. After oral administration, peptides are degraded into harmless amino acids, making them natural and safe alternatives to synthetic antioxidants [6]. Enzymatic hydrolysis is considered an efficient method of obtaining peptides capable of antioxidant activity [7]. In comparison to chemical methods, enzymatic hydrolysis offers several advantages. These include mild reaction conditions, low production of undesirable byproducts, and high product quality and yield [8]. Antioxidant peptides can be found in various sources such as plants, spices, herbs, and even animals [9]. Edible insects, in particular, are a great source of bioactive peptides due to their high protein content [10]. As a result, edible insects are increasingly being considered as an alternative source of animal proteins.

Bombyx mori pupae are a great source of quality proteins with a total protein content of 55.60% by dry weight [11]. These pupae have been consumed traditionally in Asian countries and are now gaining popularity as a novel food in Western countries [12]. Recent studies have shown the potential health benefits of consuming these pupae [13]. These benefits include the treatment for type 2 diabetes [14], reduced risk of Alzheimer's disease [15], and protection of endothelial cells damaged by oxidized low-density lipoprotein [16]. Previous studies have found that proteins from *B. mori* have antioxidant properties when tested in vitro [17]. Enzymatic hydrolysis of silkworm proteins has been

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found to produce more potent antioxidant hydrolysates than crude protein. Moreover, the use of different enzymes during hydrolysis can result in the production of hydrolysates with varying antioxidant potencies [18].

In this study, we explored novel antioxidant peptides from enzymatically hydrolyzed *B. mori* pupae. The peptides with antioxidant activity were prepared from crude protein hydrolyzed with trypsin or pepsin. This protein was then purified through the ultramembrane. The antioxidant peptides were purified by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC), and the amino acids sequences were identified by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). The identified peptides were tested for their antioxidant property. Antioxidant ability was evaluated by measuring DPPH radical scavenging and ferrous ion chelating activities.

2. Materials and methods

2.1. Materials and reagents

B. mori pupae were obtained from Kasetsart University Smart Silk Centre, Kasetsart University, Thailand. Chemicals and reagents were purchased from the following organizations: Acetonitrile - from VWR International, LLC. (Fontenay-sous-Bois, France), Phenylmethylsulfonyl fluoride (PMSF) and Hydrochloric acid (HCl) - from Sigma-Aldrich, Inc. (Munich, Germany), Pepsin - from ELITechGroup, Inc (D.C, USA), 1,10-Phenanthroline monohydrate - from Univar Solutions Inc. (Australia and New Zealand), Sodium chloride (NaCl), Ethylenediaminetetraacetic acid (EDTA) and Sodium dodecyl sulfate (SDS) - from Bio Basic Inc. (Markham, ON, Canada), Trifluoroacetic acid (TFA) - from Thermo Fisher Scientific Inc. (Leics., UK), TritonX-100 - from Scharlau Chemie S.A. Co. (Barcelona, Spain). All other reagents used were of analytical grade and were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), or were available commercially.

2.2. Protein extraction of B. mori pupae

B. mori pupae were homogenized with cold extraction buffer containing 50 mM Tris-HCl with a pH of 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS, and 4 mM PMSF. The extracts were centrifuged at 14,000×*g* at 4 °C for 10 min. Once the centrifugation was complete, the supernatant was collected and dialyzed overnight in distilled water. The crude protein concentrations were collected and the total protein contents were measured using the Bradford assay (Bradford, 1976) [19]. The crude protein extracts were then stored at a temperature of −20 °C before further use.

2.3. Preparation of protein hydrolysates

To prepare hydrolysates, a solution of the extracted protein (25 mg/ml) in distilled water was subjected to enzymatic hydrolysis for 12 h using trypsin at pH 8.0 at 37 °C, or pepsin at pH 2.2 at 37 °C. The ratio of protein substrate to each enzyme was 25:1. After incubation, the enzymatic hydrolysis was stopped by heating in boiling water for 10 min. The hydrolysate was centrifugated at 9000×*g* at 4 °C for 15 min; the supernatant solution was then stored at −20 °C for further analysis.

2.4. Peptide purification

The prepared hydrolysates were filtered through an ultramembrane (Millipore, Darmstadt, Germany) with 3, 10, and 30 kDa cut-offs to isolate low-molecular-weight compounds. The resulting filtrates were collected as six fractions: >30 kDa, <30 kDa, 30-10 kDa, <10 kDa, 10-3 kDa, and <3 kDa. These fractions were then purified using a C18 semi-preparative RP-HPLC column (4.5 × 250 mm, 5 μm, Thermo Fisher Scientific Inc. USA). Elution was performed with a solution of 0.1% trifluoroacetic acid (TFA) in distilled water (v/v) combined with 100% acetonitrile. Mobile phase channel A was 0.1% (v/v) formic acid and 2% (v/v) acetonitrile, and channel B was 0.1% (v/v) formic acid and 100% (v/v) acetonitrile. The linear gradient of the ratio of solution A and solution B was 100% A for 2 min, 30–80% B for 2–25 (24.8) minutes, and 100% A for 4 (3.5) minutes. The flow rate was set at 0.3 mL/min, measured at wavelengths of 220 nm, and 6 fractions were stored at −20 °C for further analysis.

2.5. Identification and synthesis of the purified peptide

After identifying the HPLC fractions with the highest antioxidant activity, they were further purified using the same RP-HPLC conditions. The purified peptides were then subjected to analysis by the 6420 Triple Quadrupole LC/MS (Agilent Technologies, Inc., USA), coupled with an HPLC system using a nanoelectrospray ionization source, which recorded the mass spectral range of 50–1506 *m/z*. To determine the amino acid sequence, the de novo sequencing method was employed, and the MS/MS spectra were analyzed by PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions Inc., Waterloo, ON, Canada] for peptide identification. Three purified peptides were synthesized by Smart Science Co., Ltd. (Pathum Thani, Thailand), with a final purity of ≥98% verified by HPLC (Gilson L7420, Hitachi Limited, Tokyo, Japan). The synthesized peptides were used for further analysis.

2.6. Antioxidant activity assay

2.6.1. DPPH radical scavenging activity

The study aimed to determine the antioxidant activity of various compounds in terms of their ability to donate hydrogen or scavenge radicals, using a stable radical called 2,2-diphenyl-2-picrylhydrazyl (DPPH•), following the Blois method [20]. The hydrolysates and isolated fractions were dissolved in distilled water at 1 mg/mL and added to a 50 mM potassium phosphate buffer (pH 7.0), making a total volume of 150 μl. Then, 50 μl of 0.125 mg/mL 2,2-diphenyl-1-picrythyhydrazyl radical (DPPH) in 70% ethanol was added. The resulting mixture was incubated at 25 °C for 30 min, and the absorbance of the sample was measured at 517 nm. BHA and BHT were used as positive controls, and the assay was performed in triplicate. The percentage oxidation inhibition was calculated using the following formula:

$$\% \text{ Radical scavenging activity} = [(A_{\text{control (without extract)}} - A_{\text{sample}}) / A_{\text{control (without extract)}}] \times 100$$

Where A is the absorbance reading measured at 517 nm.

The EC50 value was defined as the concentration of the compound required to reduce 50% of the DPPH radical under the assay conditions.

2.6.2. Iron chelating activity

The test is based on the ability to remove the color from the iron-ferrozine complex. Ferrozine can quickly form a colored complex with iron. The iron chelating activity was tested according to the procedure described by Chaudhary et al. [21], with a slight modification. To prepare the 1,10-phenanthroline-iron (III) reagent, 0.198 g of 1,10-phenanthroline monohydrate, 2 ml of 1 M hydrochloric acid, and 0.16 g of ferric ammonium sulfate were mixed in 100 ml water. The hydrolysates and isolated fractions were mixed with deionized water to a total volume of 150 μl. Then, 20 μl of the prepared 1,10-phenanthroline-iron (III) reagent and 30 μl of methanol were added. After incubating at 50 °C for 30 min, the absorbance of the complex was measured at 510 nm. BHA and BHT were used as positive controls. The assay was performed in triplicate. The percentage inhibition was calculated using the following formula:

$$\% \text{ Chelating activity} = [(A_{\text{control (without extract)}} - A_{\text{sample}}) / A_{\text{control (without extract)}}] \times 100$$

Where A is the absorbance reading measured at 510 nm.

The EC50 (Effective Concentration) value was defined as the concentration of the compound required to chelate 50 % of ferrous ions, under the assay conditions.

2.7. Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test to determine the differences between untreated and treated groups (Prism version 4.0 software, GraphPad Software Inc). Data were represented as the mean ± SEM. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Preparation of protein hydrolysates from B. mori pupae and antioxidant activity

B. mori pupae proteins were separately hydrolyzed with trypsin and pepsin at optimal conditions. The antioxidant activities of crude protein and protein hydrolysates were evaluated by measuring their DPPH radical scavenging activities and ferrous ion chelating activities. The DPPH radical scavenging activity presented as EC_{50} values is shown in Table 1. The DPPH radical scavenging activities of crude protein, trypsin, and pepsin hydrolysate were 1801.8 ± 54.9 μg/ml, 1322.1 ± 68.9 μg/ml, and 1054.1 ± 58.9 μg/ml, respectively. The highest antioxidant activity was observed in the pepsin hydrolysate, which was significantly higher than positive controls; BHA and BHT (1302.9 ± 42.7 μg/ml and 1461.9 ± 53.8 9 μg/ml, respectively).

Table 2 demonstrates the ferrous ion chelating activities of the crude protein and prepared hydrolysates from *B. mori* pupae in comparison with BHA and BHT as positive controls. The EC_{50} of ferrous ion chelating activities of crude protein, trypsin, and pepsin hydrolysate were 336.7 ± 18.6 μg/ml, 398.9 ± 24.3 μg/ml, and 149.3 ± 19.3 μg/ml, respectively. The results showed that pepsin hydrolysate exhibited higher ion chelating activities than BHA (302.6 ± 22.5 μg/ml) and BHT (317.9 ± 19.4 μg/ml).

3.2. Purification and identification of antioxidant peptide

3.2.1. Fractionation of pepsin hydrolysate by ultrafiltration

In the experiment, pepsin hydrolysate showed the highest antioxidant activity. Therefore, pepsin hydrolysate was further selected to fractionate with 3 kDa, 10 kDa, and 30 kDa MWCO membranes. The resulting filtrates were collected as P1 (MW > 30 kDa), P2 (10 < MW < 30 kDa), P3 (3 < MW < 10 kDa), and P4 (MW < 3 kDa), respectively. Each fraction was pooled, lyophilized and its antioxidant activity was assayed. DPPH radical scavenging and ferrous ion chelating activity were used to evaluate the antioxidant activities of the four fractions in comparison with BHA and BHT as positive controls. As shown in Fig. 1A, fraction P2 exhibited the highest DPPH radical scavenging activity (43.26%), which was significantly higher than P1, P3, P4, and positive controls. Furthermore, fraction P2 also showed significantly higher ferrous ion chelating activity (40.92%) compared with other fractions and positive controls. Therefore, fraction P2 was subjected to RP-HPLC for further purification.

Table 1
DPPH radical scavenging activity of crude protein and protein hydrolysates from *B. mori* pupae.

Samples	DPPH radical scavenging activity (EC_{50} μg/mL)
Crude protein	1801.8 ± 54.9[c]
Trypsin hydrolysate	1322.1 ± 68.9[a]
Pepsin hydrolysate	1054.1 ± 58.9[b]
BHA	1302.9 ± 42.7[a]
BHT	1461.9 ± 53.8[a]

The values are given as mean ± SD (n = 3). The values followed by different superscripts (a–c) are significantly different from each other (P < 0.05; ANOVA, followed by Dunnett's multiple comparison test).

Table 2
Ferrous ion chelating activity of crude protein and protein hydrolysates from *B. mori* pupae.

Samples	Ferrous ion chelating activity (EC_{50} μg/mL)
Crude protein	336.7 ± 18.6[a]
Trypsin hydrolysate	398.9 ± 24.3[a]
Pepsin hydrolysate	149.3 ± 19.3[b]
BHA	302.6 ± 22.5[a]
BHT	317.9 ± 19.4[a]

The values are given as mean ± SD (n = 3). The values followed by different superscripts (a–b) are significantly different from each other (P < 0.05; ANOVA, followed by Dunnett's multiple comparison test).

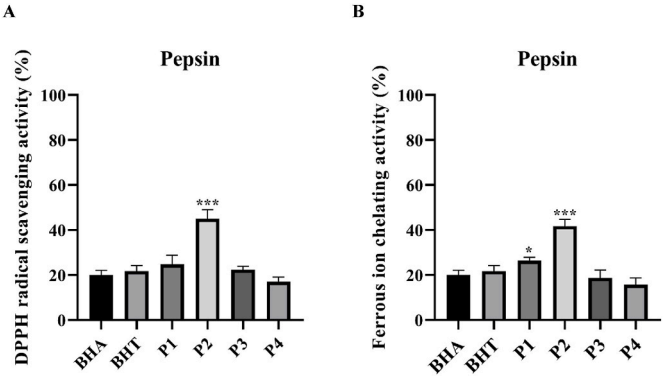

Fig. 1. DPPH radical scavenging and ferrous ion chelating activities of fractions from ultrafiltration. A: DPPH radical scavenging activity of fractions from pepsin hydrolysate. B: Ferrous ion chelating activity of fractions from pepsin hydrolysate. All samples were tested at a concentration of 1.0 mg/mL. Data are presented as mean ± standard deviation (SD) (n = 3, *P < 0.05; **P < 0.01; ***P < 0.001, unpaired Student *t*-test).

3.2.2. Isolation of peptide by reversed-phase high-performance liquid chromatography (RP-HPLC)

The reversed-phase high-performance liquid chromatography (RP-HPLC) was used for further separation and purification of fraction P2. As seen in Fig. 2, fraction P2 was subsequently separated into four fractions (F1–F4). Each fraction was pooled, and its antioxidant activity was assayed. As shown in Fig. 3A and B, all fractions displayed antioxidant activity and the F3 peptide exhibited the highest DPPH radical scavenging and ferrous ion chelating activity values of 66.78% and 58.74% respectively, which was significantly higher than that of the other 3 fractions. The antioxidant activity of the identified peptides was compared with that of synthetic antioxidants BHA and BHT, which were used as references. The identified peptide had stronger antioxidants than BHA and BHT.

3.2.3. Sequence identification of antioxidant peptide by liquid chromatography with tandem mass spectrometry (LC-MS/MS)

The F3 peptide with the highest antioxidant activity was further subjected to LC-MS/MS and the results were analyzed by a combination of Mascot searching and MassLynx V4.1 software. Three major novel peptides contributing to the antioxidant behaviors of fraction F3 were identified. The three MS/MS spectra were shown in Fig. 4 and were identified as Glu-Asn-Ile-Ile-Leu-Phe-Arg (MW = 903.52 Da), Leu-Asn-Lys-Asp-Leu-Met-Arg (MW = 904.48 Da), and Met-Leu-Ile-Ile-Ile-Met-Arg (MW = 904.52 Da), respectively.

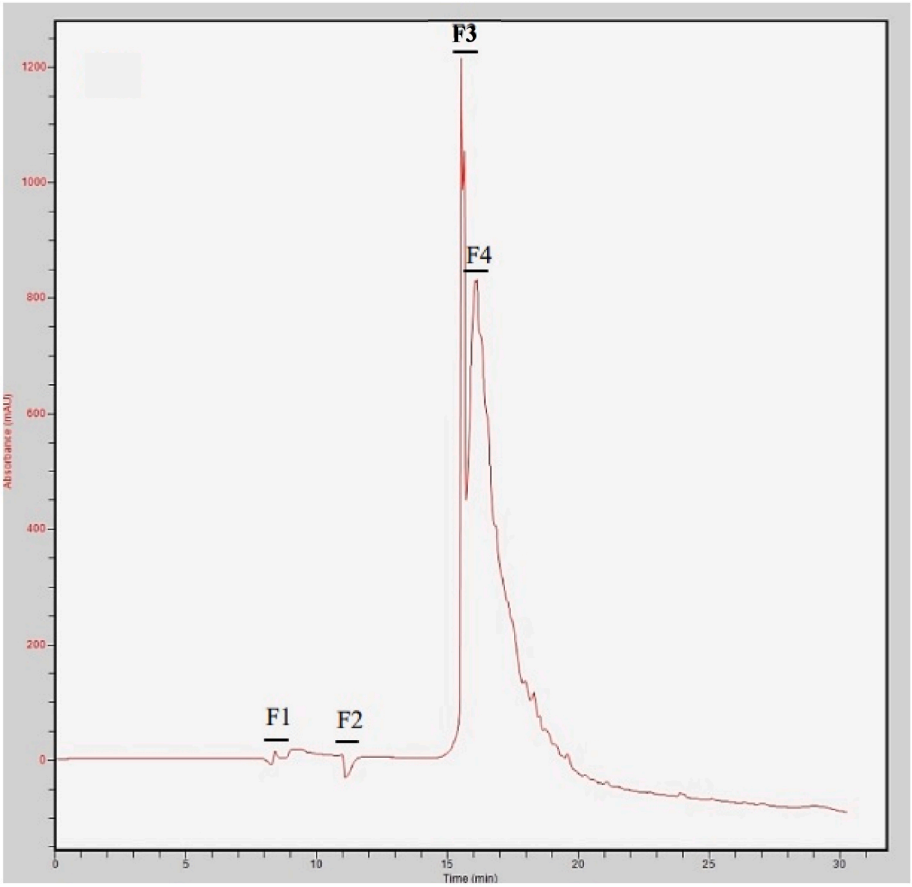

Fig. 2. RP-HPLC chromatogram of fraction P2 from pepsin hydrolysate. P2 was separated into four fractions (F1–F4) by a Thermo C18 column.

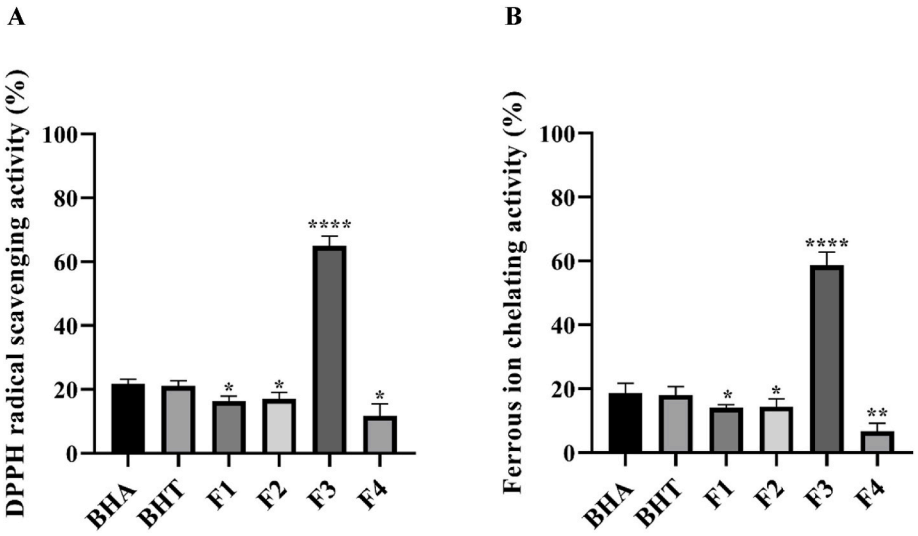

Fig. 3. DPPH radical scavenging and ferrous ion chelating activities of peptides from Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). A: DPPH radical scavenging activity of fractions from fraction P2. B: Ferrous ion chelating activity of fractions from fraction P2. All samples were tested at a concentration of 1.0 mg/mL. Data are presented as mean ± standard deviation (SD) (n = 3, *P < 0.05; **P < 0.01; ***P < 0.001, unpaired Student *t*-test).

3.3. Antioxidant activity of the synthesized peptides

Three identified peptides derived from *B. mori* pupae were chemically synthesized. The antioxidant activity of the synthesized peptides is shown in Fig. 5. All three novel peptides exhibited stronger antioxidant activity than BHA and BHT. The peptide ENIILFR (Glu-Asn-Ile-Ile-Leu-Phe-Arg) had the highest DPPH radical scavenging activity (63.12%). Furthermore, peptide ENIILFR also showed the highest ferrous ion chelating activity (65.03%).

4. Discussion

Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in the food

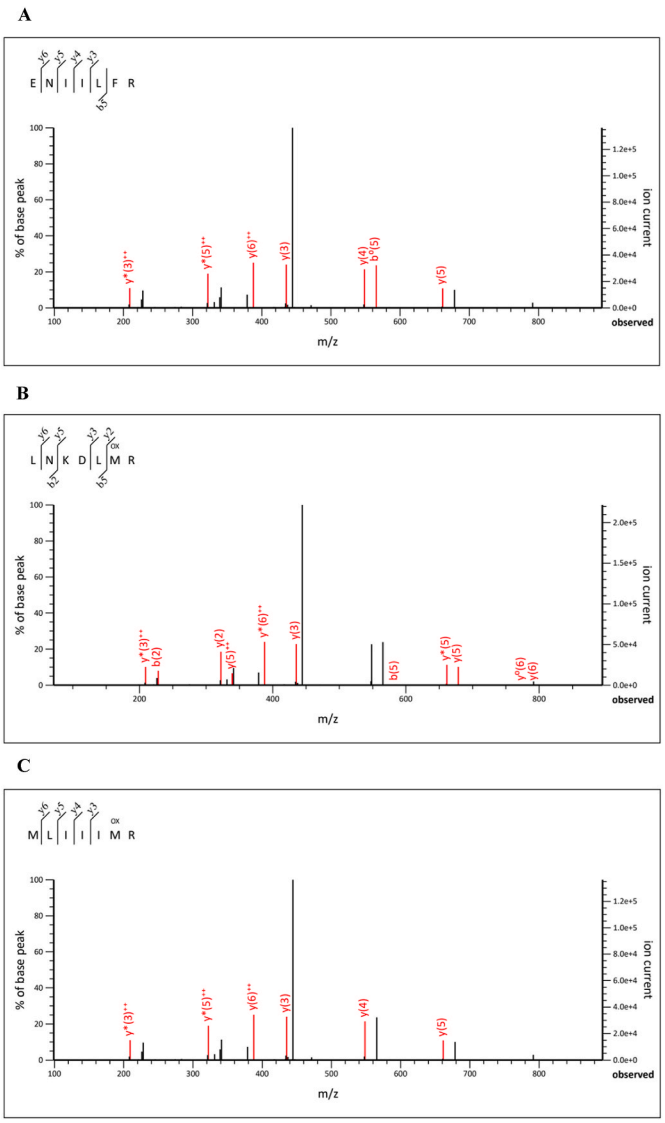

Fig. 4. The mass spectrogram of the identified peptides. A: Mass spectrum analysis of the antioxidant peptide Glu-Asn-Ile-Ile-Leu-Phe-Arg (MW = 903.52 Da). B: Mass spectrum analysis of the antioxidant peptide Leu-Asn-Lys-Asp-Leu-Met-Arg (MW = 904.48 Da). C: Mass spectrum analysis of the antioxidant peptide Met-Leu-Ile-Ile-Ile-Met-Arg (MW = 904.52 Da).

industry. However, the extended use and high doses of these synthetic antioxidants in food are strictly limited due to their potential risks to human health. These risks include cytotoxicity, oxidative stress induction, and carcinogenicity. Therefore, it is necessary to regulate the use of these synthetic antioxidants in food products [22,23].

B. mori pupae are a widespread type of edible insect, particularly popular in Asia [24]. They are known for their high nutritional values, containing significant amounts of proteins, carbohydrates, and lipids [25]. Recent studies have shown that *B. mori* pupae have functional properties, such as anti-cancer [26], antioxidant [27], anti-inflammation, and α-glucosidase inhibitory activity [28] making them a valuable food source with potential health benefits.

Antioxidant peptides found in edible insect protein can be used as bioactive ingredients in food products to help prolong shelf-life [29]. Several studies have been conducted on the antioxidant properties of hydrolysates or bioactive peptides derived from animal by-products, such as tilapia (*Oreochromis niloticus*) [30], muscle and by-products from vertebrates and invertebrates [31], Pectoral fin protein from salmon processing by-products [32], and by-products of common carp (*Cyprinus carpio*) [33]. In this study, we identified the antioxidant peptides from *B. mori* pupae, the edible insect representing a valuable by-product of the silk-reeling industry. The protein hydrolysates were prepared by trypsin or pepsin. The highest antioxidant activity was observed in the pepsin hydrolysate, which was significantly higher than crude protein, trypsin hydrolysate, and synthetic antioxidants; BHA and BHT. Enzymatic hydrolysis is dependent on the chemical structure, molecular size, amino acid composition, hydrophobicity, and polar groups of protein hydrolysates [34]. Pepsin is a crucial industrial enzyme with applications in various industries such as the food and pharmaceutical industry [35]. Pepsin's specificity is potentially affected by the amino acid residues with a preference for cleavage in hydrophobic residues, like tyrosine, phenylalanine, or tryptophan [36]. Recent studies have shown that pepsin hydrolysates and peptides obtained by pepsin hydrolysis exhibited high antioxidant activity [37–39]. This enzyme has a preference for breaking the bonds that involve hydrophobic amino acids [40]. Therefore, the released peptides will have hydrophobic amino acids in their end position, including leucine, isoleucine, or valine. These peptides seem to have better antioxidant activity than peptides without the hydrophobic end groups [41].

Ultrafiltration with MWCO membranes can be utilized for the separation of peptides based on their molecular mass [42]. In our study, we found that fraction P2 exhibited the highest levels of DPPH radical scavenging activity and ferrous ion chelating activity. Previous research has suggested that small molecular weight fractions possess better antioxidant potential than high molecular weight protein fractions [43,44]. However, some studies were in contrast with these observations, such as those on whey protein [45], yellowstripe trevally (*Selaroides leptolepis*) [46], barley glutelin [47], and poultry viscera protein hydrolysate [48]. The high level of DPPH radical scavenging activity of higher molecular weight peptides may be attributed to their increased hydrophobicity, which promotes interaction with DPPH scavenging [49]. Additionally, peptides with lower molecular weights can result in increased hydrophilic peptide concentrations that are not available to DPPH free radicals, leading to reduced efficiency in inhibiting free radicals [50]. Peptides with higher molecular weight containing hydrophobic clusters containing proline, leucine, phenylalanine, and valine, also have increased negative charge, resulting in higher ferrous ion chelating capacity [51].

Among the four fractions separated by RP-HPLC, fraction F3 showed the strongest antioxidant activity. Each fraction peak eluted in correlation with sample polarity due to the properties of the C18 column. Typically, hydrophobic components remain trapped in the column and are difficult to elute [52], indicating that fraction F3 may contain a relatively high concentration of hydrophobic amino acids. Liquid chromatography followed by MS/MS (LC–MS/MS) is commonly used to identify peptide sequences [53]. The three peptides were identified as Glu-Asn-Ile-Ile-Leu-Phe-Arg (ENIILFR) (MW = 903.52 Da), Leu-Asn-Lys-Asp-Leu-Met-Arg (LNKDLMR) (MW = 904.48 Da), and Met-Leu-Ile-Ile-Ile-Met-Arg (MLIIIMR) (MW = 904.52 Da), respectively. As previously mentioned, the antioxidant properties of peptides are determined by the amino acid compositions, sequences, Molecular Weight, and structures [54]. Here, the identified peptides with antioxidant activity contain 7 residues with an MW ranking of 903–905 Da. This is consistent with other studies, that is, the MW of antioxidant peptides typically ranges from 250 to 1800 Da [55].

All three identified peptides showed higher antioxidant activity than the reference controls. The peptide ENIILFR exhibited the strongest antioxidant capacity. The peptides that were found to have antioxidant properties contained aromatic amino acids like Phe (F), His (H), Trp (W), and Tyr (T). These amino acids provide protons for electron-deficient radicals, which helps in scavenging radicals [56]. Additionally, acidic amino acids like Asp (D) and Glu (E), as well as basic amino acids such as Arg (R) and Lys (K), have the ability to neutralize unpaired electrons and radicals by using carbonyl and amino groups in their side chains as chelators of metal ions [57]. It was also observed that the

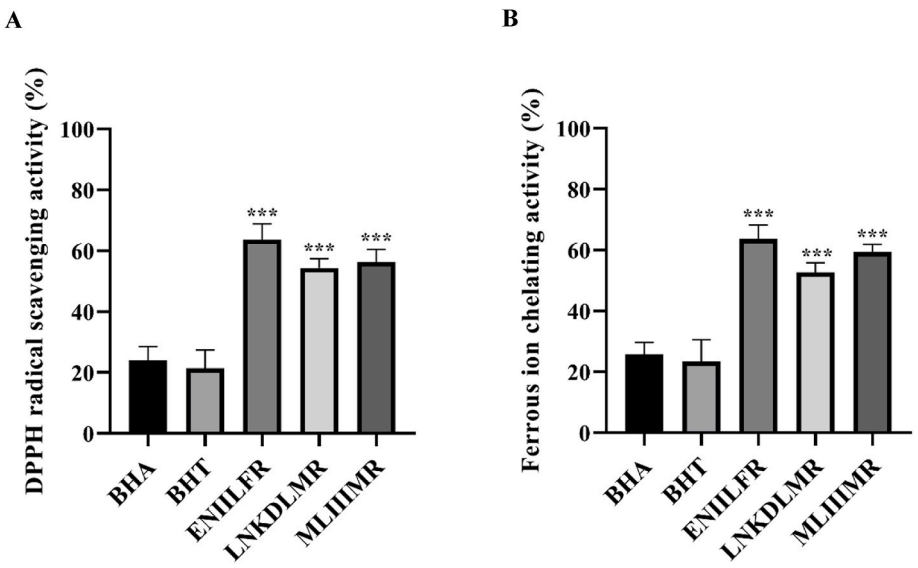

Fig. 5. DPPH radical scavenging and ferrous ion chelating activities of the identified peptides. A: DPPH radical scavenging activity of the identified peptides. B: Ferrous ion chelating activity of the identified peptides. All samples were tested at a concentration of 1.0 mg/mL. Data are presented as mean ± standard deviation (SD) (n = 3, ***P < 0.001, unpaired Student t-test).

presence of repeating amino acids in peptide sequences, like the dipeptide and tripeptide components Ile (I) -Ile (I) and Ile (I) – Ile (I) – Ile (I), have high biological properties [58]. Therefore, the amino acid sequences of the purified peptides play a crucial role in their antioxidant abilities.

According to our previous research, the protein hydrolysate from *Samia ricini* pupae showed antioxidant activity. We also identified three antioxidant peptides: Met-Ley-Ile-Ile-Ile-Met-Arg, Leu-Asn-Lys-Asp-Leu-Met-Arg, and Glu-Asn-Ile-Ile-Leu-Phe-Arg [59]. Recently, a study reported that two peptides from *B. mori* pupae hydrolysates prepared by alcalase, namely SWFVTPF, and NDVLFF, had the potential to reduce cellular oxidation in HepG2 cells [27]. Khammuang et al. also discovered two antioxidant peptides, AAEYPA and AKPGVY, from *B. mori* pupae hydrolysates prepared by alcalase. These peptides showed the highest ABTS and DPPH radical scavenging activities [60]. We searched for the sequences of the antioxidant peptides in the BIOPEP-UWM database, but no corresponding bioactive peptide sequences were found. Therefore, it can be concluded that the peptides obtained in this study are three novel peptides.

5. Conclusion

In this study, two different enzymes, pepsin, and trypsin, were used to generate *B. mori* pupae protein hydrolysates. The pepsin hydrolysate showed more potent DPPH radical scavenging and ferrous ion chelating activity than crude protein and trypsin hydrolysate. The activity assessment for fractions separated by ultrafiltration showed that fraction P2 (10 < MW < 30 kDa) possessed the highest antioxidant activities. Three peptides were subsequently identified using LC-MS/MS: Glu-Asn-Ile-Ile-Leu-Phe-Arg (ENIILFR), Leu-Asn-Lys-Asp-Leu-Met-Arg (LNKDLMR), and Met-Leu-Ile-Ile-Ile-Met-Arg (MLIIIMR), respectively. All three antioxidant peptides showed high antioxidant activity. ENIILFR had the best activity. These results suggest that *B. mori* pupae could be used as natural antioxidants for preventing oxidation reactions in food processing and for enhancing the antioxidant properties of functional foods. The applications of these peptides in the food industry will be further studied to confirm our hypotheses.

CRediT authorship contribution statement

Suttida Chukiatsiri: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization. **Nattakarn Wongsrangsap:** Methodology, Investigation, Formal analysis. **Pichamon Kiatwuthinon:** Supervision. **Wannarat Phonphoem:** Supervision.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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