CHEMICAL SCIENCES

STUDY ON EXTRACTION AND BIOACTIVITY OF SHORT-CHAIN PEPTIDES DERIVED FROM BEE PUPAE

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Abstract

The objective of this study was to extract and purify low molecular weight peptides from bee pupae using a combination of solvent extraction, ion exchange chromatography, and gel filtration. Chromatographic analysis indicated that the obtained peptides were of high purity, comprising 11 hydrophilic and positively charged peptide fractions. Electrophoresis confirmed that the purified peptides had molecular weights below 5 kDa. Amino acid composition analysis revealed the presence of 16 amino acids, including 45.9% hydrophobic, 48.26% essential, 9.34% acidic, 12.0% basic, and 12.34% cyclic amino acids. The antibacterial activity of the purified peptides was evaluated and demonstrated inhibitory effects against several microbial strains, including *S. aureus*, *E. coli*, *P. aeruginosa*, *S. enteritidis*, and *K. pneumoniae*.

Keywords: Bee pupae; peptide; antibacterial; amino acid; chromatography.

Introduction

Peptides are known to play essential roles in regulating physiological and biochemical processes in humans, animals, and plants. Their diverse structures and functions have attracted considerable scientific interest [1].

Research on low molecular weight peptides is of great importance due to both fundamental scientific value and potential practical applications, particularly in the discovery of bioactive peptides from natural sources [2-5].

Products of bee origin, such as royal jelly, honey, beeswax, and bee pupae, have demonstrated various biological activities. The antioxidant properties of honey have been attributed to plant-derived phenolic compounds [3]. Moreover, studies have shown that honey, royal jelly, and propolis exhibit antibacterial effects against a wide range of microbial strains [6-8]. These antimicrobial properties may be partially due to the presence of antimicrobial peptides in their composition. Additionally, peptides derived from bee products have been reported to influence neurotransmitter systems, animal behavior, and enzymatic activity, including enzymes such as carboxypeptidase E and peptidyl-dipeptidase A. [2, 9].

The increasing prevalence of antibiotic-resistant microorganisms, driven by the misuse of antibiotics, underscores the urgent need to identify alternative antimicrobial agents. Bioactive peptides represent a promising solution [10]. Therefore, this study aimed to extract low molecular weight peptides from bee pupae and evaluate their biological activities to provide a foundation for practical applications.

Materials and Methods

Research object

Apis mellifera bee pupae were collected from a beekeeping household in Hai Duong province.

Collection and purification of peptides from bee pupae

Put 50g of bee pupae into 100ml of 90% ammonium sulfate solution. Put the mixture into a blender (Hanil, Korea) for 15 minutes. Then the mixture is centrifuged cold at 4200 rpm for 20 minutes at 10oC. Use a pipette to carefully retain the suspended suspension. Add 50ml of 0.1 M Tris, pH 7.0. Then the mixture is mixed and centrifuged cold again for 15 minutes at 4200 rpm at 10oC, remove the precipitate and collect the liquid.

The extracted peptide will be purified on an AKTA FPLC purification system (Amersham, Sweden) using an SP Sepharose Fast Flow ion exchange column (Sigma Aldrich). The column was first equilibrated with an initial buffer of 20 mM acetate solution pH 5.0 with 1 M (NH4)2SO4. The column was eluted with the initial buffer at a rate of 0.5 ml/min, each fraction being automatically recovered in a volume of 0.5 ml. The presence of peptides in the fractions was determined spectrophotometrically by absorption at 220 nm. The fractions purified in this way were pooled and used in subsequent experiments. Peptides were identified by RP-HPLC, SUPELCOSIL LC-18-T column 250×4.6 mm, particle size 5 μm. Separation was carried out following an elution gradient from buffer A - 0.1% trifluoroacetic acid solution, to buffer B - 0.1% trifluoroacetic acid solution in 50% acetonitrile. Detection was performed by absorption at 220 and 280 nm. Chromatogram analysis was performed using ChromLab software.

Amino acid content of bee pupa peptides was determined using an amino acid analysis system, using a ZORBAX Eclipse-AAA separation column (3.5µm; L x id=150 x 4.6 mm), and a fluorescence detector at Ex = 445 nm, Em = 465 nm with Clarity 2015 program for amino acid analysis.

Determination of peptide molecular weight

The molecular weight of peptides was determined by electrophoresis. The preparation of the buffer solution for electrophoresis began with weighing glycine - 28.80 g; tris - 6.05 g, then adding 20% sodium disulfate solution - 20 ml, adding distilled water to 2000 ml. To obtain a 2% buffer solution for the sample, the following reagents were added to the Eppendorf tube: mercaptoethanol - 100 µl, 10% sodium dodecyl sulfate solution (SDS) - 40 µl, 50% glycerin solution - 40 µl, bromophenol blue - 50 µl, 1 M Tris HCl pH 6.8 - 50 µl. The mixture was thoroughly mixed. The solution for fixing and staining the gel was prepared using: 125 ml isopropanol, 50 ml acetic acid, 325 ml distilled water, 1.25 g Coomassie R250. Washing solution: 50 ml acetic acid, 50 ml isopropanol, 400 ml distilled water. For sample preparation, 20 µL of sample, 10 µL of sample buffer and 10 µL of distilled water were added to an Eppendorf tube. The samples were then mixed in a Vortex apparatus and boiled for 5 min, after which they were mixed again and added to the sample wells. After electrophoresis, the gel was stained and washed. For each separation, a set of Sigma Aldrich ultra-low mass markers (1,060-26,600 Da) was used as a molecular weight marker.

Evaluation of antibacterial activity of obtained peptides

Prepare NB agar medium according to the formula: 10 g peptone; 5 g NaCl; 5 g meat extract; 1000 ml distilled water, distribute into conical flasks, close tightly and sterilize in an autoclave at 121oC for 20 minutes. When the agar medium is still warm (about 45–50oC), pour the bacterial suspension into the conical flask containing the medium at a ratio of 5 ml of suspension/100 ml of medium, shake gently to disperse the bacteria evenly in the medium, then pour into sterile 9 cm diameter petri dishes (16 ml of medium/dish), let cool until the agar solidifies. When the agar has solidified, use a sterile metal rod (3 mm in diameter) to drill wells on the agar medium in the petri dish so that each well is 2-3 cm apart. Using an Eppendorf pipette, add 10µL of peptide sample to each agar well, 10µl of 0.9% NaCl solution as negative control and ceftriaxone as positive control (10 µg/plate). Then incubate in an aerobic environment at 37°C for 24 h. After incubation, measure the diameter of the inhibition zone according to the formula:

Inhibition zone diameter = D – d.

In which:

+ D – diameter of the outer ring after incubation, mm

+ d – diameter of the well hole, mm.

Tested microorganisms include *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis, Streptococcus pyogenes, Klebsiella pneumoniae, Proteus mirabilis* and *Enterobacter cloacae*.

Results and discussions

Results of peptide extraction and purification from bee pupae

The chemical composition of bee pupae is complex, they contain high levels of carbohydrates and phenolic compounds, so the process of isolating peptides from them is very difficult. In bee pupae, the peptide content is low due to the structural characteristics of bee pupae cells (most bioactive peptides do not accumulate but are synthesized in small amounts to serve physiological and biochemical functions). Therefore, classical sample preparation and isolation methods (solid phase extraction, precipitation with TCA, acetone, ethanol, filtration, ultrafiltration, gel filtration) do not allow the rapid and efficient isolation of peptides from bee pupae. The combination of solvent extraction, ion exchange chromatography and gel filtration methods was developed in this study to be able to isolate and purify peptides from bee pupae.

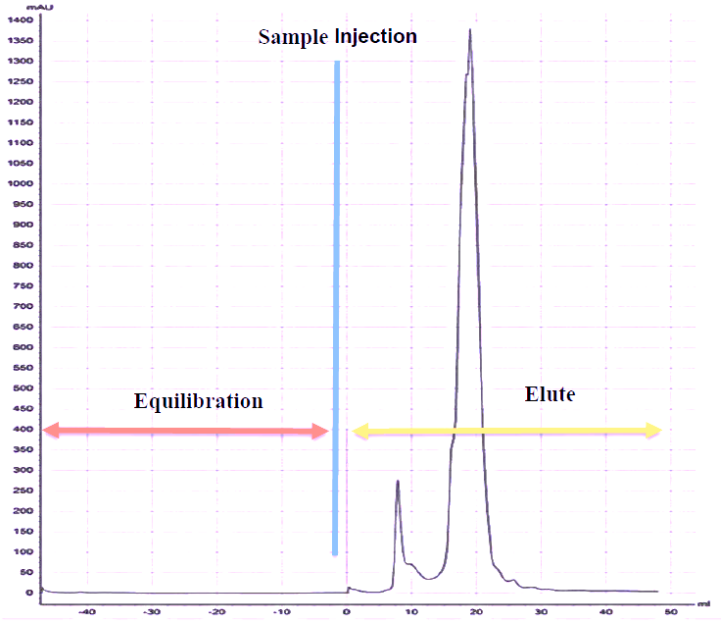

Figure 1. *Chromatogram of peptide purified by AKTA device*

The results of the purification of peptides from bee pupae are given in Figure 1, showing that there is 1 peak in the eluate representing the peptide purified from the protein extract, the sample with the highest absorbance is 1400 mAU.

The elution characteristics of the bee pupae peptide sample purified by RP-HPLC are shown in Figure 2. The chromatogram shows that the peptide composition of bee pupae is very diverse.

In the unpurified solution, the presence of 11 peptide groups was observed. The distribution of peak retention times in the chromatogram shows that most of the isolated peptides are hydrophilic, because they leave the column at acetonitrile concentrations in the eluate below 40%. The difference in the absorbance intensity of individual peaks at 220 and 280 nm wavelengths indicates the presence of amino acids with aromatic groups in the composition of the isolated peptides. Most of the peptides of bee pupae at pH = 10 were positively charged, because after ion exchange chromatography, most of the peptide groups (9 groups) were retained on the chromatographic column.

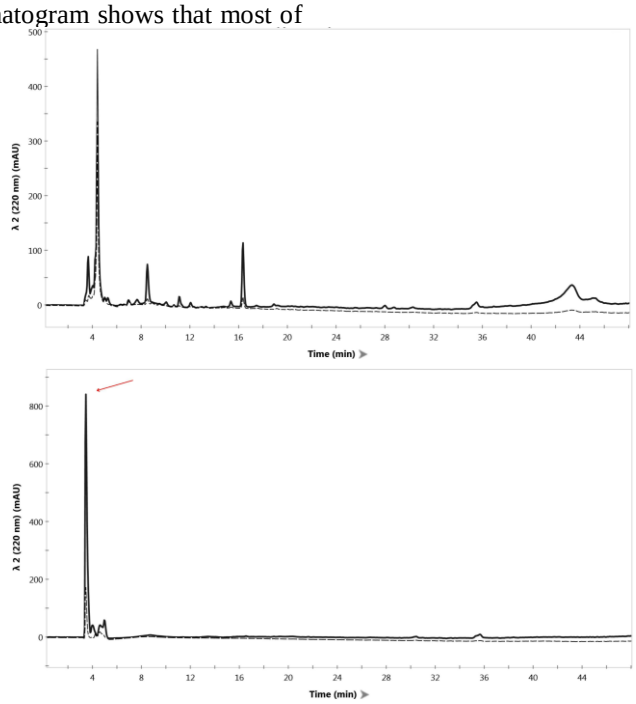

Figure 2. Chromatograms of peptide solutions before (a) and after (b) purification; dashed line – absorption at 280 nm, solid line – absorption at 220 nm

The results of molecular weight assessment of purified bee pupa peptide are shown in Figure 3. The results show that purified bee pupa peptide has a mass of less than 5 kDa. With a small size, peptides will exhibit stronger and more obvious biological activities.

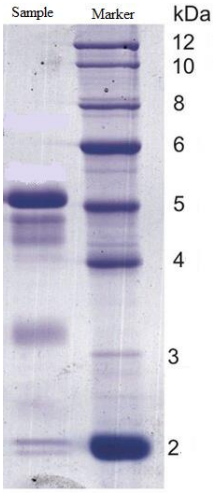

Figure 3. *Electrophoresis results of purified bee pupa peptide solution*

Table 1 shows that the purified bee pupa peptide contains up to 16 amino acids: alanine, arginine, aspartic, glutamic, glycine, proline, cysteine, methionine, leucine, phenylalanine, serine, valine, lysine, isoleucine, tyrosine and threonine with contents of 6.61, 6.17, 3.97, 5.37, 6.38, 4.72, 7.70, 6.62, 7.21, 7.60, 6.07, 6.62, 5.83, 7.74, 4.74 and 6.64%, respectively. Hydrophobic amino acids account for 45.9%, essential amino acids are 48.26%, acidic is 9.34%, basic is 12.0% and cyclic is 12.34%.

The presence of non-polar (hydrophobic) amino acids in the peptide chain such as: methionine, valine, leucine and isoleucine contributes to enhancing the antioxidant activity of the peptide.

Table 1.

Amino acid composition of purified bee pupa peptide

No.	Amino acid	Retention time, minutes	Content, %
1	Alanine	2,1	6,6
2	Arginine	2,9	6,2
3	Aspartic acid	4,1	4,0
4	Glutamic acid	5,9	5,4
5	Glycine	6,9	6,4
6	Proline	8,9	4,7
7	Cysteine	9,3	7,7
8	Methionine	11,2	6,6
9	Lucien	12,3	7,2
10	Phenylalanine	14,3	7,6
11	Serine	14,9	6,1
12	Valine	17,1	6,6
13	Lysine	17,9	5,8
14	Isoleucine	18,2	7,7
15	Tyrosine	20,1	4,7
16	Threonine	21,1	6,6

Biological activity results of purified bee pupa peptide

The results of determining the antibacterial activity of purified bee pupa peptide against the tested microbial strains by disc diffusion method and the minimum inhibitory concentration are presented in Table 2.

Table 2.

Antibacterial activity of purified bee pupa peptides

Test microorganisms	Refined bee pupa peptide			
	Diameter of antibacterial ring, mm	Minimum inhibitory concentration, µg/ml		
		50	20	10
S. aureus	20±1	x	x	x
E. coli	18±1	x	x	+
P. aeruginosa	10±1	x	+	+
S. enteritidis	8±1	x	+	+
S. pyogenes	0	+	+	+
K. pneumoniae	8±1	x	+	+
P. mirabilis	0	+	+	+
E. cloacae	0	+	+	+
Note: x - *no growth signs* + - *growth signs*				

Based on the data analysis in Table 2, it was found that the purified bee pupa peptide had a strong inhibitory effect on the growth of two tested strains, S. aureus and E. coli, with the formation of antibacterial diameters of 20±1 and 18±1 mm, respectively, with moderate activity against P. aeruginosa, S. enteritidis and K. pneumoniae strains with the formation of antibacterial diameters from 8±1 to 10±1 mm and no antibacterial activity against S. pyogenes, P. mirabilis and E. cloacae strains. The minimum inhibitory concentration for S. aureus was 10 µg/ml, for E. coli was 20 µg/ml, and for P. aeruginosa, S. enteritidis, K. pneumoniae strains was 50 µg/ml.

Conclusions

In this study, a solvent extraction, ion exchange chromatography and gel filtration method was developed to isolate and purify peptides from bee pupae. The obtained peptides were pure, hydrophilic and positively charged. The molecular weight of the obtained peptides was less than 5 kDa, containing most of the amino acids, including hydrophobic amino acids, essential amino acids, acidic amino acids, basic amino acids and cyclic amino acids. The preliminary evaluation of the biological activity of the purified bee pupae peptides showed that they were active against various strains of microorganisms and the minimum inhibitory concentration was from 10-50 µg/ml.

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