



Evaluation of the biochemical constituents and antioxidant potential of *Heterotrigona itama* bee bread extracted using the bromelain-assisted method

Norhazniza Aziz^{1*}, Koh Soo Peng¹, Nur Diyana Alyas¹ and Fadzilah Puteh¹

¹ Food Science & Technology Research Centre, MARDI Headquarters, 43400 Serdang, Selangor, Malaysia

*Corresponding author: nhazniza@mardi.gov.my

Received 9 July 2025
Revised 3 February 2026
Accepted 8 April 2026

Abstract

Bee bread is a nutrient-dense substance produced through the microbial fermentation of pollen, nectar, and bee secretions, serving as the primary nourishment for stingless bee colonies. This study aimed to evaluate the biochemical constituents of *Heterotrigona itama* bee bread, specifically investigating how bromelain-assisted extraction influences its antioxidant potential and nutrient solubility. Nutritional profiling of the fresh bee bread revealed a high content of carbohydrates (57.0%) and proteins (23.3%). The results demonstrated that bromelain treatment significantly improved the biochemical profile of the enzymatically-treated bee bread (EBB) compared to the raw (RBB) and water-extracted (WBB) controls. Post-treatment, the total soluble protein content increased substantially from 195.8 mg/mL in RBB to 456.7 mg/mL in EBB. Furthermore, enzymatic hydrolysis significantly retained and enhanced the antioxidant capacity, showing a 26.5% to 32.2% increase in radical-scavenging activity through DPPH and ABTS assays relative to the raw samples. The main phenolic acids found in all samples analyzed using HPLC were gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid, and rutin. Proline was identified as the predominant free amino acid, with all nine essential amino acids present, alongside non-protein amino acids such as gamma-aminobutyric acid (GABA). These findings indicate that bromelain-assisted extraction effectively enhances the nutritional composition and antioxidant potential of *H. itama* bee bread.

Keywords: Amino Acid, Antioxidant, Bee Bread, Enzyme Hydrolysis, Phenolics

1. Introduction

Bee bread is a unique, bioactive substance produced by bees through the natural fermentation of pollen mixed with nectar and bee secretions. Within the colony, this specialized product serves as the primary protein source and is essential for the development and health of larvae and worker bees [1]. Traditionally recognized for its complex nutritional profile, bee bread is rich in proteins, carbohydrates, lipids, and a diverse range of micronutrients, including minerals and vitamins. In Malaysia, bee bread from the *Heterotrigona itama* species has been found to be particularly high in carbohydrates (57.1%–58.9%) and protein (21.7%–23.3%) [2]. As a fermented product, it serves as a nutrient-dense source of antioxidants and probiotics that support immune function, digestive health, and energy levels while potentially offering anti-inflammatory and neuroprotective benefits [3].

While the raw nutritional value of bee bread is high, its bioactive compounds, specifically phenolics and flavonoids, are often bound within the complex pollen wall matrix, which can limit their extractability and biological availability. Enzymatic hydrolysis has emerged as a critical method for overcoming these limitations. By breaking down complex protein molecules into smaller peptides and free amino acids, enzymes can enhance the solubility and functional properties of the substrate [4]. Bromelain, a proteolytic enzyme derived from pineapple (*Ananas comosus*), is particularly effective for this purpose due to its ability to remain active across a broad range of pH (3.0–8.0) and temperature (35°C–70°C). In other food substrates, bromelain treatment has been shown to significantly boost antioxidant activity and improve the release of bioactive phytochemicals [5].

Although recent studies, including an extensive overview by [6] has detailed various biotechnological techniques for degrading the pollen exine to enhance nutrient bioavailability, these investigations have

predominantly focused on honeybee (*Apis mellifera*) pollen. In contrast, existing research on *H. itama* bee bread has largely been restricted to its raw nutritional profiles across different geographical regions [2,7–9]. Consequently, there remains a critical research gap concerning the impact of specific proteolytic modifications on the biochemical liberation of nutrients from stingless bee products. Understanding this process is scientifically essential for maximizing the functional potential of bee bread and optimizing its extraction efficiency. To address this gap, the current study examines how bromelain-assisted extraction influences specific biochemical markers, including total soluble protein, phenolic and flavonoid concentrations, and the detailed profiles of both amino acids and phenolic compounds. Additionally, the research assesses changes in antioxidant performance through DPPH, ABTS, and ferric reducing antioxidant power (FRAP) assays.

2. Materials and methods

2.1 Sample preparation

Bee bread from *H. itama* was collected from a stingless bee colony located at Universiti Putra Malaysia (UPM), Serdang. The samples were dried at 45°C for 30 hours and subsequently ground into a fine powder, which was then stored at 4°C until use. For enzymatic hydrolysis, 10 g of bee bread powder was dissolved in 100 mL of phosphate buffer (pH 7) and treated with 1% bromelain (CAS 9001-00-07) [10]. The mixture was incubated in a shaker incubator at 40°C and 120 rpm for one hour. Enzyme inactivation was carried out by heating the solution at 90°C for 15 minutes in a water bath. After centrifugation, the supernatant was collected and stored at –20°C for further analysis, labelled as enzymatically-treated bee bread (EBB). The control sample consisted of bee bread processed under the same conditions but without the addition of bromelain. Additionally, a raw bee bread extract was prepared by macerating the sample in water for 15 minutes, with the control and raw extracts labelled as WBB and RBB, respectively.

2.2 Proximate analysis

Proximate analysis of bee bread for moisture, ash, crude protein, crude fat, total dietary fiber, and carbohydrates was conducted using the AOAC standard methods. Moisture was measured by drying the samples to a constant weight, while ash content was determined by incineration at 550°C for 6 hours. The crude protein content was determined by multiplying the measured nitrogen value by a conversion factor of 6.25, according to the 955.04 method. Meanwhile, crude fat was extracted using petroleum ether in accordance with the 920.39 method. Total dietary fiber was assessed using the Megazyme assay (method 985.29) [11].

2.3 Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method, with minor changes, by mixing 1 mL sample extract with the Folin-Ciocalteu reagent (Sigma, USA) for 5 minutes. After adding 4 mL of 7.5% sodium carbonate, the mixture was incubated in the dark for 2 hours. Absorbance was read at 765 nm. Gallic acid was used for the standard curve, and results were expressed as mg GAE/mL. All samples were analyzed in triplicate [12].

2.4 Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was measured using the aluminum chloride colorimetric method [13]. About 1.0 mL of sample extract was mixed with 0.3 mL of 5% sodium nitrite and left at room temperature for 5 minutes. Then, 0.3 mL of 10% aluminum chloride was added and incubated for another 6 minutes, followed by 2.0 mL of 1 M sodium hydroxide and 2.4 mL of distilled water. The mixture was mixed well, and absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Varian Cary 50, Australia). Quercetin was used to create the calibration curve, and results were expressed as mg quercetin equivalents per mL of sample (mg QUE/mL). All tests were done in triplicate.

2.5 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to [14], with a few adjustments. A stock solution of 300 mM acetate buffer at pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 20 mM FeCl₃.6H₂O solution was made. The FRAP working reagent was prepared by combining 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of FeCl₃.6H₂O solution. The sample extract (150 µL) was allowed to react with 2850 µL of the working reagent for 30 minutes in the dark. The absorbance was measured at 593 nm, and ascorbic acid was employed as the standard. Results were expressed as mg ascorbic acid equivalent per mL of sample (mg AAE/mL).

2.6 DPPH and ABTS free radical scavenging activity

The DPPH assay was performed with slight modifications from the method described by [14]. A stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol and stored at -20°C . A working solution was made by diluting 10 mL of the stock with 45 mL of methanol, then adjusted to an absorbance of 1.17 ± 0.02 at 515 nm using a spectrophotometer. For the assay, 0.15 mL of sample extract was added to 2.85 mL of the DPPH working solution and incubated in the dark for 30 minutes. After incubation, absorbance was recorded at 515 nm.

The ABTS assay was performed according to a modified method [15]. ABTS was prepared at 7 mM in distilled water and reacted with 2.45 mM potassium persulfate to generate $\text{ABTS}^{\cdot+}$ radicals. The mixture was kept in the dark at room temperature for 12–16 hours. Prior to use, the solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. For analysis, 190 μL of the sample was mixed with 10 μL of $\text{ABTS}^{\cdot+}$ solution and incubated for 20 minutes before measuring absorbance at 734 nm. Ascorbic acid was used as the reference, and radical scavenging was calculated using Equation (1) below.

$$\text{Inhibition (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (1)$$

Where the Abs control represents the absorbance of DPPH/ABTS without the extract, while the Abs sample denotes the absorbance of DPPH/ABTS with the extract.

2.7 Total soluble protein

The total soluble protein content was determined using the Bradford assay [16]. Bovine serum albumin (BSA) was used as the standard to generate a calibration curve, and results were expressed in μg of protein per mL of extract ($\mu\text{g/mL}$). All measurements were performed in triplicate, and blanks were prepared using distilled water in place of the sample extract.

2.8 Phenolics compound profile

Phenolic compounds in the extract were analyzed using the Acquity UPLC system (Waters, Milford, MA, USA) operating at a flow rate of 0.4 mL/min. Separation was carried out on a Kinetex C18 100A column (100 mm $\times$ 2.1 mm; 1.7 μm), maintained at 30°C . UV detection was performed at wavelengths of 280, 330, and 360 nm. A gradient elution was employed using mobile phase A (3% acetic acid in water) and mobile phase B (methanol). Quantification was based on calibration curves prepared from known concentrations of reference phenolic standards, identified by their retention times and characteristic UV absorbance profiles [17].

2.9 Free amino acid profile

The free amino acid profile was determined using the procedure provided by [17]. The filtrated extract was initially treated with 70 μL of AccQ-TagTM Ultra borate buffer and 20 μL of AccQTM Fluor reagent. The analysis was carried out using Ultra Performance Liquid Chromatography (UPLC) with a UV-PDA detector set to 260 nm (Waters, USA). The Acquity UPLC Taq Ultra Column (2.1 mm internal diameter, 100 mm $\times$ 1.7 mm particle size) was used to analyze amino acids individually. Waters EmpowerTM 2 software was used for data interpretation and quantification. Calibration curves were developed by injecting reference amino acids with known retention times.

2.10 Statistical analysis

The experiment data were analyzed with MINITAB Version 17. A one-way ANOVA was conducted, and a significance threshold was determined using Tukey's test ($p < 0.05$). Results are shown as the mean $\pm$ standard deviation.

3. Results and discussion

3.1 Proximate analysis of bee bread

The proximate composition of the fresh bee bread was determined to establish a nutritional baseline and ensure the quality of the starting material (Table 1). These foundational results confirm that the fresh bee bread used in this study met the standard macronutrient profile required for subsequent enzymatic processing. The analysis revealed that carbohydrates were the major component (57.00 g/100 g), followed by significant amounts of protein (23.30 g/100 g) and total dietary fiber (14.05 g/100 g). These values are consistent with previous findings that reported carbohydrate concentrations ranging from 57.06 to 58.89 g/100 g and protein contents between 21.10 and

23.33 g/100 g in Malaysian stingless bee bread [2,19]. Total dietary fiber is a key nutritional component of bee bread, contributing significantly to its associated health benefits. While bee bread generally contains a moderate amount of dietary fiber, the reported values vary widely (0.50% to 18.18%) depending on factors such as botanical origin, environmental conditions, and analytical methods [20,21]. To date, there are limited data on the dietary fiber content of bee bread produced in Malaysia. In the present study, bee bread was found to contain a relatively high dietary fiber content of 14.05%. These findings underscore the potential of bee bread as a rich source of dietary fiber, complementing its content of proteins, carbohydrates, lipids, vitamins, and minerals.

Table 1 The proximate composition of fresh dried bee bread

Parameters	Values
Carbohydrate (g/100 g)	57.00 ± 3.40
Protein (g/100 g)	23.30 ± 1.20
Moisture (%)	11.45 ± 0.85
Total dietary fiber (g/100 g)	14.05 ± 3.45
Crude fat (g/100 g)	5.45 ± 1.35
Ash (%)	2.80 ± 0.01

Among the macronutrients analyzed, ash was the least abundant, averaging 2.80 g/100 g in *H. itama* bee bread. This result is in agreement with the guideline suggested by [21], which states that ash content in bee bread should not exceed 6 g/100 g. Comparable ash values have been reported for stingless bee bread from different species and regions, including *Tetragonula biroi* from the Philippines (3.58 g/100 g) [22], *H. itama* from Malaysia (1.70%) [23], and *Lepidotrigona flavibasis* from Thailand (2.20 g/100 g) [24]. The moisture content of the dried bee bread analyzed in this study was 11.45%, aligning with observations by [2] who reported moisture values ranging from 11.09% to 12.51% for dried bee bread collected from various districts in Malaysia. In contrast, fresh bee bread exhibits considerably higher moisture levels due to the hygroscopic nature of pollen, which readily absorbs water from the environment. The moisture contents in fresh pot pollen from stingless bees, *Melipona seminigra* and *Melipona interrupta* in Brazil, can reach up to 53.39% [25]. Consequently, proper drying conditions are crucial for lowering moisture to acceptable levels, thereby enhancing the product's shelf-life and minimizing the risk of microbial spoilage during storage.

3.2 Total soluble protein and antioxidant properties of bee bread extract

Bromelain, a proteolytic enzyme, hydrolyzes proteins into smaller peptides and amino acids, enhancing solubility by reducing molecular size and altering structure. This improves functional properties like emulsification and antioxidant activity. In this study, bromelain treatment significantly increased ($p < 0.05$) the soluble protein content of bee bread up to 456.67 µg/mL, more than double that of the untreated samples (WBB and RBB), as shown in Table 2. These findings are consistent with previous research on bromelain-treated plant proteins, such as soy, pea, rice, oat, and hemp, where extensive hydrolysis led to improved solubility and functionality [28,29].

Table 2 Total soluble protein, total phenolic and flavonoid content, and antioxidant properties of enzymatically treated bee bread (EBB), water-extracted bee bread (WBB), and raw bee bread (RBB).

Biological properties	EBB	WBB	RBB
Total soluble protein (µg/mL)	456.667 ± 36.717 ^a	124.889 ± 10.814 ^b	195.778 ± 6.890 ^c
Total phenolic (mg GAE/mL)	0.719 ± 0.129 ^a	0.313 ± 0.042 ^b	0.222 ± 0.028 ^b
Total flavonoid (mg QUE/mL)	0.214 ± 0.012 ^a	0.148 ± 0.007 ^b	0.151 ± 0.004 ^b
FRAP (mg AAE/mL)	0.062 ± 0.004 ^a	0.094 ± 0.003 ^b	0.101 ± 0.002 ^b
DPPH inhibition (%)	62.477 ± 1.709 ^a	52.405 ± 2.624 ^b	49.819 ± 2.631 ^b
ABTS inhibition (%)	93.068 ± 0.238 ^a	77.409 ± 1.593 ^b	70.393 ± 5.457 ^b

Values denoted by different letters within the same row are significantly different ($p < 0.05$).

Enzymatic hydrolysis enhances the nutritional profile and bioavailability of bee bread by liberating phenolic and flavonoid compounds from the pollen matrix. This release of bioactive constituents significantly amplifies antioxidant capacity, offering improved protection against oxidative stress-related diseases [29]. Furthermore, the generation of low-molecular-weight peptides via bromelain treatment further bolsters these effects, as smaller peptides exhibit superior free radical scavenging and metal ion chelation properties [4].

This study demonstrated that enzymatic hydrolysis significantly retained a higher amount of TPC and TFC of EBB, surpassing the levels found in WBB and RBB. Correspondingly, antioxidant activities measured via DPPH and ABTS assays showed increases of 26.5% and 32.2%, respectively, relative to RBB.

These findings align with previous research, which showed that extraction processes using bromelain can increase the antioxidant capacity of the substrate. Corn gluten meal and mung bean protein treated with bromelain also exhibited enhanced antioxidant effects in DPPH and ABTS assays, particularly in peptide fractions ranging from 1 to 10 kDa [10]. The use of bromelain for enzymatic hydrolysis boosts the antioxidant capacity of hempseed

protein isolate (BHPI-12) by making the protein more soluble, increasing its ability to neutralize DPPH radicals, and improving its oxygen radical antioxidant capacity (ORAC) [28]. Likewise, bromelain hydrolysis increased the release of polyphenols and flavonoids from materials like roasted butterfly pea seeds, which led to higher phytochemical concentrations and greater antioxidant potential [29].

Interestingly, the FRAP assay in this study revealed a significant ($p < 0.05$) decline in FRAP values following bromelain hydrolysis. This observation may be attributed to biochemical changes during enzymatic treatment, which release bound phenolic compounds that, although more bioavailable, may exhibit lower ferric ion-reducing power compared to their polymer-bound forms. Additionally, changes in molecular structure and interactions can shift antioxidant mechanisms, resulting in increased radical scavenging (DPPH and ABTS) but decreased ferric reducing activity. However, a reduction in FRAP does not necessarily indicate diminished antioxidant potential, as hydrolysates may still retain or even improve their biological effectiveness through different antioxidant pathways [14].

3.3 Phenolic compounds of bee bread extract

Bee bread is a potent fermented product of pollen, nectar, and bee secretions, valued for its high nutritional and bioactive potential. Among its diverse constituents, phenolic compounds are the primary contributors to its health-promoting properties [1]. In this study, gallic acid was identified as the predominant phenolic across all samples, with concentrations ranging from 62.57 to 68.16 $\mu\text{g/g}$ (Figure 1). Other key bioactive compounds detected include protocatechuic acid, caffeic acid, and the flavonoid rutin. These compounds are valued for their possible health benefits. For example, gallic acid and caffeic acid are well-known for their potent ability to reduce inflammation and protect nerve cells. Additionally, rutin and protocatechuic acid greatly improve heart health and shield cells from damage caused by oxidation [31]. Our results demonstrate that bromelain-mediated hydrolysis is an advanced extraction technology that far outperforms conventional techniques. While standard extraction primarily recovers surface-level or unbound phenolics, the enzymatic approach successfully modifies the phenolic profile by penetrating the complex structural matrix of the bee bread [34]. The substantial increase in phenolic and flavonoid concentrations post-treatment suggests that these compounds are naturally tethered to the protein-carbohydrate matrix of the pollen exine. As a proteolytic enzyme, bromelain facilitates the structural degradation of pollen wall proteins, liberating sequestered phenolic acids that remain insoluble and unrecoverable during simple water extraction.

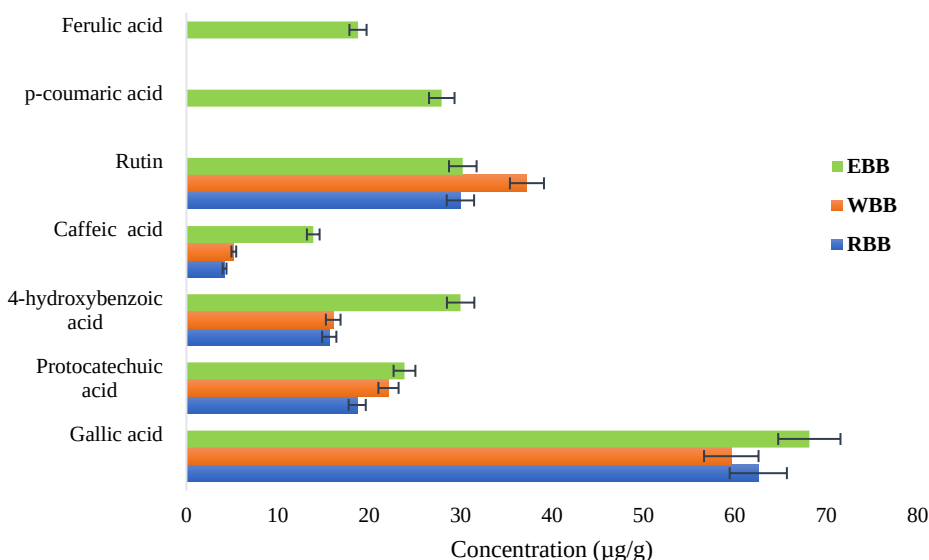


Figure 1 Phenolic compounds composition of enzymatically treated bee bread (EBB), water-extracted bee bread (WBB), and raw bee bread (RBB).

The baseline phenolic profile of bee bread is typically dictated by its botanical and geographic origins, with natural fermentation acting as a preservative for these compounds [7]. However, the extraction technique is the decisive factor in the variety of phenolics detected. While previous research in Turkey [8] and Malaysia [2] identified prevalent acids like gallic and protocatechuic, this study demonstrated that a more rigorous enzymatic approach substantially broadens the diversity of the recovered profile.

Most notably, ferulic acid and p-coumaric acid were identified exclusively after enzymatic hydrolysis. Their absence in the raw samples confirms that these compounds are chemically bound to cell wall components or

proteins via ester, ether, or hydrophobic bonds. The release of these particular acids holds biological importance. Ferulic acid acts as a potent antioxidant, recognized for its capacity to reduce cholesterol levels and guard against skin damage caused by UV radiation. In parallel, p-coumaric acid has been correlated with a decreased likelihood of stomach cancer and reduced levels of inflammation [33]. Bromelain works by breaking down the cell walls and cutting the chemical bonds that keep phenolic acids “locked” inside the bee bread. Although the process might slightly alter some compounds or cause them to bind with peptides [9], it significantly increases the total amount of bioactive compounds recovered. This proves that enzymatic hydrolysis is a superior extraction method that captures high-value nutrients usually lost during standard processing.

3.4 Free amino acid profile of bee bread extract

This study identified 20 free amino acids in the bee bread samples that were enzymatically broken down, revealing all 9 essential amino acids, as illustrated in Figure 2. The specific composition of amino acids in each sample evaluated is detailed in Table 3.

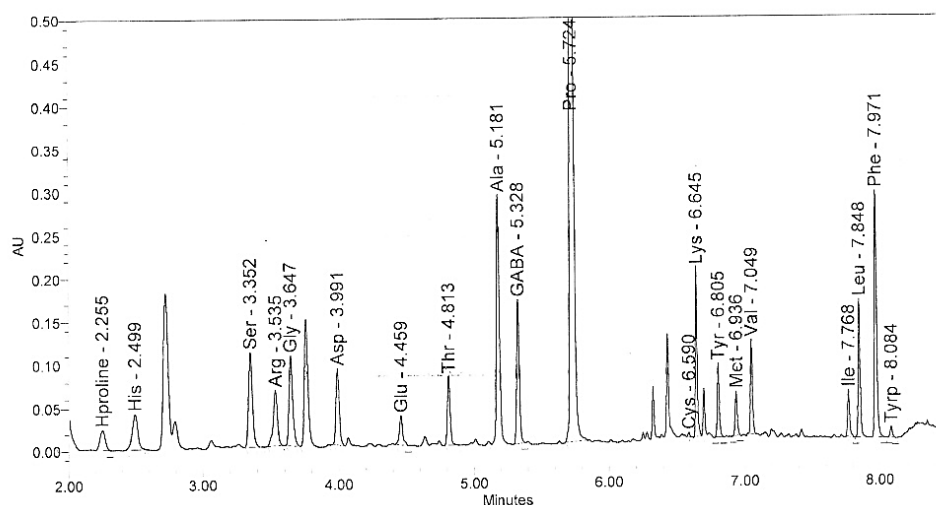


Figure 2 The chromatographic profiles from amino acid analysis of enzymatically hydrolyzed bee bread (EBB).

The free amino acid profile is a vital indicator of both the nutritional accessibility and functional potential of bee bread. Table 3 shows that amino acid concentrations vary significantly depending on the extraction method used, with bromelain-assisted extraction (EBB) showing a selective enrichment of specific components compared to water extraction (WBB) and raw bee bread (RBB). Proline was identified as the predominant amino acid across all samples, with levels ranging from 619.677 to 719.618 mg/100 g. Although proline is a non-essential amino acid, it is considered conditionally essential during periods of physiological stress or illness, playing a critical role in collagen synthesis, wound healing, and immune support [34]. Notably, EBB significantly increased the concentrations of arginine (53.260 ± 3.216 mg/100 g), tryptophan (5.440 ± 0.701 mg/100 g), and tyrosine (32.322 ± 2.434 mg/100 g) compared to WBB. This suggests that bromelain specifically targets and cleaves peptide bonds within the pollen matrix or interior proteins. While water extraction is limited to recovering pre-existing free amino acids, the proteolytic action of bromelain actively generates a new pool of bioavailable nutrients. This is further evidenced by isoleucine reaching its highest concentration in the EBB group (17.318 ± 0.773 mg/100 g), indicating that enzymatic treatment successfully disrupts the cell wall to release sequestered essential amino acids. The liberation of tryptophan and tyrosine is particularly significant, as these aromatic amino acids donate protons to free radicals, directly contributing to the higher antioxidant activity observed in enzymatic samples compared to water-extracted controls [35].

However, enzymatic hydrolysis did not result in a uniform increase for all amino acids; for instance, glutamate and proline levels remained lower in EBB than in RBB. This may occur because certain amino acids remain bound within unhydrolyzed peptide chains or form complexes with liberated peptides during processing, reducing their detection as free monomers. Despite these variations, the overall EBB profile includes all nine essential amino acids and significant levels of gamma-aminobutyric acid (GABA), which ranged from 70.240 to 78.790 mg/100 g. As a key inhibitory neurotransmitter, GABA plays a crucial role in reducing neuronal activity, promoting relaxation, and alleviating anxiety [17]. Its presence, along with the improved amino acid profile, confirms that bromelain-assisted extraction effectively enhances the functional value, muscle recuperation potential, and overall bioavailability of bee bread nutrients.

Table 3 Amino acid composition in enzymatically treated bee bread (EBB), water-extracted bee bread (WBB), and raw bee bread (RBB).

Amino acid content (mg/100 g)	RBB	WBB	EBB
<i>Essential amino acid</i>			
Histidine (His)	46.853 ± 3.298 ^a	41.725 ± 1.038 ^b	43.150 ± 1.745 ^b
Isoleucine (Ile)	15.035 ± 1.504 ^a	13.067 ± 1.115 ^b	17.318 ± 0.773 ^c
Leucine (Leu)	54.100 ± 1.727 ^a	48.420 ± 3.968 ^b	53.657 ± 1.452 ^a
Lysine (Lys)	41.348 ± 0.798 ^a	35.198 ± 6.213 ^b	40.570 ± 1.043 ^{ab}
Methionine (Met)	17.730 ± 1.157 ^a	16.035 ± 0.608 ^b	18.653 ± 0.698 ^a
Phenylalanine (Phe)	100.537 ± 4.985 ^a	91.477 ± 3.558 ^b	105.925 ± 10.417 ^a
Threonine (Thr)	34.162 ± 1.586 ^a	30.232 ± 2.442 ^b	31.972 ± 1.572 ^{ab}
Tryptophan (Tyrp)	4.115 ± 0.396 ^a	3.547 ± 0.207 ^a	5.440 ± 0.701 ^b
Valine (Val)	32.503 ± 1.124 ^a	26.863 ± 2.222 ^b	30.857 ± 0.344 ^a
<i>Non-essential amino acid</i>			
Arginine (Arg)	44.818 ± 2.769 ^a	41.573 ± 1.758 ^a	53.260 ± 3.216 ^b
Cysteine (Cys)	1.020 ± 0.141 ^a	0.893 ± 0.147 ^{ab}	0.762 ± 0.099 ^b
Aspartate (Asp)	63.343 ± 2.105 ^a	56.530 ± 6.828 ^a	57.952 ± 3.369 ^a
Alanine (Ala)	114.727 ± 2.276 ^a	98.362 ± 11.653 ^b	101.698 ± 0.730 ^b
Glutamate (Glu)	30.852 ± 1.262 ^a	25.742 ± 2.339 ^b	22.903 ± 1.668 ^c
Glycine (Gly)	38.045 ± 1.825 ^a	33.822 ± 1.333 ^b	35.867 ± 1.098 ^b
Proline (Pro)	719.618 ± 25.000 ^a	627.227 ± 69.000 ^b	619.677 ± 20.736 ^b
Serine (Ser)	62.247 ± 2.914 ^a	54.653 ± 4.250 ^b	55.333 ± 2.382 ^b
Tyrosine (Tyr)	29.437 ± 1.229 ^a	27.978 ± 0.484 ^a	32.322 ± 2.434 ^b
Hydroxyproline (Hproline)	21.957 ± 1.469 ^a	19.618 ± 0.851 ^b	20.618 ± 1.129 ^{ab}
Gamma-Aminobutyric Acid (GABA)	78.790 ± 2.456 ^a	70.240 ± 9.273 ^b	72.760 ± 3.013 ^{ab}

Values denoted by different letters within the same row are significantly different ($p < 0.05$).

4. Conclusion

This study demonstrates that bromelain-assisted extraction is a superior method for enhancing the functional value of *H. itama* bee bread. Treating bee bread with bromelain more than doubled the amount of soluble protein. This process also efficiently broke down the pollen structure, releasing trapped phenolic compounds. Notably, ferulic and p-coumaric acids were recovered exclusively after enzymatic hydrolysis. The bee bread further comprised all essential amino acids, as well as non-protein amino acids like GABA. In conclusion, enzymatic hydrolysis facilitated by bromelain improved both the antioxidant capabilities and the preservation of bioactive compounds within the bee bread.

5. Acknowledgements

The authors are grateful to the research team and the laboratory staff who contributed to this study. This project was fully funded by the IMAT-KPKM Product Development and Branding Project Grant (K-RF288-1001-KSR999) under the Ministry of Agriculture and Food Security, Malaysia.

6. Author Contributions

Norhazniza Aziz: Conceptualization, Investigation, Methodology, Writing – original draft, Supervision; Koh Soo Peng: Data curation, Formal analysis; Nur Diyana Alyas: Resources; Fadzilah Puteh: Formal analysis

7. References

- [1] Mărgăoan R, Stranț M, Varadi A, Topal E, Yücel B, Cornea-Cipcigan M, et al. Bee Collected Pollen and Bee Bread: Bioactive Constituents and Health Benefits. *Antioxidants*. 2019;8(12):568.
- [2] Mohammad SM, Mahmud-Ab-Rashid NK, Zawawi N. Botanical Origin and Nutritional Values of Bee Bread of Stingless Bee (*Heterotrigona itama*) from Malaysia. *J Food Qual*. 2020;(1):2845757.
- [3] Bakour M, Laaroussi H, Ousaaïd D, El Ghouizi A, Es-Safi I, Mechchate H, et al. Bee Bread as a Promising Source of Bioactive Molecules and Functional Properties: An Up-to-Date Review. *Antibiotics*. 2022;11(2).
- [4] Wouters AGB, Rombouts I, Fierens E, Brijs K, Delcour JA. Relevance of the Functional Properties of Enzymatic Plant Protein Hydrolysates in Food Systems. *Compr Rev Food Sci Food Saf*. 2016;15(4):786–800.
- [5] Manzoor Z, Nawaz A, Mukhtar H, Haq I. Bromelain: Methods of Extraction, Purification and Therapeutic Applications. *Braz Arch Biol Technol*. 2016(5):16150010.

- [6] Alcalá-Orozco M, Lobo-Farfan I, Tirado DF, Mantilla-Escalante DC. Enhancing the Nutritional and Bioactive Properties of Bee Pollen: A Comprehensive Review of Processing Techniques. *Foods*. 2024;13(21).
- [7] Ćirić J, Haneklaus N, Rajić S, Baltić T, Lazić IB, Đorđević V. Chemical composition of bee bread (perga), a functional food: A review. *J Trace Elem Miner*. 2022(2):100038.
- [8] Kaplan M, Karaoglu Ö, Eroglu N, Silici S. Fatty acid and proximate composition of bee bread. *Food Technol Biotechnol*. 2016;54(4):497–504.
- [9] Aksoy A, Altunatmaz SS, Aksu F, Tokatlı Demirok N, Yazıcı K, Yıkılmış S. Bee Bread as a Functional Product: Phenolic Compounds, Amino Acid, Sugar, and Organic Acid Profiles. *Foods*. 2024;13(5):795.
- [10] Damulienė V, Kaškonienė V, Kaškonas P, Maruška A. The Influence of Enzymatic Hydrolysis on Bee Pollen Antioxidant and Antibacterial Activities. *Foods*. 2023;12(19).
- [11] AOAC International. Official methods of analysis of AOAC International. 19th ed. Gaithersburg, MD; 2012.
- [12] Singleton VL, Rossi JA. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am J Enol Vitic*. 1965;16(3).
- [13] Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem*. 1999;64(4):555–559.
- [14] Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Hawkins Byrne D. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Compos Anal*. 2006;19(6–7):669–675.
- [15] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*. 1999;26(9–10):1231–7.
- [16] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72(1–2):248–254.
- [17] Kahar AA, Koh SP, Danial AM, Long K. Effect of soaking conditions on gamma-aminobutyric acid (GABA) and amino acids content in mung bean. *Food Res*. 2022(6):109–116.
- [18] Mat Rashid Z, Abd Rashid KA, Rois Anwar NZ. Heterotrigona Itama Bee Bread Extracts: Effect of Solvent Polarity on Extraction Yield, Chemical Characteristics and Antioxidant Activity. *J Agrobiotechnol*. 2023;14(1):44–53.
- [19] Aylanc V, Falcão SI, Vilas-Boas M. Bee pollen and bee bread nutritional potential: Chemical composition and macronutrient digestibility under in vitro gastrointestinal system. *Food Chem*. 2023(413):135597.
- [20] Svečnjak L, Bošković K, Prđun S, Mrkonjić Fuka M, Tanuwidjaja I. Metric and Spectral Insight into Bee-Pollen-to-Bee-Bread Transformation Process. *Foods*. 2023;12(22):4149.
- [21] Campos MGR, Bogdanov S, de Almeida-Muradian LB, Szczesna T, Mancebo Y, Frigerio C, et al. Pollen composition and standardisation of analytical methods. *J Apic Res*. 2008;47(2):154–161.
- [22] Bakour M, Fernandes Â, Barros L, Sokovic M, Ferreira ICFR, Badiaa Iyousi. Bee bread as a functional product: Chemical composition and bioactive properties. *Lebenson Wiss Technol*. 2019(109):276–282.
- [23] Ismail WIW, Hussin NN, Mazlan SNF, Hussin NH, Radzi MNFM. Physicochemical Analysis, Antioxidant and Anti Proliferation Activities of Honey, Propolis and Beebread Harvested from Stingless Bee. *IOP Conf Ser Mater Sci Eng*. 2018;440(1):012048.
- [24] Chuttong B, Phongphisutthinant R, Sringarm K, Burgett M, Barth OM. Nutritional composition of pot-pollen from four species of stingless bees (Meliponini) in southeast Asia. In: *Pot-Pollen in Stingless Bee Melittology*. Cham: Springer International Publishing; 2018. p. 313–324.
- [25] Rebelo KS, Ferreira AG, Carvalho-Zilse GA. Physicochemical characteristics of pollen collected by Amazonian stingless bees. *Ciência Rural*. 2016;46(5):927–932.
- [26] Shahbal N, Jing X, Bhandari B, Dayananda B, Prakash S. Effect of enzymatic hydrolysis on solubility and surface properties of pea, rice, hemp, and oat proteins: Implication on high protein concentrations. *Food Biosci*. 2023(53):102515.
- [27] Marinova M, Cuc NTK, Tchorbanov B. Enzymatic Hydrolysis of Soy Protein Isolate by Food Grade Proteinases and Aminopeptidases of Plant Origin. *Biotechnol Biotechnol Equip*. 2008;22(3):835–838.
- [28] Martins IM, Roberto BS, Blumberg JB, Chen CYO, Macedo GA. Enzymatic biotransformation of polyphenolics increases antioxidant activity of red and white grape pomace. *Food Res Int*. 2016(89):533–539.
- [29] Ee KY, Khoo LY, Ng WJ, Wong FC, Chai TT. Effects of Bromelain and Trypsin Hydrolysis on the Phytochemical Content, Antioxidant Activity, and Antibacterial Activity of Roasted Butterfly Pea Seeds. *Processes*. 2019;7(8):534.
- [30] Benzie IFF, Devaki M. The ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: concepts, procedures, limitations and applications. In: *Measurement of Antioxidant Activity & Capacity*. Chichester, UK: John Wiley & Sons, Ltd; 2017. p. 77–106.

- [31] Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutat Res.* 2005;579(1–2):200–213.
- [32] Arshad ZIM, Amid A, Yusof F, Jaswir I, Ahmad K, Loke SP. Bromelain: An overview of industrial application and purification strategies. *Appl Microbiol Biotechnol.* 2014;98(17):7283–7297.
- [33] Cosme P, Rodríguez AB, Espino J, Garrido M. Plant Phenolics: Bioavailability as a Key Determinant of Their Potential Health-Promoting Applications. *Antioxidants* 2020;9(12):1263.
- [34] Meng C, Wang J, Zeng Y, Yao X, Meng J. Dietary Proline Supplementation Promotes Growth and Development in Weaned Foals by Modulating Gut Microbial Amino Acid Metabolism. *Microorganisms.* 2025;13(11):2598.
- [35] McCaslin TG, Pagba C V., Chi SH, Hwang HJ, Gumbart JC, Perry JW, et al. Structure and Function of Tryptophan–Tyrosine Dyads in Biomimetic β Hairpins. *J Phys Chem B.* 2019;123(13):2780–2791.