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ORIGINAL PAPER

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THE ENHANCEMENT OF BIOACTIVE PEPTIDES, DIETARY POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN *RHIZOPUS OLIGOSPORUS* FERMENTED SOYBEAN TEMPEH THROUGH PROTEIN HYDROLYSIS

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ABSTRACT

Background. There is a growing interest in harnessing bioactive peptides derived from food-grade raw materials, especially for the development of functional foods. Tempeh milk protein hydrolysates (TMPHs) are interesting sources of bioactive peptides. In the current study, TMPHs were characterized by their bioactive peptides, dietary polyphenols and antioxidant activity.

Material and methods. TMPHs were made from *Rhizopus oligosporus* fermented tempeh with fermentation times of 0, 1, 2, 3 and 4 days, designated as TMPH0, TMPH1, TMPH2, TMPH3 and TMPH4, respectively. The morphological appearance of tempeh was examined by scanning electron microscopy. The analyzed characteristics of TMPHs were physicochemical properties, total soluble proteins (TSP), trichloroacetic acid (TCA)-soluble peptides, bioactive peptides, antioxidant activity, total phenolic compounds (TPCs) and total flavonoid compounds (TFCs).

Results. The morphological appearance and microstructure of tempeh with varied fermentation times were different under SEM due to the growth of *R. oligosporus*. The pH of TMPHs remained constant at 7 for samples with fermentation times of 0, 1 and 2 days and continuously decreased for those fermented for 3 and 4 days. However, total acidity continuously increased from the first day of fermentation. TMPH3 had the highest level of total soluble proteins and trichloroacetic acid (TCA)-soluble peptides. SDS-PAGE analysis demonstrated that TMPHs consist of various protein fractions with large molecular weights from 25 kDa and with lower molecular weights. Their SDS-PAGE patterns were different, indicating protein alteration during fermentation. In DPPH and ABTS assays, TMPH3 exhibited the strongest antioxidant activity. These results agreed with those from the analysis of total phenolic compounds (TPCs) and total flavonoid compounds (TFCs) showing that TMPH3 had the highest TPC and TFC.

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Conclusion. This study demonstrates that TMPHs possess the capability to yield bioactive peptide-derived proteins with active biological properties, a favorable degradation mechanism, and enhanced bioaccessibility, making them suitable for use in the production of functional foods.

Keywords: fermented soybeans, soybean milk, vegan food, plant protein hydrolysate, antioxidant peptides, isoflavones

INTRODUCTION

Various animal- and plant-derived fermented foods are integral components of daily diets across numerous regions worldwide. The practice of food fermentation, employed for centuries, serves to preserve perishable food items. Fermentation can potentially improve the digestibility and bioavailability of proteins, carbohydrates, lipids, and minerals. It can enhance nutritional value, reduce cooking time, and contribute to microbial safety. Processes of pathogen reduction in preserved foods such as non-heat treated pork salami (McKinney et al., 2004) show that fermented foods can slow down the growth of pathogens. For fermented food in Thailand such as Thai Traditional fermented fish (Pla-Som), which is fermented in the traditional Thai style, *Kazachstania* Yeast supports the fermentation process (Punyauppa-Path et al., 2022). The microorganisms employed in fermentation include bacteria, yeasts, and filamentous fungi such as molds. Notably, naturally occurring lactic-acid bacteria (LAB) assume a significant role in these processes (Lei et al., 2013).

Soybean (*Glycine max* L.) is the most extensively cultivated plant globally, renowned for its substantial protein content. As with most legume seeds, soybeans contain a variety of proteins, many of which significantly contribute to nutritional well-being. Soybeans notably have high protein content and are abundant in vitamins, minerals and insoluble fiber including isoflavones (Saha and Mandal, 2019). Soybeans are not suitable for raw consumption and are processed to create a variety of food products, with fermentation being the most common method. Various Asian countries use different names for fermented soybean products, reflecting variations in preparation techniques and the microbial strains involved. This diversity leads to differences in the texture and form of the resulting fermented products. For instance, in Japan, fermented soybean products are referred to as natto and miso (Chan et al., 2021). In Korea, they are known as cheonggukjang (Piao and Eun, 2020), while in traditional Chinese fermented food, the term is douchi (Zhou et al., 2024). Another example is tempeh or tempe, a solid cake-like fermented soybean product produced using Indonesian fermented soybean and *Rhizopus oligosporus* (Tamang et al., 2022). It has gained global popularity, particularly among vegetarians and in developing nations, due to its ease of preparation and cost-effectiveness. Overall, it is an economical, nutritious, healthy, easy-to-prepare food (Mani and Ming, 2017).

Tempeh has been noted for its robust antioxidant effects, antimicrobial properties, and favorable impact on intestinal microbiota. These benefits are attributed to its rich concentrations of isoflavone, folate, vitamin B12, and various other compounds (Cao et al., 2019). Nevertheless, tempeh faces challenges in terms of consumer acceptance. Its bitter taste and unpleasant odor are its primary drawbacks, largely restricting its commercialization in the market to whole food products. Hence, there is a necessity for alternative technologies that can harness tempeh to produce functional ingredients. Soybeans have protein content ranging from approximately 30% to 48% on a dry basis (Syah et al., 2015). This substantial protein content makes soybeans an excellent reservoir of parent proteins suitable for the production of bioactive peptides through various approaches. Singh et al. (2014) examined the functional significance of bioactive peptides from soybeans, encompassing antihypertensive, antioxidative, antiobesity, immunomodulatory, anti-diabetic, hypocholesterolemic, and anticancer properties. The bioactive peptides isolated from soy-fermented foods, such as natto and tempeh, have been documented to have high nutritional value, high fiber,

carbohydrate, energy, and antioxidant properties (Canaan et al., 2022).

Bioactive peptides are those peptides characterized by the presence of 2–20 amino acids within the structures of the parent proteins (Agyei, 2015; Sanjukta and Rai, 2016). For these peptides to exhibit beneficial health effects, they must be liberated from their parent proteins through at least one of three methods: (i) *in vivo* hydrolysis by gastrointestinal proteolytic enzymes, (ii) microbial fermentation, and (iii) *in vitro* hydrolysis facilitated by microorganism- or plant-based proteases (Daliri et al., 2017). In this context, bioactive peptides demonstrate physiological functionalities which are particularly beneficial for the cardiovascular system (antioxidative, antithrombotic, and antihypertensive effects), nervous system, gastrointestinal system (mineral binding, antimicrobial activity), and immune system (immunomodulatory properties) (Chalamaiah et al., 2019; Sanjukta and Rai, 2016).

Isoflavone consumption has been shown to positively impact antioxidant status in *in vivo* studies. For example, dietary soybean isoflavones administered for 30 days exhibited favorable effects on the antioxidant status of male Wistar rats (Barbosa et al., 2011).

Sitanggang et al. (2020) highlighted a dual method incorporating fermentation and *in vitro* hydrolysis for the production of bioactive peptides from tempeh. To the best of our knowledge, the continuous production of bioactive peptides in automated membrane reactors is predominantly employed for milk proteins (Sitanggang et al., 2021). It is intended to improve the market feasibility of functional ingredients sourced from tempeh by employing safer technological methods.

The aims of this work are to demonstrate a microwave extraction technique for tempeh milk protein hydrolysates (TMPHs) to elicit useful antioxidant and bioactive properties. Tempeh milk protein hydrolysates (TMPHs) were tested for total soluble proteins (TSP) and trichloroacetic acid (TCA)-soluble peptides. TMPHs were analyzed using gel electrophoresis analysis and antioxidant assays. Morphological analyses categorized their physical and morphological characteristics under scanning electron microscopy (SEM). We focused on determining the antioxidant and bioactive properties from tempeh milk protein hydrolysates (TMPHs) during the fermentation process.

MATERIALS AND METHODS

Microorganisms, culture conditions and inoculum preparation

A pure culture of *R. oligosporus* TISTR 3098 (from the Thailand Institute of Scientific and Technological Research) and soybean seeds (purchased from a local market in Ubon Ratchathani province, Thailand) was used in the current study. The work was conducted in the Department of Biological Science, Ubon Ratchathani University, Ubon Ratchathani, Thailand. Fungal growth on potato dextrose agar (PDA) incubated at 28 ±2°C for 96–120 h was carried out to obtain single colonies. Then, spores (10^5 spores/mL) were harvested and diluted in sterile DI water for future use as a starter culture inoculum.

Process of tempeh fermentation and tempeh milk protein (TMP) production

After being submerged overnight in 1% acetic acid at room temperature, approximately 500 g of soybeans were boiled at a low heat by using a temperature of 55–60°C for 5–10 minutes.

They were soaked for thirty minutes at a ratio of one volume of soybeans to three volumes of clean water, drained and allowed to cool to room temperature. Then, they were inoculated with a starter culture that consisted of a 1 mL suspension of *R. oligosporus* containing 10^5 spores/mL. Next, the mixture (50 g) was weighed and packaged into plastic bags with holes for airflow and incubated at 28–30°C for 96 h (4 days). During fermentation, samples were taken every day (0, 1, 2, 3, and 4 days) and stored at –20°C for preservation against protein degradation. Subsequently, TMP was prepared from tempeh samples mixed with DI water at a ratio of 1:3 in an extractor using Frutelia equipment (Tefal, Thailand) model no. ZE370138 at speed number 2, and a power of 350 W for 20 min, followed by pasteurization in an autoclave at 110°C for 10 min to obtain optimized conditions (Phupaboon et al., 2023).

Preparation of tempeh milk protein hydrolysates (TMPHs)

The TMPH samples were extracted using microwave-assisted extraction following the method described by Phupaboon et al. (Phupaboon et al., 2023; Phupaboon

et al., 2022a), with some modifications. Briefly, a supernatant (10 g) was homogenized and extracted using microwave equipment under optimal conditions at 100 W and a maximum temperature of 60ºC for 10 minutes. After that, a homogenate from each TMP sample was centrifuged at 10,000 rpm and 4ºC for 5 min and lyophilized to produce TMPH powders. The temperature of the powders was maintained at –20°C until the investigation of their physicochemical characteristics and bioactivity.

The Tempeh milk protein hydrolysates (TMPHs) were subjected to two methods of anti-oxidation analysis, namely DPPH and ABTS.

Determination of total phenolic compounds

Chemical preparation

1. 7% sodium carbonate (Na_2CO_3): Weight Na_2CO_3 3,500 mg dissolved in distilled water (Dw) and final volume adjusted to 50 mL.
2. (1:10) Folin-Ciocaltue reagent: Added 1 mL of Folin-Ciocaltue reagent to 9 mL of Dw in a total volume of 10 mL.
3. (1 mg/mL) Gallic acid dissolved in 1.0 Dw.

Methodology

The determination of total phenolic content was conducted by the method of Al-Duais et al. (2009) with slight modifications. 20 µL of sample extract (1 mg/mL) or standard solution in methanol was mixed with 100 µL of 1:10 diluted Folin–Ciocalteu reagent in a 96-well microplate and then incubated for 6 min. Then, 80 µL of 7% sodium carbonate solution was added and the mixture was kept at room temperature in dark conditions for 30 min. Gallic acid (1 µg/mL) was used as standard and two-fold serial dilution was prepared for a calibration curve in the range of 10–1,000 µg/mL. The absorbance was measured at 765 nm using the microplate reader (PerkinElmer, Germany). Measurements of every sample were taken in triplicate. The results were expressed as mg gallic acid equivalents (GAE)/g dry matter (mg GAE/g DM).

The antioxidant activity of TMPHs was determined by the free radical scavenging activity of the total phenolic compounds (TPC). The alteration of the color by Folin-Ciocalteu reagent's reaction with phenolic compounds was investigated and compared to a standard solution of gallic acid. The results were expressed as milligrams of gallic acid equivalents per liter (mg/L GAE).

Determination of total flavonoid compounds

Chemical preparation

1. 10% Aluminum Chloride ($AlCl_3 \times 6H_2O$): Weight $AlCl_3 \times 6H_2O$ 1.00 mg dissolved in distilled water (Dw), total final volume adjusted to 10 mL.
2. 1 M Sodium acetate (NaOAc): Weight 1360.8 mg of NaOAc dissolved into 9 mL of Dw; total final volume adjusted to 10 mL.
3. (1 mg/mL) Quercetin dissolved in 1.0 Dw.

Methodology

Total flavonoid content was determined as reported by Topçu et al. (2007), with some modification of the methods. 20 µL of extracts (1 mg/mL) or standard solution (Quercetin, 1-1000 µg/mL) and 20 µL of 10% aluminum chloride solution were poured into a 96-well plate. After that, 180 µL of Dw and 20 µL of 1 M NaOAc solution were added and mixed well. After incubation for 30 min at room temperature, the absorbance was measured at 415 nm with a microplate reader (PerkinElmer, Germany). Total flavonoid content was evaluated as mg quercetin equivalent (QUE)/g dry extract (mg QUE/g DM).

Characterization of morphological, physicochemical and TMPH change

Tempeh morphology

Each tempeh sample was cross sectioned before observing its fungal mycelium, surface structure, and soybean morphology. The dried samples were attached on stubs using a metallic tape, degassed using nitrogen gas, and sputtered with gold-palladium before visualization under field emission-scanning electron microscopy (FE-SEM) (TESCAN, Model: Mira, PA, USA) with a 15 kV accelerating voltage (Phupaboon et al., 2022a).

pH and total acidity (TA) analyses

Both pH and TA were determined following AOAC methodology (Association of Official Analytical Chemists, 2000). A filtered supernatant was directly analyzed using a pH meter and TA values were titrated using a 0.1 N NaOH solution. The results are presented as the percentage of lactic acid equivalent in units of g/100 mL. These analyses were done in duplicate.

Total soluble protein (TSP) and trichloroacetic acid (TCA)-soluble peptide analyses

The TSP and TCA-soluble peptide content of TMPHs (1 mg) were homogeneously dissolved in 1 mL of DI water and a 10% (w/v) TCA solution according to a previously reported procedure (Phupaboon et al., 2020; Phupaboon et al., 2022b). The relevant values were determined using the Lowry method (Lowry et al., 1951) and the TSP content was expressed as mg/g protein hydrolysates (PH) as bovine serum albumin (BSA) read at 660 nm. TCA-soluble peptide levels were determined from the tryptophan content as mg/g PH in spectroscopic measurements using Folin--Ciocalteu reagent at 595 nm obtained from triplicate experiments.

Examination of TMPH alteration by gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate the change in TMPHs during tempeh fermentation using a Mini-Protein II-unit (Bio-Rad, CA, USA) employing an acrylamide gel consisting of stacking (5%) and resolving (12%) gels with slight modifications (Phupaboon et al., 2023; Phupaboon et al., 2022a). SDS--PAGE gel was prepared from distilled water, 1.5 M Tris HCl pH 8.8, 10% SDS, 30% acrylamide-bis, 10% ammonium persulfate, and TEMED. All chemical reagents of molecular biology grade were purchased from Merck, USA. The protein marker used was Chromatein Prestained Protein Ladder, purchased from Vivantis, Malaysia. The extractor (Frutelia equipment) was a Tefal (Thailand) brand, model no. ZE370138.

Antioxidant activity of TMPHs

Two approaches described in the protocol (Phupaboon et al., 2023; Phupaboon et al., 2020; Phupaboon et al., 2022b) were used to determine the antioxidant activity obtained from TMPHs by measuring free-radical scavenging inhibition: the DPPH method at 517 nm (Arnao, 2001) and the ABTS method at 734 nm (Binsan et al., 2008). These methods used a 96-well microplate and a PerkinElmer microplate reader (PerkinElmer, Massachusetts (USA) and Germany). Each analysis was performed in triplicate, and the results are expressed as percentage inhibition of DPPH and/or ABTS radical scavenging compared with vitamin C (ascorbic acid) as a positive control. The experiments were done in triplicate.

Statistical analysis

In this study, each assessment was completed in triplicate. The data are presented as mean ±SD. Analysis of variance (ANOVA) was done using SPSS-KKU Statistics v.27 to assess the differences between the treatments using Duncan's multiple range tests (DMRTs) with significant p-values < 0.05.

RESULTS AND DISCUSSION

Morphological characteristics during tempeh fermentation and TMP samples

After subjecting tempeh to a controlled fermentation process for 4 days, starting from day 0, a comparative study was conducted, examining the mycelial effects. As depicted in Figure 1 (a–e), on day 0, there was no *R. oligosporus* TISTR 3098 mycelium on the soybeans. However, after 1 day of fermentation, white mycelium with a fluffy appearance began to appear on the soybeans. By day 2, more extensive white mycelium with a fluffy texture was evident, consolidating the soybeans into a unified mass. On the third day, the white mycelium continued to proliferate, further uniting the soybeans into a cohesive mass. By day 4, the white mycelium had significantly increased, enveloping the soybeans entirely and rendering the individual beans indistinguishable. These findings provide a detailed visual representation of the progression of mycelium development on the soybeans throughout the four-day fermentation process (images a–e in Fig. 1).

The morphology of the mycelium during four-day tempeh fermentation was examined using scanning electron microscopy (SEM). As depicted in Figure 1 (f–j), on day 0, no mycelium was observed on the soybeans. On day 1, the beginnings of a network-like structure of mycelium became evident. On day 2, the mycelial network exhibited a more intricate and interconnected pattern. On day 3, the mycelial network became more complex. On day 4, the mycelial network reached its maximum complexity, forming an intricate and well-developed structure. These findings provide a detailed microscopic perspective on the progressive development of mycelium on the soybeans over the four-day fermentation period (images f–j in Fig. 1).

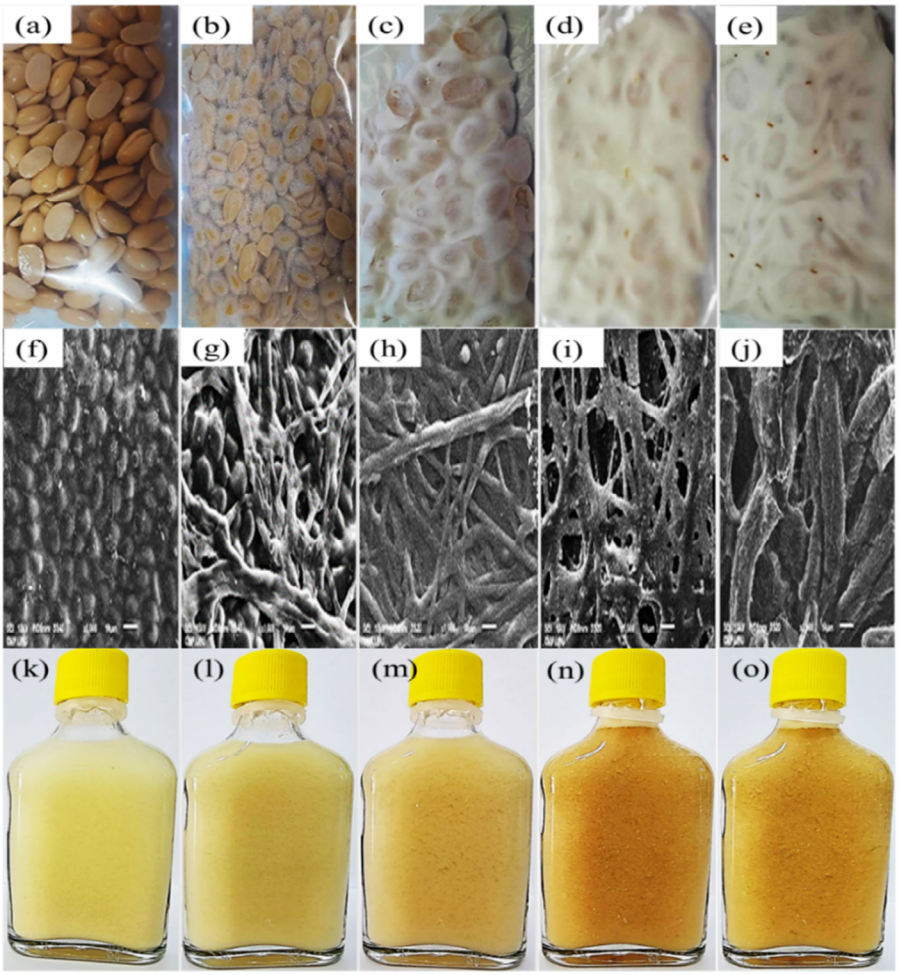

Fig. 1. The appearance of soybeans, mycelium after inoculation with *R. oligosporus* during tempeh fermentation, and TMP samples: (a–e) respectively indicate sample appearance 0 to 4 days after incubation, (f–j) show cross-sectioned tempeh samples at different times under FE-SEM micrographs, and (k–o) show prototype TMP samples at different fermentation times

Tempeh milk protein (TMP) was prepared from tempeh samples mixed with DI water in a 3:1 ratio using an extractor with Frutelia equipment (Tefal, Thailand). The color of the tempeh was clear yellow on day 0. It gradually became darker yellow on days 1, 2, 3 and 4. On day 4, the darkest yellow color was observed. The change in color is a result of the Maillard reaction, which causes a brown or dark yellow color in food and can affect taste, smell, and food characteristics (Tanthapanichakoon, 2004).

Physicochemical characteristics of TMPHs

Changes in pH and total acidity (TA) are illustrated in Figure 2a. The initial pH was 7, and the pH progressively decreased throughout fermentation. By the conclusion of fermentation on day 4, the pH had reached 2.00 ±0.01. Concurrently, the TA, measured as lactic acid, exhibited a consistent increase, from an initial 0.13% to a final 2.59%.

Changes in TMPH during tempeh fermentation were determined using SDS-PAGE, and the total soluble

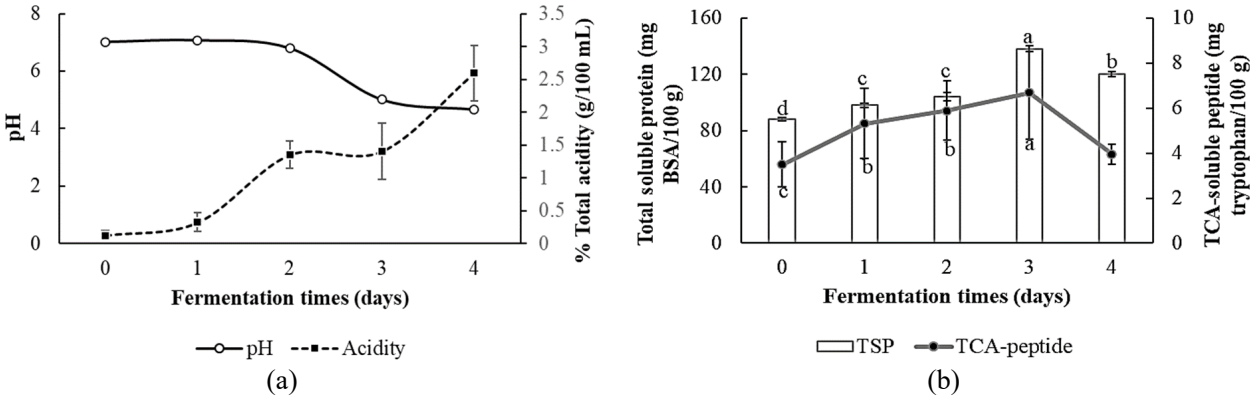

Fig. 2. Physicochemical changes during tempeh fermentation by an *R. oligosporus* starter culture; (a) pH and total acidity profiles, and (b) content of total soluble proteins and TCA-soluble peptides obtained from TMPHs. Error bars indicate standard deviation of the mean. Different letters indicate significant differences ($p < 0.05$) between means

protein of tempeh was measured at different fermentation times from day 0 to 4. The total soluble protein increased with fermentation time (Kuligowski et al., 2017; Liu et al., 2023). Until day 4, the total soluble protein decreased. The highest expression of total soluble protein occurred on day 3 of TMPH production (Fig. 2b).

Protein alteration of TMPHs

The highest level of TCA soluble tempeh peptides was recorded on day 3 of fermentation. The analysis of TMPHs during the four-day fermentation was performed using SDS-PAGE. TMPH protein profiles were different on days 0, 1, 2, 3, and 4. On day 0, a peptide at 10 kDa was present at higher levels than were found on days 1–4. However, a 20 kDa TMPH protein fraction was expressed at a higher intensity on days 1–3 than on days 0 and 4. In addition to the peptide at 10 kDa and the protein fraction at 20 kDa, 25 kDa protein fractions were present on day 0 but with lower intensities on days 1–4 (Fig. 3).

Fermented soybean tempeh retains both amino acids and protein peptides within the tempeh matrix (Teoh et

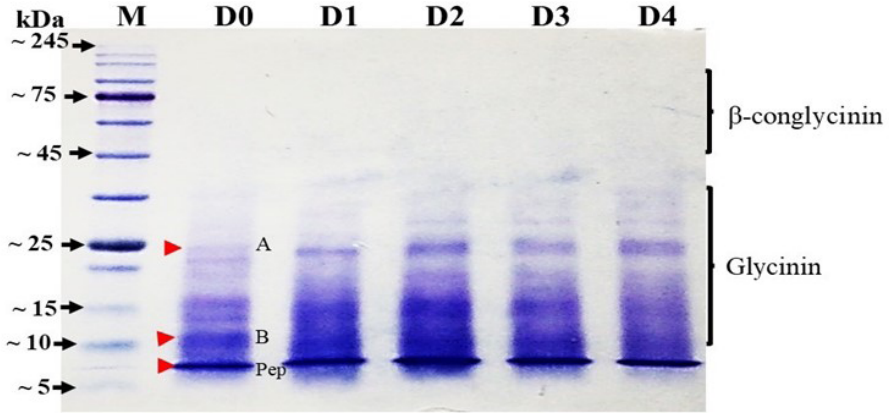

Fig. 3. Protein alteration of TMPHs determined from their SDS-PAGE profiles during tempeh fermentation at different incubation times (0 to 4 days). A, acidic subunit of 11s globulin; B, basic subunit of 11s globulin; Pep = peptide

al., 2024). This finding aligns with the research of Tahir, Anwar, Mubeen, and Raza (Tahir et al., 2018), who reported that tempeh fermentation allows for the preservation of protein content, including amino acids and protein peptides. Furthermore, studies have indicated that *Rhizopus* mold, which ferments soybeans into tempeh, may enhance protein levels during fermentation processes (Tan et al., 2024). Additionally, Bavia et al. (2012) found that tempeh exhibits increased protein content compared with fresh soybeans, along with various peptide components, including small molecular weight peptides such as GENEEEDSGAIVTVK (GK-15), which contribute significantly to tempeh's flavor profile (Amin et al., 2020).

Moreover, tempeh fermentation serves as a method of food preservation, facilitating the absorption of minerals, proteins, fibers, vitamins, and isoflavones, as well as producing bioactive peptides (Rizzo, 2024). This enhances nutrient absorption and phytochemical uptake at higher levels, thereby allowing tempeh to serve as a viable alternative protein source to animal meat. Moreover, tempeh fermentation helps maintain protein levels for extended periods, particularly during food preservation.

TPC, TFC and antioxidant activity of TMPHs

The highest amount of TPC was 227.750 ±6.594 mg GAE/100 g on day 3 of fermentation, while the lowest amount was 105.667 ±5.744 mg GAE/100 g found in unfermented tempeh. The total flavonoids were examined and quantified in addition to TPC. The lowest quantity of total flavonoids was 5.045 TFC (mg QUE/100g) on day 2 in unfermented tempeh, while the highest amount was 7.697 TFC (mg QUE/100g) on day 3 in fermented tempeh. Furthermore, the efficiency of free radical scavenging of fermented tempeh was shown using the DPPH and ABTS assays. From the ABTS assays, a higher scavenging effect of 90.305% was shown from days 2 to 4 than on days 0 to 1, and similar results were obtained using DPPH assays. In the DPPH assays, the highest scavenging activity of 82.23% was recorded on day 3 for fermented TMPH. On days 2 and 4, scavenging activity as assessed using DPPH assays was significantly lower at 76.174% and 75.55%, respectively (Kuligowski et al., 2017; Liu et al., 2023).

From the results of the antioxidant activity assays, fermented TMPHs had activity similar to that reported by Barus et al. (2019), such that Tempeh fermented using *Rhizopus* spp. (ATH 35, ATH 24, and ATH 53) presented the highest antioxidative effect, 84%, in the ATH 35 treatment. Fermented tempeh produced using *Rhizopus stolonifera* exhibited a higher antioxidative effect than that produced using *R. oligosporus* and *R. oryzae*. Watanabe et al. (2023) suggested that the antioxidant activity of tempeh results from substances in fermented tempeh such as isoflavone, aglycone and hydroxylate compounds. Furthermore, Liu et al. (2023) found the highest antioxidative effect after 48 h of tempeh fermentation by *R. oligosporus*, which was

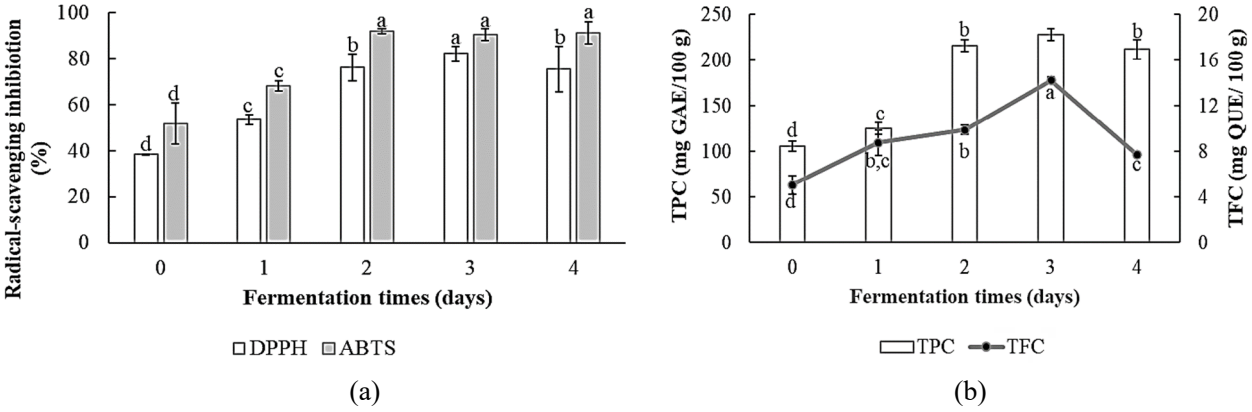

Fig. 4. Biological activity of TMPHs obtained from different tempeh fermentations, (a) indicated by total polyphenolic and total flavonoid contents, and (b) by DPPH and ABTS radical-scavenging inhibition. Error bars indicate standard deviation of the mean. Different letters indicate significance ($p < 0.05$)

related to the increased amount of total phenolic compounds (TPC) in a time-dependent manner. In 2022, Wang et al. (2022) investigated the bioactivity of black bean tempeh and reported that large protein molecules in this tempeh were gradually degraded during the fermentation process, producing many smaller peptides. However, the level of protein hydrolysis, including the quantity of peptides smaller than 10 kDa, was higher than for unfermented black bean tempeh.

CONCLUSIONS

This study described the characterization of tempeh milk protein hydrolysates (TMPHs). TMPHs were shown to be a source of bioactive peptides derived from proteins, including total soluble proteins, TCA--soluble peptides and small molecular weight peptides. Additionally, they contained TPC and TFC with antioxidant activity. The current study provides supporting information for the further development of tempeh milk protein hydrolysates (TMPHs) to catalyze protein hydrolysis and produce peptides with higher bioactivity that can be used as functional food ingredients.

DECLARATIONS

Data statement
All data supporting this study has been included in this manuscript.

Ethical Approval
Not applicable.

Competing Interests
The authors declare that they have no conflicts of interest.

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