Original article

Unveiling Neuroprotective Potential in Tempeh Peptide Extracts by *In Vitro* Screening of Anti-Alzheimer's Compounds

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Abstract

Background: Alzheimer's Disease (AD) incidence and prevalence increase every year, commonly related to neuron inflammation and degeneration conditions. Tempeh, a traditional fermented product from Indonesia, was reported to have anti-inflammatory, antioxidant, and anti-Alzheimer properties. However, anti-Alzheimer properties of tempeh peptide extracts have not been extensively examined. This research studied the effect of the extracted peptide from tempeh in preventing and delaying Alzheimer's disease.
Methods: Tempeh peptide was extracted using water maceration and quantified using HPLC and spectrophotometry. Anti-Alzheimer properties of tempeh were analyzed with Ellman's assay of anticholinesterase and in vitro gene expression analysis using LPS-induced neural Schwann cells.
Results: As a result, tempeh contained 19.27% of GABA, which is reported to have anti-Alzheimer properties, and other amino acids. Tempeh peptide extract at 12.5 µg/mL had strong inhibition activity toward acetylcholinesterase at 12.61%, and 100 µg/mL of tempeh peptide extract had 8.97% butyrylcholinesterase inhibition activity. Tempeh peptides extract also altered the expression of various genes related to Alzheimer's disease, such as TNF-α, BACE 1, Ntrk 1, BDNF 2, and APP.
Conclusion: This research proved that various peptides from tempeh have anti-Alzheimer properties.

Keywords: Alzheimer disease, Gene expression, Peptides, Soy Foods.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that attacks and damages neurons in the brain (1). It is the primary cause of dementia, which is characterized the by loss of memory and a decline in cognitive function (2). The incidence of AD increased by up to 147.95% from 1990 to 2019 (3). Even in Indonesia, more than 1.2 million Indonesians were suffering from AD in 2016 (4). The exact cause of AD remains unknown, but most novel research indicates that AD is caused by the formation of lesions called senile plaques (SP) and neurofibrillary tangles (NFT), which

promote neuron inflammation, degeneration, and death (5). Cholinergic system dysfunction and oxidative stress could also cause AD (6). AD cure research has been conducted for decades, but AD therapies are usually targeted at a single specific AD pathogenesis, and most of them fail to improve late-onset AD symptoms (7). Several natural products for AD have also been studied (8).

As a native crop plant to Asia, soybean has been cultivated and processed into a wide variety of food products (9). In Indonesia, soybeans are processed into a traditional fermented food called tempeh. Tempeh is

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produced by fermenting white soybeans (*Glycine* spp.) with a microbial consortium, usually including *Rhizopus oligosporus* (10). The fermentation process of tempeh alters the bioactive compounds. Therefore, tempeh increased the amount of free amino acids, γ-aminobutyric acid (GABA), and peptides that have proven to have several beneficial properties, such as antioxidant, anti-inflammatory, and anticholinesterase activities (11). These properties make tempeh a good candidate for natural AD prevention and therapy. Several studies have also shown that tempeh consumption can prevent various diseases, such as neurodegenerative, high cholesterol, diabetes, and diarrhea, and can be increase acetylcholine production, reduce β-amyloid production (6), and improve memory function in older people (12). Extracted GABA from tempeh is also capable of increasing memory function in rat models and has strong antioxidant properties (13). Related to this study, tempeh oil and its derivative (microemulsion) have also been examined and are known to possess anti-Alzheimer's activities through in vitro studies (14, 15).

An in vitro model study is often used to simulate multiple pathological processes in humans. The enormous information given by this study makes this method wildly used in many pharmacology and biomedical studies (16). We used *in vitro* methods in this study to simulate the capability of tempeh peptide extract to alter the expression of AD-related genes in the Schwann cell model. This study also analyzed the biochemical properties of tempeh peptide extract related to multiple AD pathogenesis, providing a deeper understanding of the potential of tempeh peptide extract in preventing AD.

Materials and Methods
Sample Preparation
The tempeh was made using a modified method from Mubarok and Deden (17). The tempeh was made with white soybeans (Glycine max) using tempeh starter culture. A kilo of soybeans was macerated for 12 hours and boiled for 30 minutes. The boiled soybeans was then air-dried for 20 minutes and fermented for 2 days wrapped in banana leaves. The tempeh was freeze-dried and ground to make tempeh powder as a sample for this research.

Tempeh Peptide Extraction
The water-soluble fraction of the tempeh peptide was extracted using a modification of Koh, Jamaluddin (18) method. Distilled water was used to dissolve the peptide compounds by macerating the tempeh powder for 50 minutes with agitation at 265 rpm. Then, the mixture was centrifuged to separate the pellet from the supernatant. The supernatant containing the water-soluble fraction of tempeh peptide extract was filtered and then freeze-dried.

High-Performance Liquid Chromatography (HPLC) Analysis
HPLC was used to detect and quantify the GABA content in the tempeh peptide extract. This was done based on the method described by Le, Verscheure (19) without any derivatization process. The stationary phase used was a C18 column. The mobile phases used were solvent A and solvent B. Solvent A was 0.05 mol/L sodium acetate buffer (pH 7.2), and solvent B was a mixture of 0.1 mol/L sodium acetate buffer, acetonitrile, and methanol. The samples were measured using a UV variable wavelength detector (VWD) at 338 nm, and the flow rate was set to 1 mL/min. The standard curve was made using purified GABA.

γ-aminobutyric acid (GABA) Quantification
GABA quantification was performed using a modified spectrophotometric method described by Karladee and Suriyong (20). The sample was diluted with 80% ethanol solution and centrifuged at 3000 x g at 22 °C for 15 minutes, then the supernatant was separated and mixed with 0.2 mL borate buffer, 1 mL 6% phenol reagent, and 0.4 mL NaCl 9% in a test tube. Then the mixture was boiled for 10 minutes and ice-cooled for 15 minutes. The absorbance was measured using a spectrophotometer at 645 nm. GABA concentration was quantified by calculating the linear model equation result from the standard curve made with purified GABA.

Anticholinesterase Activity Analysis

This method was used to measure the inhibitory activity of tempeh peptide extract against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). This test was done using modified Ellman's method described by Rhee *et al.* (2021). This method was performed using 5,5'- dithio-bis- [2-nitrobenzoic acid] (DNTB) and a mixture of multiple buffer solutions. Buffer A contained 50 mM Tris-HCl (pH 8), buffer B contained 50 mM Tris-HCl (pH 8) and 0.1% w/v bovine serum albumin (BSA), and buffer C contained 50 mM Tris-HCl (pH 8), 0.1 M NaCl, and 0.02 M $MgCl_2 \cdot 6H_2O$.

The test was carried out on a 96-well microplate using a mixture of 25 μL ACTI, 125 μL DNTB, 50 μL B buffer, and 25 μL of sample, incubated at 30 °C for 10 minutes. Then, the mixture was added with 25 μL of enzyme (AChE and BChE) and measured with a microplate reader at 412 nm at 30 °C for 15 minutes. Commercial AD drugs galanthamine and donepezil were used as positive controls. Inhibition activity values were expressed as percentages and calculated using this formula:

$$\text{Inhibition activity (\%)} = \frac{negative\ control\ absorbance - sample\ absorbance}{negative\ control\ absorbance} \times 100\%$$

Cytotoxicity Analysis

Cytotoxicity was assessed using a method described by Tolosa, Donato (21) to test the toxicity of the boiled soybean peptide extract, tempeh peptide extract, and lipopolysaccharide on neural Schwann cells (RSC96) ATCC CRL-2765. The test was done using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) reagent to measure cell viability after exposure to the samples. About 25 μL of cells were mixed with 25 μL of sample on a 96-well microplate, then incubated for 24 hours at 37 °C with 5% CO_2. Then, the cells were washed with 25 μL MTT and incubated for 2 hours at 37 °C with 5% CO_2. DMSO was then added and incubated for another 10 minutes at room temperature, then the color change was measured using a microplate reader at 550 nm of wavelength.

Gene Expression Analysis

This study also analyzed the potential of tempeh peptide extract to alter the expression of multiple genes that were related to AD pathogenesis. The gene study was done using an *in vitro* model of neural Schwann cells from *Rattus norvegicus* (RSC96) ATCC CRL-2765. The cells were exposed to lipopolysaccharide (LPS) from *Salmonella typhosa* to stimulate neural inflammation in AD patients (22). Then, healthy and LPS-exposed cells were further exposed to samples.

Total cellular RNA was extracted using GENEzol™ Reagent (Geneaid), and the cDNA synthesis was carried out using the SensiFAST™ cDNA Synthesis Kit (Bioline). The obtained cDNA was then measured with RT-qPCR using a mixture of 10 μL SsoFast™ EvaGreen® Supermix, 1 μL primer, 1 μL template, and 8 μL nuclease-free water. The list of primers used in this test is shown in Table 1.

Table 1. List of primers.

Gene	Function	Sequences	Size (bp)	Accession Number
BDNF 2	Cholinergic system	F:GTGTGACCTGAGCAGTGGGCAAAGGA R: GAAGTGTACAAGTCCGCGTCCTTA	554	NM_012513.4
*NTrk*1	Cholinergic system	F: TTCAATGGCTCCGTGCTCAATG R: GGTCTCCAGATGTGCTGTTAGTGT	274	BC062580.1
BACE 1	β-amyloid Production	F: GGATTATGGTGGCCTGAGCA R: CCAGGATGTTGAGCGTCTGT	667	NG_029372.2
APP	β-amyloid Production	F: AGCAGAAGGACAGACAGCAC R: AGTGGTCAGTCCTCGGTCAG	455	XM_006248008.5
TNF-α	Inflammation Response	F: GGCAGGTCTACTTTGGAGTCATTG R: ACATTCGAGGCTCCAGTGAATTCGG	319	AF269159.1
β-actin	Internal control	F: TGGAATCCTGTGGCATCCATGAAAC R: TAAAACGCAGCTCAGTAACAGTCCG	439	XM_032887061.1

Statistical Analysis

The results were analyzed with IBM SPSS Statistics Data Editor using an independent sample t-test and Tukey's one-way ANOVA to determine differences and homogeneity between sample groups. The result of anticholinesterase activity analysis was calculated using an independent sample t-test to test the significance of inhibition between boiled soybean peptide extract and tempeh peptide, extract with $p = 0.05$. Gene expression data were analyzed statistically using one-way ANOVA.

Results

Tempeh Extract Peptide Contents and GABA Profiling using HPLC

Extraction of water-soluble peptide from tempeh yielded more than 33% of solid peptide mass, and 17% from the boiled soybean extract.

HPLC analysis for GABA profiling showed that purified GABA control was detected in two peaks at 32 minutes of retention time, like purified GABA control (Table 4). The first peak was glutamate, and it showed that each 1 g/mL of tempeh peptide extract and boiled soybean peptide extract contains 1.40 mg (0.14% $^{w}/_{w}$) and 7.02 mg (0.7% $^{w}/_{w}$) glutamate. The second peak was the GABA peak, and it also showed that tempeh peptide extract contained 1.65 mg (0.17% $^{w}/_{w}$) GABA, while boiled soybean contained 2.14 mg (0.21% $^{w}/_{w}$) of GABA (Table 2). Quantification of GABA was also done with a spectrophotometric method using the phenol reagent. The test showed that every 1 g/mL of boiled soybean contained 52.91 mg/mL (5.29% $^{w}/_{w}$) of GABA, while every 1 g/mL of tempeh showed that it contained 192.73 mg/mL (19.27% $^{w}/_{w}$) of GABA, with a linear equation of $y = 0.0011x + 0.0355$ with $R^2 = 0.9987$.

Table 2. Glutamate and GABA content in boiled soybean and tempeh peptide extracts.

Substances	Sample	Retention time (mins)	Peak area (mAU*s)	Content (mg)
Glutamate	Soybean	32.66	1883.56	7.02*
	Tempeh	32.62	535.84	1.40*
GABA	Soybean	32.88	611.42	2.14**
	Tempeh	32.81	503.05	1.65**

Anticholinesterase Activity of Tempeh Peptide Extract

*Content in 1g/mL of sample, concentration was calculated using a linear equation of: $y = 0.00479x + 201.43$ with $R^2 = 0.1959$. **Content in 1 g/mL of sample, concentration was calculated using a linear equation of: $y = 0.00445x + 135.31$ with $R^2 = 0.940$.

Anticholinesterase (AChE) analysis of tempeh showed that the tempeh peptide extract has more AChE inhibition activity than boiled soybean peptide extract, especially at 12.5 µg/mL, which showed the highest inhibition activity toward AChE at 12.61%. Meanwhile, boiled soybean peptide extract showed more than 1% inhibition activity at most concentrations, except at 100 µg/mL, which showed 3.23% inhibition activity toward AChE. Moreover, at 100 µg/mL, boiled soybean peptide extract showed the highest inhibition activity toward BChE at 6.67% and

tempeh peptide extract also showed highest inhibition activity toward BChE at 8.97%. The results also showed that tempeh peptide has more inhibition activity toward both AChE and BChE (Tables 3 and 4). Positive controls, galanthamine showed the highest inhibition activity toward both AChE (87.48%) and BChE (74.47%), while donepezil showed higher inhibition activity toward AChE than BChE.

Statistical analysis using an independent sample t-test showed that there was a significant difference of AChE inhibition activity between tempeh peptide extract and

boiled soybean extract, while on BChE, there was no significant difference between tempeh peptide extract and boiled soybean extract. Meanwhile, control groups, galanthamine and donepezil were also significantly different from each other and when compared to the tempeh peptide extract and boiled soybean extract.

Table 3. Acetylcholinesterase inhibition activity value of tempeh peptide extract at various concentrations (Mean ± SD, *n* = 4).

Sample (µg/mL)	Inhibition activity (%)				
	12.5	25	50	75	100
Soybean	1.68 ± 0.53	1.99 ± 0.54	1.55 ± 0.57	1.08 ± 0.64	3.23 ± 0.72
Tempeh*	12.61 ± 0.18	3.43 ± 0.44	5.05 ± 0.45	3.87 ± 0.11	9.41 ± 0.23
Galanthamine**	71.69 ± 0.61	79.69 ± 0.63	86.47 ± 0.47	87.40 ± 0.62	87.48 ± 0.60
Donepezile**	43.82 ± 1.32	59.13 ± 1.58	67.22 ± 1.61	70.72 ± 1.65	73.66 ± 1.67

* p < 0.05 compared to boiled soybean peptide extract. ** p < 0.05 compared to other samples.

Table 4. Butyrylcholinesterase inhibition activity value of tempeh peptide extract at various concentrations (Mean ± SD, *n* = 4).

Sample (µg/mL)	Inhibition activity (%)				
	12.5	25	50	75	100
Soybean	4.29 ± 0.18	3.61 ± 0.23	2.91 ± 0.17	2.87 ± 0.09	6.67 ± 0.40
Tempeh	3.75 ± 0.05	4.91 ± 0.10	5.49 ± 0.06	6.29 ± 0.08	8.97 ± 0.27
Galanthamine**	38.14 ± 0.51	55.00 ± 0.20	64.28 ± 0.08	73.23 ± 0.10	72.47 ± 0.16
Donepezile**	11.19 ± 0.80	10.88 ± 0.87	23.66 ± 0.87	22.06 ± 0.65	17.80 ± 0.85

** p< 0.05 compared to other samples.

Cytotoxicity Analysis of Tempeh Peptide Extract on Schwann Cells (RSC96)

The test showed due to Table 5, that all samples exhibited only minor cytotoxicity toward Schwann cells, especially the boiled soybean peptide extract, which slightly reduced the number of cells. Meanwhile, the addition of LPS and increased concentrations only caused small changes in cell viability across all samples. These results showed that exposure to LPS and higher concentrations of tempeh peptide extract and boiled soybean peptide extract did not reduce cell viability by more than 50%.

Table 5. Cytotoxicity analysis result of LPS-induced boiled soybean extract, tempeh peptide extract on Schwann cells (RSC96).

Sample	Cells Viability (%)
Positive control	100
S50	99
S100	82
T50	100
T100	89
S50 + LPS	66
S100 + LPS	61
T 50 + LPS	100
T 100 + LPS	100

S: soybean, T: tempeh.

Effect of Tempeh Peptide Extract on Alzheimer's Disease-Related Gene Expression in LPS-Induced Schwann Cells (RSC96)

This analysis was done using tempeh peptide extract and boiled soybean peptide extract as the samples, LPS-induced cells as a positive control, and healthy Schwann cells as the negative control. The RT-qPCR cycle threshold (CT) value was then used to calculate $2^{-\Delta\Delta Ct}$ and analyzed using Tukey's one-way ANOVA. The result showed that *TNF*-α and *BACE*1 genes were significantly downregulated compared to the positive control (Fig. 1). Meanwhile, *Ntrk*1 and *BDNF*2 genes were significantly upregulated compared to the positive control. The *APP* gene showed mixed expression, as it was significantly upregulated when treated with 50 µg/mL of tempeh extract and downregulated when treated with 100 µg/mL.
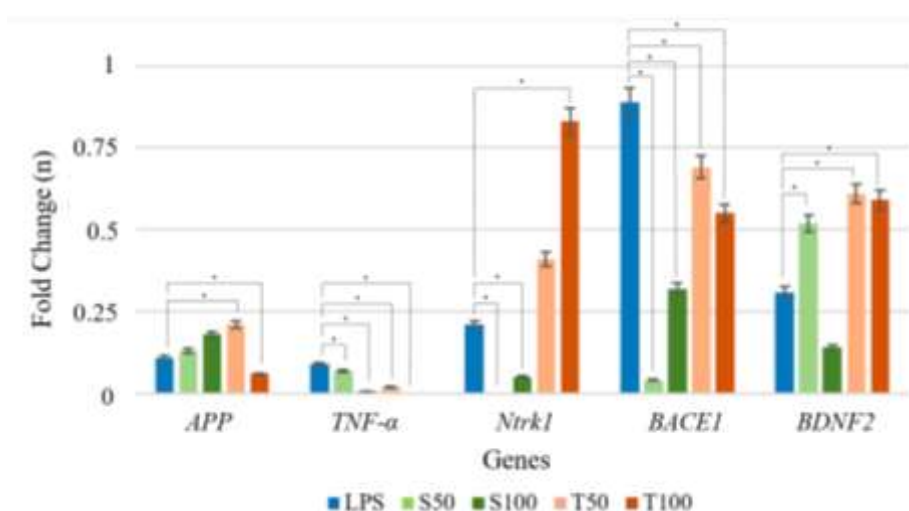

Fig. 1. Gene expression level of Schwann cells treated with boiled soybean peptide extract (S) and tempeh peptide extract (T). Meanwhile, LPS-induced cells (LPS) were used as a positive control. *Data was significantly different compared to the positive control (LPS), p< 0.05.

Discussion

Extraction of water-soluble peptides from tempeh showed that tempeh has more water-soluble peptide mass than boiled soybeans. The altered content of peptides and other compounds in tempeh could be the result of the fermentation process, which involves many enzymes produced by the inoculum (23). Hydrolysis of proteins by proteases and an increase in amino acid content in tempeh during fermentation have been documented in numerous studies (24, 25). Unfortunately, it is difficult to determine the exact effect of the fermentation process on tempeh because there are too many variables involved, such as fermentation conditions, substrate conditions, inoculum strain, type of wrapping used, and many more. HPLC results showed that tempeh contained lower amounts of glutamate and GABA than the boiled soybean peptide extract. GABA and glutamate are small aliphatic amino acids that do not naturally possess strong electroactivity or UV absorbance. These properties can affect the accuracy and precision of crude GABA and glutamate quantification using HPLC (26), and may partly explain the low regression value observed for the glutamate results. Various derivatizing reagents for GABA, such as o-phthalaldehyde (OPA) and naphthalene-2,3-dicarboxaldehyde (NDA), are often used to increase GABA stability, electroactivity, and UV absorbance (27). The low resolution of the chromatogram data was likely due to the absence of a GABA-specific derivatization step in this method, as the main goal of the research was to screen various peptides and amino acids. Further optimization is needed to detect GABA with greater accuracy. Moreover, traditional tempeh fermentation has been documented to reduce GABA content in the substrate due to the presence of oxygen,

which inhibits GABA production during solid-state aerobic fermentation. In contrast, the absence of oxygen promotes cellular acidification, creating optimal conditions for GABA and amino acid synthesis in during soybean fermentation (28). Meanwhile, spectrophotometric analysis showed that tempeh contains a higher concentration of GABA, which contradicts the HPLC results. This contradiction likely arises because the spectrophotometric method uses Folin's phenol reagent, which reacts non-specifically with amino acids. As a result, this method has been reported to overestimate GABA levels, often 6-45 times higher than HPLC results, due to the presence of other amino acids in tempeh that interfere with the reaction (19), as well as heat-induced degradation of GABA (29). However, this still proves that tempeh contains higher GABA and other amino acids compared to boiled soybean extract.

Human cholinesterase consists of two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). These enzymes play important roles in the cholinergic nervous system of the human brain. The main difference between them lies in their substrate preference. AChE primarily hydrolyzes acetylcholine, while BChE is more effective at hydrolyzing butyrylcholine. Both acetylcholine and butyrylcholine function as key neurotransmitters in the cholinergic system, which is involved in many brain processes, particularly those related to cognition and memory (30). The cholinergic hypothesis of Alzheimer's disease (AD) suggests that elevated levels of AChE and BChE can lead to reduced availability of cholinergic neurotransmitters, thereby disrupting synaptic transmission and impairing brain function (31). Cholinesterase inhibitors are compounds that block the activity of these enzymes. Several of these inhibitors, including galantamine, donepezil, and rivastigmine, are currently used as treatments for AD (32). Many of these synthetic substances are reported to have low effectiveness and side effects, such as hepatotoxicity and gastrointestinal disturbances (33). In addition, there are only a few natural substances that are known to have cholinesterase inhibitor properties.

In this research, tempeh peptide extract showed more significant inhibitory activity toward AChE than boiled soybean peptide extract. Fermented products usually show elevated peptide and amino acid contents due to protein breakdown from the substrate by the inoculum (24). Peptides and amino acids contained in fermented soybean products have been documented to exhibit strong inhibitory properties toward acetylcholinesterase (34). On the other hand, tempeh peptide extract did not show significant inhibitory activity toward BChE. BChE is commonly found in blood plasma and usually exists at a lower concentration than AChE. However, a recent study reported that BChE is also involved in AD pathogenesis (35). Both tempeh peptide extract and boiled soybean peptide extract showed lower inhibitory activity toward BChE compared to AChE. This could be the result of peptide inhibitor selectivity, since BChE and AChE have different molecular conformations (36). Enzyme kinetic analysis and molecular computational modeling may be performed to gather more detailed information.

The APP gene is involved in amyloid protein synthesis and the cleavage process (37). The APP gene mutations are usually found in AD patients and cause amyloid plaque formation in the brain. A mutated APP gene alters the building blocks of the amyloid precursor protein, leading to the formation and accumulation of toxic β-amyloid, which forms plaques in the brain (38). APP is generally overexpressed in AD patients, as it can lead to the induction of β-amyloid protein and neurodegeneration (39). In this research, a low concentration of tempeh peptide extract caused significant upregulation of the APP gene, and significant downregulation was observed at higher concentrations. The expression also showed a downward trend as the concentration of tempeh peptide extract increased. This effect could be further tested to analyze the trend of APP gene regulation with increasing tempeh peptide extract concentrations. On the

other hand, boiled soybean peptide extract showed insignificant upregulation of the APP gene expression compared to the tempeh peptide extract and control, with an upward trend as the concentration increased. The trend of tempeh peptide extract showed better inhibitory potential toward APP gene expression than the soybean peptide extract, especially at higher concentrations.

Another gene involved in AD pathogenesis related to the cellular inflammation pathway is tumor necrosis factor alpha (TNF-α). TNF-α is a proinflammatory cytokine that has been documented to cause neuroinflammation in the brain and induce AD-like symptoms (40). The expression of TNF-α also affects the regulation of β-amyloid protein and promotes the formation of senile plaques (41). Tempeh is also known to have a strong anti-inflammatory effect by activating certain anti-inflammatory genes (6). This research showed that tempeh peptide extract caused significant downregulation of TNF-α and a downward trend with increasing concentrations. On the other hand, boiled soybean peptide extracts also showed a significant downregulation trend of TNF-α with increasing concentration. This indicates that both boiled soybean peptide extract and tempeh peptide extracts have anti-AD potential by inhibiting the expression of the TNF-α gene and limiting the inflammatory reaction. Several studies have reported immunomodulatory effects of GABA and other amino acids in fermented foods and their ability to reduce cytokine secretion (25, 42).

Neurotrophic tyrosine kinase receptor (NTRK) is a tyrosine kinase receptor involved in the cellular tyrosine kinase signaling pathway, which regulates cellular development, communication, apoptosis, and plasticity. The NTRK group consists of TRKA, TRKB, and TRKC, which are the binding sites for nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin-3, respectively. Many AD patients and mouse models show significant downregulation of the Ntrk1 gene expression (43). In this study, cells treated with tempeh peptide extract showed a significant upregulation trend of Ntrk1 expression, while all boiled soybean peptide extract-treated cells showed significant downregulation of the Ntrk1 gene. Other clinical trial studies have also reported that consumption of fermented soybean increased serum BDNF levels and improved cognitive function, likely because the GABA content in tempeh peptide extract can alter BDNF expression (44).

This research concludes that tempeh peptide extract can be utilized to prevent and delay the progression of AD-related diseases. Tempeh peptide extract contains GABA and peptides that exhibit strong cholinesterase inhibition activity, which can be used to prevent cholinergic dysfunction, cellular oxidative stress, and inflammation in AD patients. Moreover, extracted tempeh peptide also showed promising results and trends in regulating APP, TNF-α, Ntrk1, BDNF2, and BACE1 genes to lower the risk of AD. Further research and molecular modeling may be needed to identify each potential peptide and to simulate its anti-AD properties.

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Conflicts of Interest
The authors certify that there is no conflict of interest.

Authors' contribution
Gilbert Kurnia contributed to all the data acquired above for the research. Dionysius Subali and Yanti contributed for the research design and data analysis. Revelo Eved Christos and Yang-Chia Shih contributed for the writing and data presentation. All authors read and approved the final manuscript.

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