



## Proteomic study of bioactive peptides from tempe

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**Tempe is a traditional Indonesian fermented soybean mostly produced in small industries and sold locally throughout the country. Studies on the bioactive peptides in tempe are rare. Here, we studied bioactive peptides in samples from three tempe producers with different degrees of sanitation. The peptide sub-fractions of tempe from each producer were collected following water extraction, ultrafiltration (<3 kDa), gel filtration chromatography, and reversed phase-high performance liquid chromatography (RP-HPLC) separation followed by liquid chromatography-mass spectrometry (LC-MS). The MS spectra were then predicted using FindPept tools, and their biofunctionalities were confirmed with BIOPEP databases. There were few similar peptides found in tempe from the three producers. Peptides Val-His and Ala-Leu-Glu-Pro were found in tempe from all producers. Producers having a good sanitation level had more bioactive peptides than those with moderate or poor sanitation levels (58%, 43% and 35%, from good to poor sanitation). This work showed that the tempe from the three producers had antihypertensive, antidiabetic, antioxidative and antitumor peptides.**

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[**Key words:** Bioactive peptide identification; Bioinformatic; Fermented soybean; Gel filtration chromatography; Proteomic; Tempe]

Tempe is an indigenous Indonesian fermented soybean product rich in nutritive components. During fermentation, microorganisms generate several vital bioactive components and decrease anti-nutrition agents. Biochemical changes occur during soybean fermentation in tempe that improve human health. There is an increase in soluble protein, folate, vitamin B12, tocopherols, free isoflavones, and superoxide dismutase (SOD) with decreases in lipids, phytic acid, oligosaccharides, trypsin inhibitor, and tannins (1–3). Fermentation allows microorganisms to secrete proteolytic enzymes capable of converting the proteins in soybean into peptides (such as dipeptides, tripeptides, and oligopeptides) possessing many biofunctional properties (4,5). Some studies revealed that the peptides in fermented soybean products such as *doenjang* (Korea), *douchi* (China), *natto* (Japan), *thua nao* (Thailand), and tempe (Indonesia) are associated with biofunctional properties such as angiotensin I-converting enzyme (ACE) inhibition, antioxidant, anti-diabetic, anticancer, antithrombotic, hypocholesterolemic, and immunomodulatory activities (6–11).

Most tempe uses soybeans (*Glycine max* L.) as the substrate for microflora during fermentation. Soybeans are rich in proteins and are up to 35–40% protein in dry basis (12). The main proteins in

soybean include glycinin (11S globulin), lipophilic proteins (LP), and  $\beta$ -conglycinin (7S globulin) accounting for 46%, 31%, and 23% of the total protein (13). Other storage proteins in soybean are *Kunitz* and *Bowman-Birk* trypsin inhibitors, lectins (hemagglutinins), lip-oxygenase, sucrose-linked protein, and  $\alpha$ -amylase (14,15). Glycinin (molecular weight/MW 320–375 kDa) has five subunits (G1, G2, G3, G4 and G5), while  $\beta$ -conglycinin (MW 150–200 kDa) has four subunits ( $\alpha$ ,  $\alpha'$ ,  $\beta$  and  $\gamma$ ). Bioactive peptides are usually encrypted in their storage proteins and are commonly composed of 2–20 amino acid residues. These peptides are functionally active after protein cleavage by proteolytic enzymes during fermentation. The conversion of the storage protein into bioactive peptides occurs via two major mechanisms, i.e., fermentation and enzymatic hydrolysis (16).

Tempe fermentation involves *Rhizopus* spp. molds and other microorganisms such as lactic acid bacteria and yeasts (17,18). There are four important steps to making tempe including soaking, boiling the soybeans, mold inoculation using starter called *ragi* (containing many microorganisms, mainly *Rhizopus* spp.), and incubation at room temperature for 24–36 h (19). Many types of molds are involved in the manufacture of tempe in Indonesia including *Rhizopus oligosporus*, *R. oryzae*, *R. arrhizus*, *R. stolonifer*, *R. microsporus*, *R. rhizopodiformis*, *R. chinensis*, and *Mucor* sp. (1,20). Tempe production in Indonesia may differ among regions and producers regarding process and fermentation conditions (21). In short, there is no standard for making tempe in Indonesia. This allows one to produce various peptides in tempe (20) due to the differences in tempe inoculum, processing, packages, hygiene, and

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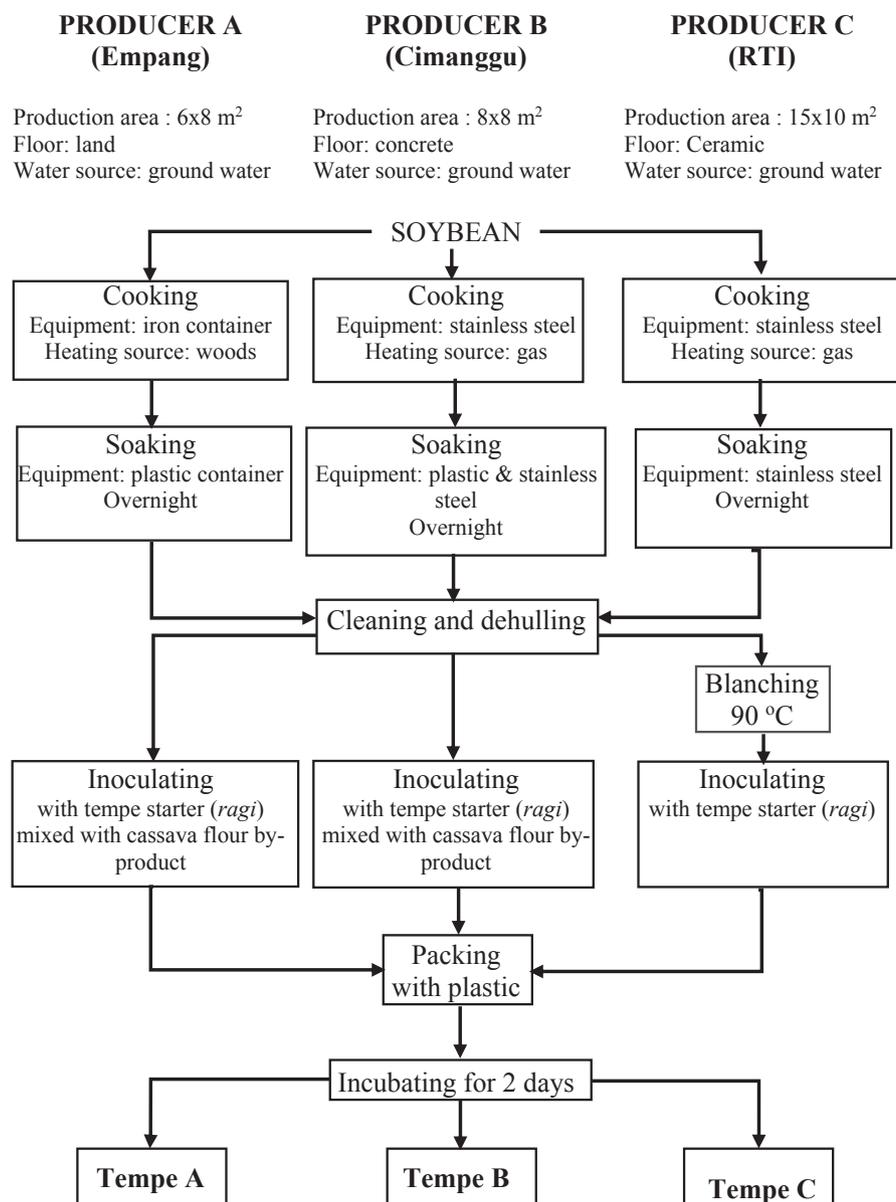


FIG. 1. Different tempe processes of three different producers.

sanitation levels. This can lead to variations in the diversity of microorganisms underlying fermentation (18). Fermentation has a remarkable ability to affect peptide sequences and the bio-functionalities of tempe, but the relationship between peptide products and fermentation details has not yet been extensively studied.

Numerous studies have reported biofunctional peptides derived from fermented foods—especially those from fermented soybeans. Dipeptidyl peptidase (DPP) IV inhibitor peptides, Lys-Leu and Leu-Arg, had been known as anti-diabetic agents from natto fermented by *Aspergillus oryzae* (8). Soy sauce fermented by *Aspergillus sojae* produced the antihypertensive peptides Gly-Tyr, Ala-Phe, Val-Pro, Ala-Ile, and Val-Gly (22). Fermentation of miso using *A. oryzae* produced two antihypertensive peptides: Val-Pro-Pro and Ile-Pro-Pro (23). In addition, antioxidative peptides in okara fermented by *A. oryzae*, *R. oligosporus*, *Actinomucor elegans*, and *Bacillus subtilis* were also found (24). Leu-Ile-Val-Thr-Gln and Leu-Ile-Val-Thr

peptides resulted from soy protein fermented by *Lactobacillus casei* spp. *pseudoplantarum* could act as an angiotensin converting-1-enzyme (ACE I) inhibitor for anti-hypertension applications (25). Thus, it is clear that many different bioactive peptides consisting of 2–5 amino acids are found in fermented soybeans.

Although tempe was first recorded in the 16th century and has been widely consumed throughout Indonesia, most producers still retain traditional methods for producing tempe. Tempe has recently been produced under better hygiene and sanitization conditions. However, there are no published reports on the different bioactive peptides in tempe as a function of sanitation conditions during production. This is important because it can affect the microorganisms involved and the bioactive peptides generated. Therefore, the main aim of this research was to identify the bioactive peptides resulting from several tempe producers with different sanitation levels using proteomic and bioinformatics approaches.

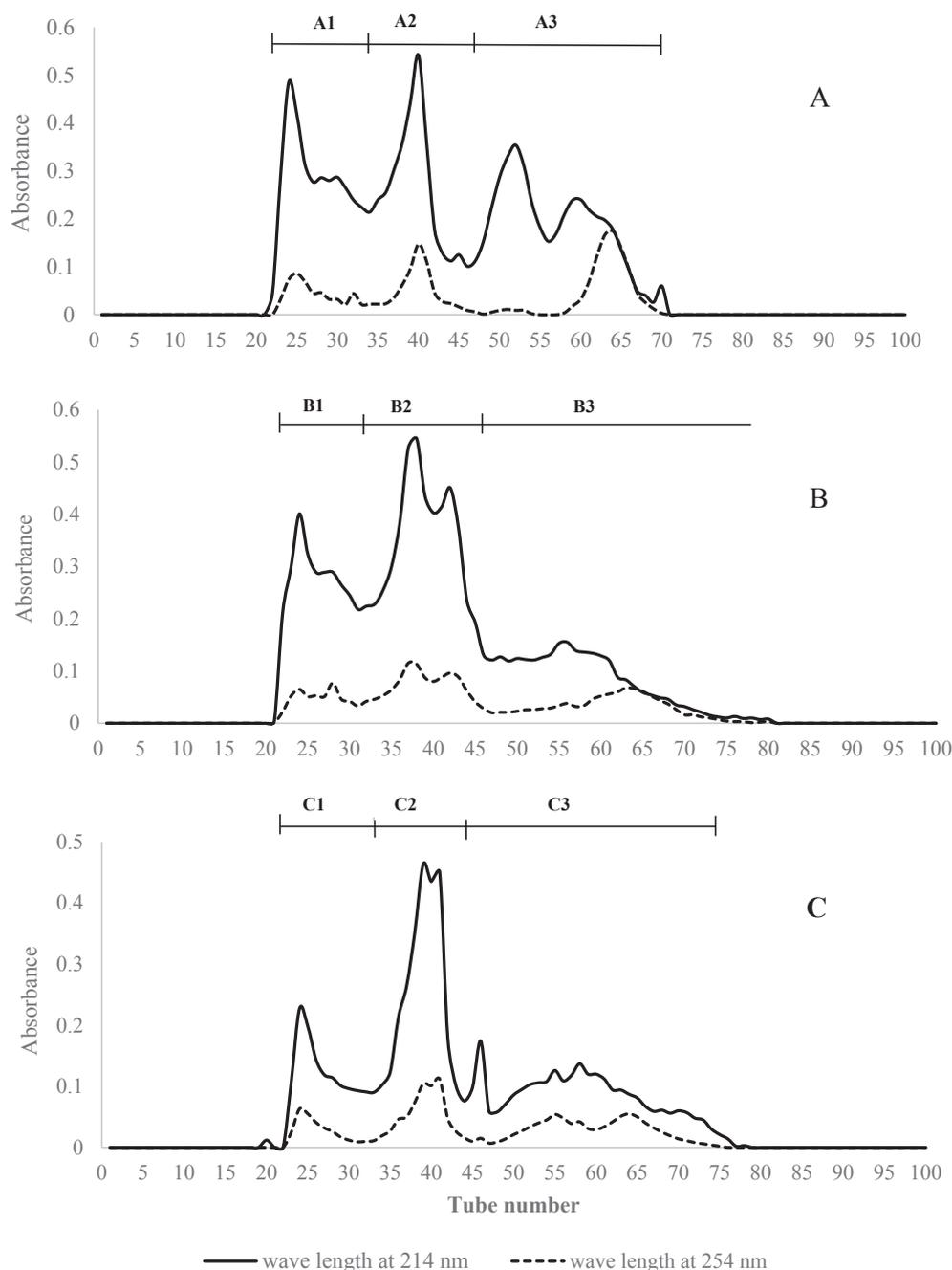


FIG. 2. Fractions of chromatograms of three different ultrafiltered samples of tempe (<3 kDa) obtained by Sephadex G-15 gel filtration chromatography (65 cm x 2.5 cm i.d.) using deionized water as an eluent. Chromatograms represented the results of tempe samples: (A) from producer with poor sanitation level, (B) with moderate sanitation level and (C) with good sanitation level. Each sample has three fractions.

#### MATERIALS AND METHODS

**Materials** Tempe samples were obtained from three different producers located in Bogor, Indonesia with different sanitation levels: tempe A from Empang (poor), tempe B from Cimanggu (moderate), and tempe C from RTI (good). The plate count agar (PCA) and eosin methylene blue agar (EMBA) were obtained from Oxoid, UK. Acetonitrile, trifluoroacetic acid (TFA), formic acid (FA), and methanol for reversed phase-high performance liquid chromatography (RP-HPLC) were from Merck (Darmstadt, Germany).

**Determination of sanitation levels of tempe producers** Tempe A was produced in a 42-m<sup>2</sup> area with limited facilities including an earth floor, iron containers for cooking, plastic containers for soaking, and wood-burning stove. Tempe B was manufactured in a 64-m<sup>2</sup> facility with a concrete floor, stainless-steel and plastic containers for cooking and soaking, and gas for heating. Tempe C was made in a 150-m<sup>2</sup> facility with a ceramic floor and equipped with stainless-

steel containers using gas for heating. The sanitation levels on these tempe producers were determined by total bacteria testing using the total plate count in their production room and water. Five sterilized petri dishes (9 cm i.d.) containing 2% PCA (Oxoid, UK) were put in five different sites of the production room; the lids were opened for 15 min and incubated at 37°C for two days. The number of total bacteria colonies was then counted and expressed as colony forming units (CFU). Meanwhile, a 200-mL sterilized glass jar was used to collect the water used at each producer. The water was then poured into two sterilized petri dishes containing PCA agar (with 10<sup>0</sup>, 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> dilutions) and EMBA agar (selective agar for *Escherichia coli*); these were incubated at 37°C for two days.

**Protein extraction** Tempe (250 g) from each producer was sliced (1-mm thick) and then oven-dried (60°C) for 7 h and ground to a fine powder. The tempe powder (1 g) was dissolved with 30 mL of acetonitrile (Merck), 10% (v/v) trifluoroacetic acid (TFA) (Merck), and distilled water (1:0.02:1, v:v:v). The solution was then sonicated for 5 min and incubated in a shaking water bath for

60 min at 30°C, and finally centrifuged at 20,000 ×g for 25 min at 4°C (Himac CR21G, Hitachi, Tokyo, Japan). The supernatant was collected and filtered using 0.45 µm regenerated N47 membrane. The filtrate was stored at –21°C for further processing.

**Ultrafiltration** The filtrate was then ultra-filtered with centrifugation using Amicon Ultra 2 Ultra Cel 3 (molecular weight cut off/MWCO 3 kDa) (Sigma Aldrich, St. Louis, MO, USA). Centrifugation lasted for 60 min at 3500 ×g and 20°C. The fraction with a molecular weight of <3 kDa was collected and stored at –21°C for the next steps.

**Fractionation by gel filtration chromatography** A volume of 10 mL of the <3 kDa ultrafiltered fraction of each sample was loaded onto a column packed with Sephadex G-15 (2.5 cm i.d. × 65 cm) (GE Healthcare Bio-Sciences AB, Sweden) and eluted with deionized water as a mobile phase at a constant flow rate of 2.14 mL/min until 100 tubes with 7.5 mL of eluate each were obtained via a fraction collector SF-100 (Advantec Toyo, Japan) The UV absorbance of each eluate was measured at wavelengths of 214 nm and 254 nm using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). The fractionation using gel filtration chromatography was repeated three times (total 30 mL of loaded sample). The UV absorbance values were plotted as chromatograms (Fig. 2). Three peak areas of chromatograms were collected and then freeze dried for subsequent analysis.

**Separation by RP-HPLC** The freeze-dried fractions were then re-constituted with deionized water and filtered using 0.45 µm cellulose acetate membrane for RP-HPLC analysis. The fractions of each sample (200 µL) were injected into HPLC LC-6A (Shimadzu) using a C18 column (4.6 mm i.d. × 150 mm, 5 µm, Zorbax, Agilent, Japan) operating at a flow rate of 1.0 mL/min, using 10% acetonitrile in water containing 0.1% (v/v) TFA as a mobile phase in isocratic conditions with UV-Vis detector at 220 nm. The fractions of the three samples were then separated into 23 sub-fractions. The sub-fractions obtained from 5 runs (running time for 15 min) of RP-HPLC were collected, freeze-dried, and stored at –21°C for liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis.

**Peptide identification by LC-ESI-MS** The freeze-dried sub-fractions were hydrated with 20% acetonitrile and then filtered using a 0.22-µm PVDF membrane (Merck) and analyzed with LC-ESI-MS. LC-ESI-MS was performed on LC-20ADXR UFLC (Shimadzu) system equipped with SPD-M20A diode array detector connected to Waters-ESCI multi-mode ionization. Each sub-fraction (50 µL) was injected and separated using Cadenza CD-C18 column (2.0 mm i.d. × 150 mm, 5 µm) employing 0.1% (v/v) formic acid in water (mobile phase A) and acetonitrile (mobile phase B) with 10% B (0 min)–100% B (30 min) linear gradient program at a flow rate of 0.2 mL/min. The MS condition was a positive mode of electrospray ionization with a capillary voltage of 5.00 kV and an ion source temperature and desolvation temperature of 120 and 350°C, respectively. The cone gas flow was 50 L/h, and the desolvation gas flow was 700 L/h. The MS analyzer was a triple quadrupole with a single scanning quadrupole at a mass scan range of 100–1100. The multiplier detector voltage of the MS was set at 650 V. The data obtained from the LC-ESI-MS were then analyzed using Mass<sup>++</sup> (64-bit) software (<http://www.mspp.ninja/>).

**Prediction of peptide sequences and biofunctionalities** Peptide sequences were identified by adopting the method of Singh and Vij (26) with modifications. The mass to charge (*m/z*) of the MS spectra was then searched using FindPept tools available on ExPASy Bioinformatics Resource Portal (<https://www.expasy.org/>). Storage proteins in soybean including glycinin components (G1, G2, G3, G4 and G5), β-conglycinin components (β-chain and α-chain), lectin and lipoxigenase were traced. The mass tolerance was ±0.12 Da using monoisotopic masses and interpreted as positive mode [M+H]<sup>+</sup> to detect the matched peptides corresponding to the *m/z* spectra values. By considering mass losses among the spectra, the matched peptides were selected to be predicted peptides. All predicted peptides were then confirmed to their biofunctionalities through BIOPEP databases ([www.uwm.edu.pl/biochemia/index.php/pl/biopep](http://www.uwm.edu.pl/biochemia/index.php/pl/biopep)) (27).

## RESULTS

The production process of tempe from three different producers is depicted in Fig. 1. The significant differences among the tempe producers A, B and C included production area, floor materials, cooking, inoculating, and soaking equipment. Based on total bacteria test in their production rooms and water, the highest number of total bacteria (140 CFU/plate and 2.3 × 10<sup>4</sup> CFU/mL) was attributed to producer A followed by producer B (40 CFU/plate and 3.8 × 10<sup>2</sup> CFU/mL) and producer C (4 CFU/plate and 9.0 × 10<sup>1</sup> CFU/mL). Moreover, the highest number of *E. coli* (7.5 × 10<sup>1</sup> CFU/mL) in production water was observed in producer A; producers B and C were negative for *E. coli*.

In general, tempe samples were produced using similar process including cooking, soaking, cleaning, dehulling, inoculating, incubating, and packing except for producer C who performed soybean blanching after cleaning and dehulling (prior to inoculation). All

**TABLE 1.** Subfractions with retention time (RT) of RP-HPLC peak chromatograms following Sephadex G-15 gel filtration.

Tempe	Fraction of gel filtration	RP-HPLC			
		Subfraction	RT (min)	% peak area	
A	A1	A11	1.425	28.81	
		A12	2.127	22.11	
		A13	12.623	6.57	
	A2	A21	1.053	33.13	
		A22	2.250	16.64	
		A23	2.680	17.90	
	A3	A31	1.543	13.88	
		A32	2.192	24.94	
		A33	3.892	15.79	
B	B1	B11	1.518	16.15	
		B12	2.170	78.23	
	B2	B21	1.538	18.05	
		B22	2.177	72.88	
	B3	B31	1.528	10.90	
		B32	2.168	80.58	
	C	C1	C11	1.528	10.51
			C12	2.158	49.87
			C13	4.025	11.83
C2		C21	1.558	15.99	
		C22	2.173	76.76	
		C23	5.523	1.89	
C3		C31	1.530	13.27	
		C32	2.165	82.07	

producers utilized soybean as the substrates for tempe. Interestingly, the tempe inoculant was different among producers, i.e., semi-pure tempe starters (*ragi*) mixed with cassava flour by-product (*onggok*, Indonesian) for producer A and B, and semi-pure tempe starter (containing *R. oligosporus* spores within rice flour) for producer C.

Elution profiles of the ultra-filtered tempe samples (<3 kDa) on Sephadex G-15 gel filtration are shown in Fig. 2. These eluates showed characteristic profiles depending on producers (tempes A, B and C) and were divided into three fractions for each sample. Three fractions obtained by Sephadex G-15 were named as fractions A1, A2, and A3 (tempe A); fractions B1, B2, and B3 (tempe B); and fractions C1, C2, and C3 (tempe C). RP-HPLC was performed after gel filtration resulting in 23 sub-fractions as presented in Table 1.

The 23 sub-fractions obtained from RP-HPLC were further analyzed with LC-ESI-MS and were followed by peptide sequence prediction using FindPept tools on ExPASy Bioinformatics Resource Portal. The relative molecular mass, accession number of protein origin, and bioactive properties reported by BIOPEP databases are presented in Table 2.

The results showed that the number of peptides containing 2 to 5 amino acid residues differed among tempe samples made from producer A, B, and C, i.e., 26 peptides, 21 peptides, and 26 peptides, respectively. Most predicted peptides were derived from glycinin, which is the major storage protein of soybean followed by β-conglycinin, lipoxigenase-1, and lectins.

The identified peptides from the three different tempes both showed similar and dissimilar features regarding amino acid sequences and biofunctionalities (Table 2). The results demonstrated that peptide sequences between tempes B and C were more identical in comparison with tempe A. According to BIOPEP database, we found some peptides that might exert biofunctional activities such as ACE inhibitors (acting as antihypertensive), DPP-IV inhibitors (acting as antidiabetic), oxidatives (acting as antioxidant), and calmodulin-dependent cyclic nucleotide phosphodiesterase (CaMPDE) inhibitor (acting as antitumor) properties (Table 2 and Fig. 3). Tempe C was made in the best production facilities and had the highest number of peptides associated with biofunctional properties (antihypertensive, antidiabetic, and antioxidant)

**TABLE 2.** Summary of peptides with their bioactivities identified in the three different tempe samples, analyzed using LC-ESI-MS then predicted using FindPep tool and BIOPEP.

No	Relative molecular mass	Accession number of protein origin	Peptides			Bioactivities <sup>a</sup>	Reference
			Tempe A	Tempe B	Tempe C		
1	189.12	P04776 (GLYG1)	Ala-Val			ACE inhibitor	35
2	189.13	P04776 (GLYG1)		Gly-Leu		ACE inhibitor	36
3	203.10	P08170 (LOX1)			Pro-Ser	DPP IV inhibitor	37
4	205.12	P04776 (GLYG1)			Ser-Val	DPP IV inhibitor	37
5	219.10	P04776 (GLYG1)	Ala-Glu		Ala-Glu	DPP IV inhibitor	37
6	219.13	P04776 (GLYG1)			Ser-Ile	DPP IV inhibitor	37
7	223.11	P04776 (GLYG1)			Gly-Phe	ACE inhibitor	36
8	229.16	P02858 (GLYG4)			Pro-Leu	ACE inhibitor	38
9	235.11	P08170 (LOX1)	Cys-Leu			uk	–
10	235.11	P04776 (GLYG1)	Leu-Cys	Leu-Cys		uk	–
11	236.07	P04776 (GLYG1)		Asn-Cys		uk	–
12	237.12	P04347 (GLYG5)	Ala-Phe			ACE inhibitor	36
13	244.17	P04776 (GLYG1)			Lys-Pro	Antioxidative	39
14	245.11	P04776 (GLYG1)	Glu-Pro		Glu-Pro	DPP IV inhibitor	40
15	246.18	P04776 (GLYG1)	Val-Lys			uk	–
16	255.14	P08170 (LOX1)		His-Val		DPP IV inhibitor	37
17	255.14	P04405 (GLYG2)	Val-His	Val-His	Val-His	DPP IV inhibitor	37
18	263.14	P11827 (GLCA)		Pro-Phe		DPP IV inhibitor	37
19	265.08	P11827 (GLCA)	Asp-Met			ACE inhibitor	41
20	283.13	P08170 (LOX1)			Thr-Tyr	Antioxidative	42
21	284.16	P08170 (LOX1)			Pro-Ala-Pro	ACE inhibitor	43
22	285.09	P08170 (LOX1)			Cys-Tyr	uk	–
23	289.16	P04776 (GLYG1)		Arg-Asn		DPP IV inhibitor	37
24	289.16	P02858 (GLYG4)		Asn-Arg		DPP IV inhibitor	37
25	295.13	P04776 (GLYG1)		Glu-Phe		CaMPDE inhibitor	44
26	297.11	P04776 (GLYG1)		Asp-Tyr		ACE inhibitor	41
27	303.14	P11828 (GLYG3)	His-Phe			DPP IV inhibitor	37
28	305.18	P25974 (GLCB)	Ala-Lys-Ser			uk	–
29	313.12	P04776 (GLYG1)			Met-Tyr	Antioxidative	45
30	316.15	P04405 (GLYG2)	Ala-Pro-Glu	Ala-Pro-Glu		uk	–
31	316.19	P11828 (GLYG3)		Ile-Ser-Pro	Ile-Ser-Pro	uk	–
32	316.19	P08170 (LOX1)		Ser-Leu-Pro	Ser-Leu-Pro	uk	–
33	331.23	P04776 (GLYG1)			Ile-Ala-Lys	ACE inhibitor	46
34	347.19	P08170 (LOX1)	Leu-Ser-Gln			uk	–
35	365.18	P11828 (GLYG3)	Ala-Gln-Phe			uk	–
36	365.18	P08170 (LOX1)		Gln-Ala-Phe		uk	–
37	429.23	P04776 (GLYG1)	Ala-Leu-Pro-Glu		Ala-Leu-Pro-Glu	uk	–
38	451.22	P04347 (GLYG5)	Val-Thr-Met-Thr			uk	–
39	429.23	P04347 (GLYG5)	Ala-Leu-Glu-Pro	Ala-Leu-Glu-Pro	Ala-Leu-Glu-Pro	ACE inhibitor	47
40	447.26	P04776 (GLYG1)	Gly-Lys-Asp-Lys			uk	–
41	456.32	P04776 (GLYG1)	Val-Ile-Lys-Pro			ACE inhibitor	47
42	429.19	P08170 (LOX1)	Ser-His-Trp	Ser-His-Trp	Ser-His-Trp	uk	–
43	456.20	P02858 (GLYG4)	His-Trp-Asn			uk	–
44	456.28	P08170 (LOX1)	Ile-Val-Gln-Pro			uk	–
45	451.17	P08170 (LOX1)	Ser-Ser-Glu-Glu			uk	–
46	429.20	P04776 (GLYG1)	Pro-Pro-Thr-Asp			uk	–
47	469.21	P04776 (GLYG1)		Ser-Leu-Cys-Phe	Ser-Leu-Cys-Phe	uk	–
48	451.19	P04405 (GLYG2)		Cys-Val-Glu-Thr		uk	–
49	451.26	P08170 (LOX1)			Arg-Ile-Tyr	ACE inhibitor	48
50	451.27	P08170 (LOX1)			Pro-His-Val-Val	uk	–
51	470.24	P25974 (GLCB)	Ser-His-Asn-Ile			uk	–
52	573.32	P05046 (LEC)	Ile-Asp-Thr-Lys-Pro			uk	–
53	573.27	P25974 (GLCB)		Pro-Phe-Tyr-Phe		uk	–
54	573.29	P02858 (GLYG4)		Leu-Asp-Gln-Thr-Pro	Leu-Asp-Gln-Thr-Pro	uk	–
55	573.31	P02858 (GLYG4)			Asn-Arg-Asn-Gly-Leu	uk	–
56	573.40	P05046 (LEC)		Lys-Val-Leu-Ile-Thr	Lys-Val-Leu-Ile-Thr	uk	–

<sup>a</sup> ACE inhibitor is for antihypertensive; DPP IV inhibitor is for antidiabetic; CaMPDE inhibitor is for antitumor; uk means unknown.

followed by tempe B (antihypertensive, antidiabetic, and anti-tumor) and tempe A (antihypertensive and antidiabetic), i.e., 58%, 43%, and 35%, respectively.

## DISCUSSION

In this study, we examined three tempe samples made from three producers and identified bioactive peptides. The producers A, B and C are known to use similar imported soybeans to produce tempe. Hence, the soybean seed proteome among the soybeans was similar (14). The equipment, floor condition, and cleanliness can indicate the sanitation level of producers. The sanitation level of producer A was low due to the use of iron containers, dirt floors

(non-concreted), and dirty conditions. The sanitation level of producer B was moderate due to the use of plastic and stainless-steel containers, a concrete floor, and better cleanliness. The sanitation level of producer C was considered good including desirable facilities such as stainless-steel equipment, ceramic floor, and high cleanliness (Fig. 1). These sanitation conditions may also influence the microflora involved in tempe fermentation. Our results suggested that differences in microflora involving fermentation could affect the formation of biofunctional peptides.

Producer C blanched the beans for 2–5 min (using hot water at 90°C) after cleaning and dehulling but producers A and B did not. The blanching inactivates enzyme (especially peroxidase and polyphenol oxidase) in soybeans and removes contaminating

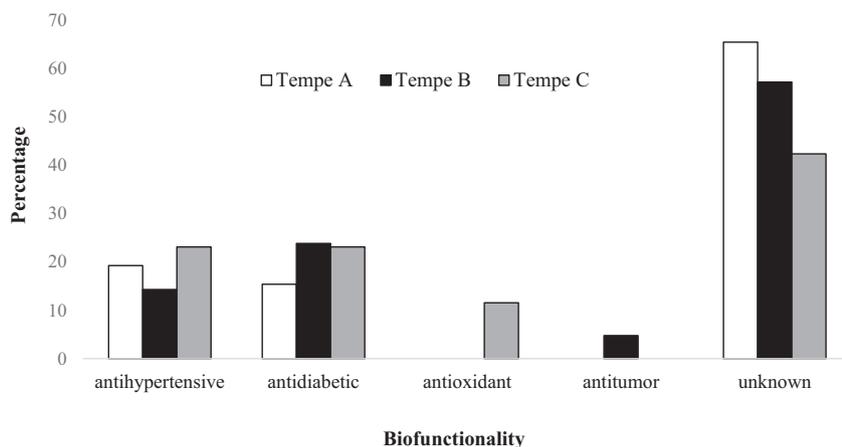


FIG. 3. Distribution of bioactivities of peptides obtained from the three different tempes, confirmed by search BIOPEP databases. Tempes A, B and C refer to tempes produced by producers A (poor), B (moderate), and C (good sanitation levels), respectively.

microorganisms. It can also enhance the extraction efficiency of bioactive components from raw materials (28). Tempe C was found to have more bioactive peptides compared to tempes A and B. Thus, this finding suggests that the blanching process of producer C may be one of important steps responsible for the availability of bioactive peptides. The contribution of blanching to the formation of peptides may relate to the reduction of microbial contaminants after 24 h of soaking (28), which can alter soybean's tissue structure including disruption of the cell membrane and increasing cell wall porosity (29). As a result, the hyphae of *Rhizopus* sp. might easily penetrate the soybean cotyledons and secrete proteases to cleave more proteins into peptides (30).

Producers A and B used a similar inoculant, i.e., semi-pure tempe starters (*ragi*) mixed with cassava flour by-product. Producer C used only a semi-pure tempe starter (only containing *R. oligosporus* spores within rice flour) as the inoculant (Fig. 1). The use of tempe starter is common in tempe production. It contains some microorganisms that play important roles in the fermentation process such as *R. oligosporus*, *R. oryzae*, *R. arrhizus*, *R. stolonifer*, *R. microsporus*, *R. rhizopodiformis*, *R. chinensis*, and sometimes *Mucor* sp. (1,20). In addition to *Rhizopus* sp., the presence of bacteria (such as *L. casei*, *Streptococcus faecium*, *Bacillus* sp. and *Klebsiella pneumoniae*) and yeasts (such as *Pichia burtonii* and *Candida didensiae*) during tempe production may also influence physical and chemical properties of tempe (17,19). The addition of *onggok* to the tempe starter (as performed by producers A and B) helped develop the starter, speed up the growth, and reduce production costs. However, the addition of the unsterilized *onggok* to the tempe starter could decrease the tempe starter quality while increasing potential contamination by undesirable microorganisms.

Fractionation using Sephadex G-15 gel filtration chromatography following <3 kDa ultrafiltration separated the fractions into three fractions. The absorbance detection of the fractions via 214 nm and 254 nm was indicated that the compounds contain peptide bonds (at 214 nm) and aromatic groups (at 254 nm) (31). Fractions A3, B2, and C2 had more aromatic groups than the other fractions (Fig. 2). The fractions containing aromatic groups were more hydrophobic due to cross-linked interaction among hydrophobic components of samples with the Sephadex G-15 gel.

Table 1 and Fig. S1 show that 23 sub-fractions were generated using RP-HPLC following fractionation with Sephadex G-15 gel filtration. The retention time of most high-concentration sub-fractions was <2.5 min; these contained hydrophilic peptides. A few sub-fractions with a retention time of >2.5 min were observed (A13, A23, A33, C13, and C23)—this might be because the

hydrophobic peptides interact with the hydrophobic stationary phase (C18 column) (32). Here, longer retention times indicate of higher hydrophobicity.

Small peptides comprised 2–5 amino acid residues (<1 kDa); these were found in all samples of tempes A, B, and C, but they existed in different numbers, i.e., 26, 21, and 26 peptides, respectively (Table 2). Some aromatic (Phe, Tyr, and Thr) and aliphatic amino acids (Pro, Leu and Val) at the C-terminals were also detected in all samples. Both types of amino acids are associated with antihypertensive properties (33). We also found that the most abundant bioactive peptides were from producer C; however, the main contributor to the abundance of the peptides has remained inconclusive.

Tempe B had fewer peptides than tempe A even though the sanitation practices in B were better than A. The similarity of peptide sequences between tempes B and C was higher than that between tempes A and B or tempes A and C (Table 2). Six common peptides (Asn-Cys, Ile-Ser-Pro, Ser-Leu-Pro, Ser-Leu-Cys-Phe, Leu-Asp-Gln-Thr-Pro, and Lys-Val-Leu-Ile-Thr) were found in both tempes B and C suggesting that the sanitation conditions in producers B and C were better than producer A.

Two similar peptides (Leu-Cys and Ser-His-Trp) were observed in tempes A and B. Tempes A and C demonstrated three similar peptides (Ala-Glu, Glu-Pro, and Ala-Leu-Pro-Glu). However, the similarity of peptide consequence between tempes B and C was higher than tempe A indicating that sanitary conditions in the production of tempes B and C may alter the peptide formation. The sanitation conditions of producers B and C were quite similar in terms of production room and equipment. These conditions can alter the growth of microorganisms, which in turn affects the proteolytic actions in tempe to produce bioactive peptides. Tempe from different producers has different chemical properties based on a metabolomics study (21). This study confirmed that sanitation conditions and the use of starter containing different microorganisms strongly impact the generation of biofunctional peptides.

All tempe samples could exert antihypertensive, antidiabetic, antioxidative, and antitumor effects (Fig. 3). Previous studies have shown that tempe can show a wide range of beneficial effects on human health including antihypertensive, antioxidant, anticancer, antimicrobial, and antithrombotic impacts (5,11). All bioactive peptides identified in tempes A, B, and C were small peptides (di-peptides, tripeptides, and oligopeptides). This demonstrates their higher potential effects—especially as antihypertensive activities (34).

Tempe C had the highest number of peptides responsible for biofunctionalities followed by tempes B and A (58%, 43%, and 35%,

respectively; Fig. 3). In tempe C, six peptides were antihypertensive (23%), six were antidiabetic (23%), and three were antioxidant (12%). In sample A, five were antihypertensive and four were antidiabetic (19% and 15%, respectively). Tempe B had three antihypertensive peptides (14%) and five antidiabetic peptides (24%); 5% had antitumor properties. The higher abundance in bio-functional properties in tempe C may be associated with less microbial contamination. The use of *onggok* in tempe starter may influence the diversities and activities of microorganisms during fermentation of tempes A and B. In addition, the blanching used by producer C can play a dual role, i.e., inactivating enzymes and alleviating microbial contamination. This increases the bioactive extraction efficiency. The advantages of blanching were also observed here because tempe C had more bio-functional peptides.

This research showed that differences in production process, equipment, and sanitation can impact the peptide profiles of tempe. This in turn impacts the products' biofunctional properties. Further studies on tempe biofunctionalities via bioassays and *in vivo* systems are clearly needed to validate these results.

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