Dear author,

Please note that changes made in the online proofing system will be added to the article before publication but are not reflected in this PDF.

We also ask that this file not be used for submitting corrections.

Journal of Bioscience and Bioengineering
VOL. xxx No. xxx, xxx, xxxx

www.elsevier.com/locate/jbiosc

Proteomic study of bioactive peptides from tempe

Badrut Tamam,[1,4] Dahrul Syah,[1,†] Maggy Thenawidjaja Suhartono,[1] Wisnu Ananta Kusuma,[2] Shinjiro Tachibana,[3] and Hanifah Nuryani Lioe[1,*]

Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, IPB Darmaga, Bogor, West Java 16680, Indonesia,[1] Department of Computer Science, Faculty of Mathematics and Natural Science, Bogor Agricultural University, IPB Darmaga, Bogor, West Java 16680, Indonesia,[2] Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Nishihara cho, Nakagado-gun, Okinawa 903-0213, Japan,[3] and Department of Nutrition, Polytechnic of Health, Denpasar, Bali 80237, Indonesia[4]

Received 14 September 2018; accepted 15 January 2019
Available online xxx

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 LC-MS. The MS spectra were then predicted using FindPept tools (https://web.expasy.org/findpept/), and their biofunctionalities were confirmed with BIOPEP databases (www.uwm.edu.pl/biochemia/index.php/pl/biopep). 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.

[**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 β-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), lipoxygenase, sucrose-linked protein, and α-amylase (14,15). Glycinin (molecular weight/MW 320–375 kDa) has five subunits (G1, G2, G3, G4 and G5), while β-conglycinin (MW 150–200 kDa) has four subunits (α, α', β and γ). 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*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Rhizopus microsporus*, *Rhizopus rhizopodiformis*, *Rhizopus 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

* Corresponding author at: Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, IPB Dramaga, Bogor, West Java 16002, Indonesia. Tel./fax: +62 251 8626725.
E-mail address: hanifahlioe@apps.ipb.ac.id (H.N. Lioe).
† Deceased on September 10th, 2017.

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.01.019

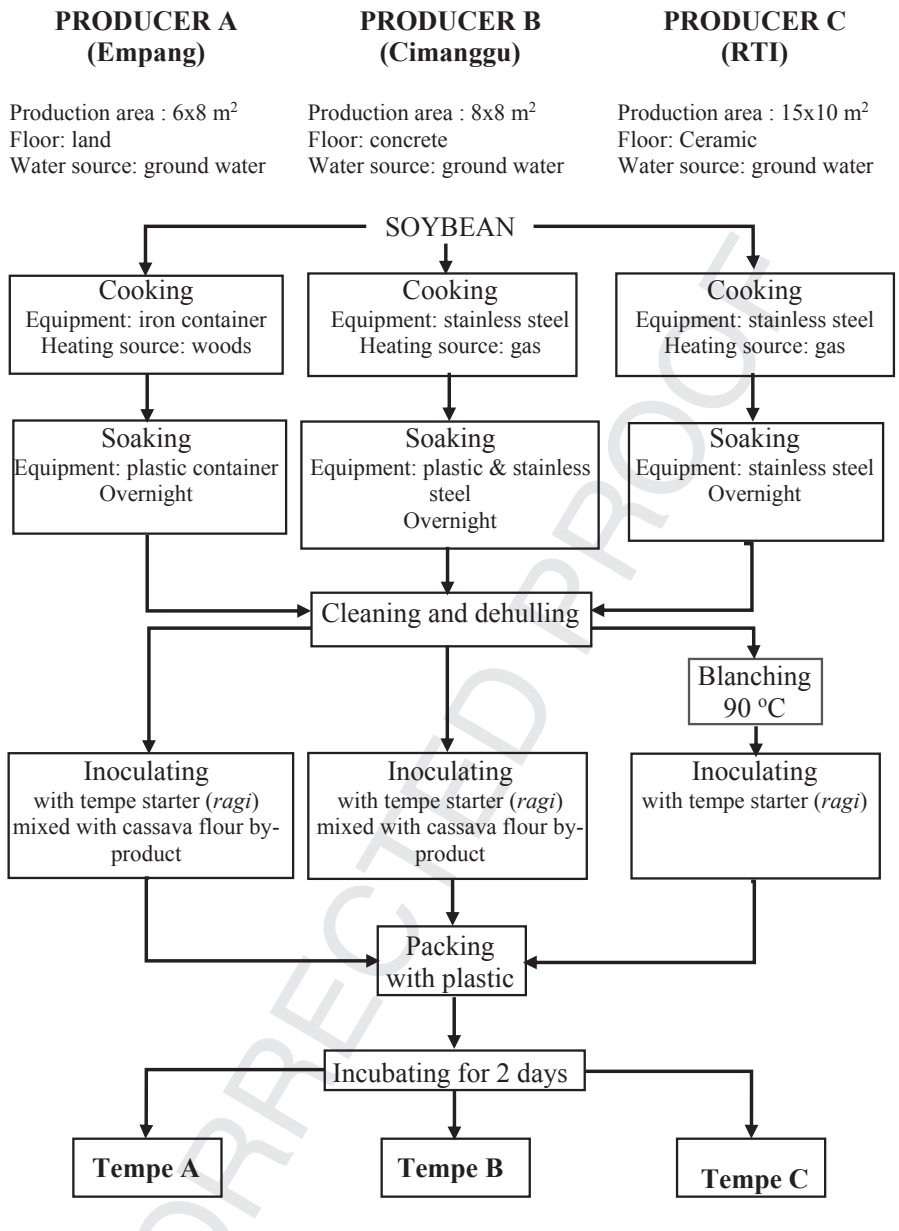

FIG. 1. Different tempe processes of three different producers.

inoculum, processing, packages, hygiene, and 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 biofunctionalities 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-I-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.

Please cite this article as: Tamam, B et al., Proteomic study of bioactive peptides from tempe, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2019.01.019

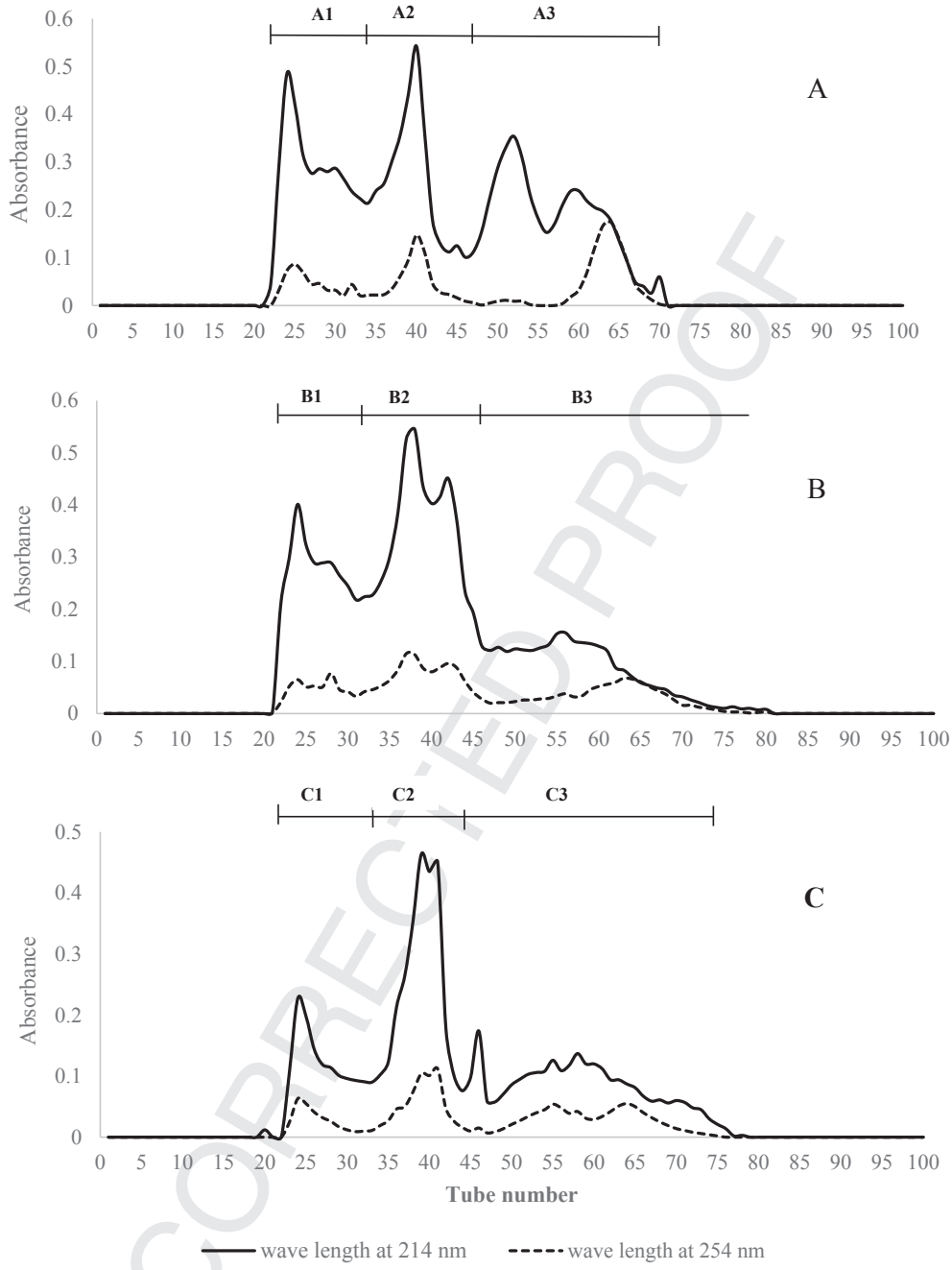

FIG. 2. Fractions of chromatograms of three different ultrafiltered samples of tempe (<3 kDa) obtained by Sephadex G-15 gel filtration chromatography (65 cm x 2.5 cm i.d) using deionized water as an eluent. Chromatograms represented the results of tempe samples: (A) from producer with poor sanitation 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^2 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^2 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^2 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 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions) and EMBA agar (selective agar for *E. 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 (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^{++} (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 lipoxygenase were traced. The mass tolerance was ±0.12 Da using monoisotopic masses and interpreted as positive mode $[M+H]^+$ 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) (27).

RESULTS

The production process of tempe from three different producers is depicted in Fig. 1. The significant differences among the producers of tempe (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 2.3×10^4 CFU/mL) was attributed to producer A followed by producer B (40 CFU/plate and 3.8×10^2 CFU/mL) and producer C (4 CFU/plate and 9.0×10^1 CFU/mL). Moreover, the highest number of *E. coli* (7.5×10^1 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 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.

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

Tempe	Fractions of gel filtration	RP-HPLC		
		Subfractions	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

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 (tempe 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, lipoxygenase-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) followed by tempe B (antihypertensive, antidiabetic, and antitumor)

Please cite this article as: Tamam, B et al., Proteomic study of bioactive peptides from tempe, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2019.01.019

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	RMM (relative molecular mass)	Accession number of protein origin	Peptides			Bioactivities	References
			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	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	

*ACE inhibitor is for antihypertensive; DPP IV inhibitor is for antidiabetic; CaMPDE inhibitor is for antitumor; uk is unknown. Q5

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 microorganisms. It can also enhance the extraction efficiency of

Please cite this article as: Tamam, B et al., Proteomic study of bioactive peptides from tempe, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2019.01.019

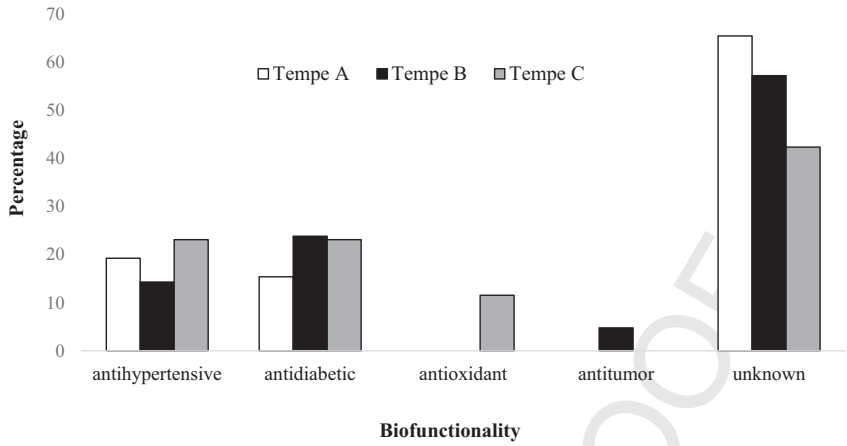

FIG. 3. Distribution of bioactivities of peptides obtained from the three different tempe producers with different sanitation levels [(A) to (C), from poor to good level], confirmed by search BIOPEP databases.

bioactive components from raw materials (28). Tempe C was found to have more bioactive peptides compared to tempe 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 *Rhyzopus* 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 byproduct. 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 tempe B and tempe C suggesting that the sanitation conditions in producer 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 B and C was higher than tempe A indicating that sanitary conditions in the production of tempe 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 (dipeptides, 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 tempe B and A (58%, 43%, and 35%, respectively; Fig. 3). In tempe C, six peptides were antihypertensive

Please cite this article as: Tamam, B et al., Proteomic study of bioactive peptides from tempe, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2019.01.019

(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 biofunctional 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 tempe 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.01.019.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Research and Technology and Higher Education Indonesia through Basic Nominated Research Scheme in 2017 (Grant no. 011/SP2H/LT/DRPM/VIII/2017). The LC-ESI-MS analyses were performed at the Center for Research Advancement and Collaboration, University of the Ryukyus, Japan. The authors have no conflict of interest to declare.

References

1. **Nout, M. J. R. and Kiers, J. L.:** Tempe fermentation, innovation and functionality: update into the third millenium, J. Appl. Microbiol., **98**, 789–805 (2005).
2. **Egounlety, M. and Aworh, O. C.:** Effect of soaking, dehulling, cooking and fermentation with *Rhizopus oligosporus* on the oligosaccharides, trypsin inhibitor, phytic acid and tannins of soybean (*Glycine max* Merr.), cowpea (*Vigna unguiculata* L. Walp) and groundbean (*Macrotyloma geocarpa* Harms), J. Food Eng., **56**, 249–254 (2003).
3. **Mo, H., Kariluoto, S., Piironen, V., Zhu, Y., Sanders, M. G., Vincken, J. P., Wolkers-Rooijackers, J., and Nout, M. J. R.:** Effect of soybean processing on content and bioaccessibility of folate, vitamin B_{12} and isoflavones in tofu and tempe, Food Chem., **141**, 2418–2425 (2013).
4. **Singh, B. P., Vij, S., and Hati, S.:** Functional significance of bioactive peptides derived from soybean, Peptides, **54**, 171–179 (2014).
5. **Sanjukta, S. and Rai, A. K.:** Production of bioactive peptides during soybean fermentation and their potential health benefits, Trends Food Sci. Technol., **50**, 1–10 (2016).
6. **Shin, D. and Jeong, D.:** Korean traditional fermented soybean products: Jang, J. Ethn. Foods, **2**, 2–7 (2015).
7. **Wang, H., Meng, F., Yin, L., Cheng, Y., Lu, A., and Wang, J.:** Changes of composition and angiotensin I-converting enzyme (ACE)-inhibitory activity during douchi fermentation, Int. J. Food Prop., **19**, 2408–2416 (2016).
8. **Sato, K., Miyasaka, S., Tsuji, A., and Tachi, H.:** Isolation and characterization of peptides with dipeptidyl peptidase IV (DPPIV) inhibitory activity from natto using DPPIV from *Aspergillus oryzae*, Food Chem., **261**, 51–56 (2018).
9. **Sanjukta, S., Rai, A. K., Muhammed, A., Jeyaram, K., and Talukdar, N. C.:** Enhancement of antioxidant properties of two soybean varieties of Sikkim Himalayan region by proteolytic *Bacillus subtilis* fermentation, Trends Food Sci. Technol., **50**, 1–10 (2016).
10. **Dajanta, K., Apichartsrangkoon, A., Chukeatirote, E., and Frazier, R.:** Free-amino acid profiles of thua nao, a Thai fermented soybean, Food Chem., **125**, 342–347 (2011).
11. **Gibbs, B. F., Zougman, A., Masse, R., and Mulligan, C.:** Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food, Food Res. Int., **37**, 123–131 (2004).
12. **Capriotti, A. L., Caruso, G., Cavaliere, C., Samperi, R., Ventura, S., Chiozzi, R. Z., and Laganà, L.:** Identification of potential bioactive peptides generated by simulated gastrointestinal digestion of soybean seeds and soy milk proteins, J. Food Compos. Anal., **44**, 205–213 (2015).
13. **Samoto, M., Maebuchi, M., Miyazaki, C., Kugitani, H., Kohno, M., Hirotsuka, M., and Kito, M.:** Abundant proteins associated with lecithin in soy protein isolate, Food Chem., **102**, 317–322 (2007).
14. **Herman, E. M.:** Soybean seed proteome rebalancing, Front. Plant Sci., **5**, 1–8 (2014).
15. **Gomes, L. S., Senna, R., Sandim, V., Silva-Neto, M. A., Perales, J. E., Zingali, R. B., Soares, M. R., and Fialho, E.:** Four conventional soybean [*Glycine max* (L.) merrill] seeds exhibit different protein profiles as revealed by proteomic analysis, J. Agric. Food Chem., **62**, 1283–1293 (2014).
16. **De Castro, J. R. S. and Sato, H. H.:** Biologically active peptides: processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries, Food Res. Int., **74**, 185–198 (2015).
17. **Suwanto, A., Rahayu, G., and Nuraida, L.:** Population dynamics of yeasts and lactic acid bacteria (LAB) during tempe production, HAYATI J. Biosci., **20**, 57–64 (2013).
18. **Barus, T., Suwanto, A., Wahyudi, A. T., and Wijaya, H.:** Role of bacteria in tempe bitter taste formation: microbiological and molecular biological analysis based on 16S rRNA gene, Microbiol. Indones., **2**, 17–21 (2008).
19. **Seumahu, C. A., Suwanto, A., Rusmana, I., and Solihin, D. D.:** Bacterial and fungal communities in tempe as reveal by amplified ribosomal intergenic sequence analysis, HAYATI J. Biosci., **20**, 65–71 (2013).
20. **Astuti, M., Meliala, A., Dalasis, F., and Wahlqvist, M.:** Tempe, a nutritious and healthy food from Indonesia, Asia Pac. J. Clin. Nutr., **9**, 322–325 (2000).
21. **Kadar, A., Aditiawati, P., Astawan, M., Putri, S., and Fukusaki, E.:** Gas chromatography coupled with mass spectrometry-based metabolomics for the classification of tempe from different regions and production processes in Indonesia, J. Biosci. Bioeng., **126**, 411–416 (2018).
22. **Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., and Uchida, R.:** Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances, J. Agric. Food Chem., **58**, 821–827 (2010).
23. **Inoue, K., Gotou, T., Kitajima, H., Mizuno, S., Nakazawa, T., and Yamamoto, N.:** Release of antihypertensive peptides in miso paste during its fermentation, by the addition of casein, J. Biosci. Bioeng., **108**, 111–115 (2009).
24. **Zhua, Y. P., Chenga, Y. Q., Wang, L. J., Fan, J. F., and Li, L. T.:** Enhanced antioxidative activity of Chinese traditionally fermented okara (Meitauza) prepared with various microorganism, Int. J. Food Prop., **11**, 519–529 (2008).
25. **Vallabha, V. S. and Tiku, P. K.:** Antihypertensive peptides derived from soy protein by fermentation, Int. J. Pept. Res. Ther., **20**, 161–168 (2014).
26. **Singh, B. P. and Vij, S.:** Growth and bioactive peptides production potential of Lactobacillus plantarum strain C2 in soy milk: a LC-MS/MS based revelation for peptides biofunctionality, LWT Food Sci. Technol., **86**, 293–301 (2017).
27. **Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., and Darewicz, M.:** BIOPEP database and other programs for processing bioactive peptide sequences, J. AOAC Int., **91**, 965–980 (2008).
28. **Xiao, H., Pan, Z., Deng, L. Z., El-Mashad, H. M., Yang, X. H., Mujumdar, A. S., Gao, Z. J., and Zhang, Q.:** Recent developments and trends in thermal blanching – a comprehensive review, Inf. Process. Agric., **4**, 101–127 (2017).
29. **Xu, G., Ye, X., Chen, J., and Liu, D.:** Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract, J. Agric. Food Chem., **55**, 330–335 (2007).
30. **Jurus, A. M. and Sundberg, W. J.:** Penetration of *Rhyzopus oligosporus* into soybeans in tempeh, Appl. Environ. Microbiol., **32**, 284–287 (1976).
31. **Belitz, H. and Grosch, W.:** Food Chemistry, 2nd ed. Springer, Berlin (1999).
32. **Aguilar, M.:** Reversed-phase high-performance liquid chromatography. Springer, Totowa, NJ (2004).
33. **Wijesekara, I., Qian, Z., Ryu, B., Ngo, D., and Kim, S.:** Purification and identification of antihypertensive peptides from seaweed pipefish (*Syngnathus schlegeli*) muscle protein hydrolysate, Food Res. Int., **44**, 703–707 (2011).
34. **Gallego, M., Mora, L., and Toldra, F.:** Health relevance of antihypertensive peptides in foods, Curr. Opin. Food Sci., **19**, 8–14 (2018).
35. **Salampessy, J., Reddy, N., Kailasapathy, K., and Phillips, M.:** Functional and potential therapeutic ACE-inhibitory peptides derived from bromelain hydrolysis of trevally proteins, J. Funct. Foods, **14**, 716–725 (2015).
36. **Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., and Cushman, D. W.:** Binding of peptide substrates and inhibitors of angiotensin-converting enzyme, J. Biol. Chem., **255**, 401–407 (1980).
37. **Lan, V. T. T., Ito, K., Ohno, M., Motoyama, T., Ito, S., and Kawarasaki, Y.:** Analyzing a dipeptide library to identify human dipeptidyl peptidase IV inhibitor, Food Chem., **175**, 66–73 (2015).
38. **Byun, H. G. and Kim, S. K.:** Structure and activity of angiotensin I-converting enzyme inhibitory peptides derived from Alaskan pollack skin, J. Biochem. Mol. Biol., **35**, 239–243 (2002).
39. **Huang, W. Y., Majumder, K., and Wu, J.:** Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interactions with phytochemicals, Food Chem., **123**, 635–641 (2010).
40. **Hikida, A., Ito, K., Motoyama, T., Kato, R., and Kawarasaki, Y.:** Systematic analysis of a dipeptide library for inhibitor development using human dipeptidyl peptidase IV produced by a *Saccharomyces cerevisiae* expression system, Biochem. Biophys. Res. Commun., **430**, 1217–1222 (2013).

41. **Wu, J., Aluko, R. E., and Nakai, S.:** Structural requirements of angiotensin I-converting enzyme inhibitory activity: quantitative structure-activity relationship study of di- and tripeptides, J. Agric. Food Chem., **54**, 732–738 (2006).
42. **Cheng, Y., Chen, J., and Xiong, Y. L.:** Chromatographic separation and tandem MS identification of active peptides in potato protein hydrolysate that inhibit autoxidation of soybean oil-in-water emulsions, J. Agric. Food Chem., **58**, 8825–8832 (2010).
43. **Meisel, H., Walsh, D. J., Murray, B., and FitzGerald, R. J.:** ACE inhibitory peptides, pp. 269–315, in: Mine, Y. and Shahidi, F. (Eds.), Nutraceutical proteins and peptides in health and disease. CRC Press, New York (2006).
44. **Li, H. and Aluko, R. E.:** Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate, J. Agric. Food Chem., **58**, 11471–11476 (2010).
45. **Erdmann, K., Grosser, N., Schipporeit, K., and Schröder, H.:** The ACE inhibitory dipeptide Met-Tyr diminishes free radical formation in human endothelial cells via induction of heme oxygenase-1 and ferritin, J. Nutr., **136**, 2148–2152 (2006).
46. **Gomez-Ruiz, J. A., Ramos, M., and Recio, I.:** Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques, Electrophoresis, **28**, 4202–4211 (2007).
47. **Vecchi, B. and Añón, M. C.:** ACE inhibitory tetrapeptides from *Amaranthus hypochondriacus* 11S globulin, Phytochemistry, **70**, 864–870 (2009).
48. **Murray, B. A. and FitzGerald, R. J.:** Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production, Curr. Pharm. Des., **13**, 773–791 (2007).

Please cite this article as: Tamam, B et al., Proteomic study of bioactive peptides from tempe, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2019.01.019