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Research Article

Bioactive Peptides from Tempeh Using PeptideCutter's Cleavage

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ABSTRACT

Tempeh is an Indonesian traditional fermented food with rich nutrition and bioactive components. *Rhizopus* sp, especially *Rhizopus oligosporus*), lactic acid bacteria (*Lactobacillus* sp.), and yeast are microorganisms involved in Tempeh fermentation. An interesting offer of Bioinformatics (*in silico* method) as a supporting tool in molecular biology studies has emerged, such as in protein cleavage. This study utilized *PeptideCutter* application on ExPASy Bioinformatics portal (https://web.expasy.org/peptide_cutter/) to cleave soy proteins glycinin G1, G2, G3, G4, G5, β-conglycinin-α chain, and β chain using available enzymes in the application with two simulations. Simulation I was conducted using enzyme complex produced by *Lactobacillus* sp. and *Rhizopus oligosporus*, while simulation II was used enzyme complex produced by *Lactobacillus* sp., *Rhizopus oligosporus*, and *Klebsiella pneumonia*. Simulation I was conducted using enzyme complex produced by *Lactobacillus* sp. and *Rhizopus oligosporus*, while simulation II was used enzyme complex produced by *Lactobacillus* sp., *Rhizopus oligosporus*, and *Klebsiella pneumoniae*. A total of 58 peptides was found from the simulation I and higher than simulation I (41 peptides). The bioactive peptides by the cleavages using *PeptideCutter* tool were dominated with dipeptides and only three peptides were in the form of tripeptides, namely *Leu-Leu-Phe* (g*lycinin* G1), *Val-Val-Phe* (g*lycinin* G5), and *Arg-His-Lys* (β-conglycinin-α chain). Bioactive peptides with antihypertensive and antidiabetic properties were mostly found in this *in silico* method of soybean cleavage.

Keywords: bioactive peptides, cleavage, *in silico*, *PeptideCutter, protein*

Introduction

Bioactive peptides are defined as protein fragments with amino acid sequences. Several biological activities are found in bioactive peptides, such as antioxidant, antihypertensive, antithrombotic, antiadipogenic, antimicrobial, anti-inflammatory, and immunomodulatory effects. Bioactive peptides are generally encrypted in the amino acid sequences of a food protein consisting of 2 to 20 amino acids or more than 20 amino acids [1]. Peptides are not active in storage proteins; however they should be released through proteolysis to activate their functional properties. Korhonen and Pihlanto reported that there are several proteolysis methods to produce bioactive peptides: 1) fermentation by proteolytic microorganisms, 2) digestion of food proteins in vivo, or 3) in vitro hydrolysis using various proteolytic enzymes [2]. One of the

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most popular fermentation products in Indonesia is Tempeh.

Tempeh is produced by the activities of several microorganisms on soybean substrate. A total of 40% glycinin (11S globulin) and 30% *b*-conglycinin (7S globulin) are soy's major proteins. Other proteins in soybeans are Kunitz and Bowman-Birk trypsin inhibitors, lectins (hemagglutinin), lipoxygenase, sucrose linked protein, and α-amylase [3,4]. Glycinin (molecular weight/MW 320-375 kDa) is composed of 5 subunits (G1, G2, G3, G4, and G5), while *β*-conglycinin (MW 150-200 kDa) consists of four subunits (*α, α, β, and γ*). *Rhizopus* sp. (especially *Rhizopus oligosporus*), lactic acid bacteria (*Lactobacillus* sp.) and yeast are microorganisms involved in soybean fermentation, especially to Tempeh [5,6]. Proteolytic enzymes secreted by these microorganisms cleave soy protein into peptides during Tempeh fermentation [7]. One of the contaminant bacteria present in Tempeh fermentation is *Klebsiella pneumoniae*. It synthesizes vitamin B12 [8]. Recently, the bioinformatics approach has grown rapidly as a tool in molecular biology for protein cleavage. Bioinformatics reduces laboratory costs and and time [9].

ExPASy *PeptideCutter* retrieved from https://web.expasy.org/peptide_cutter/ is a popular bioinformatics application in protein cleavage [10]. The *PeptideCutter* provides 27 proteolytic enzymes that plays an important rolein cutting the polypeptide chain of a protein. Peptides formed from cleavage using *PeptideCutter* are synchronized with the BIOPEP database (www.uwm.edu.pl/biochemia/index.php/pl/biopep) for bioactivity prediction. BIOPEP, developed by the University of Warmia and Mazury (UWM) has become a favoured tool in identifying bioactive peptides. Information provided by BIOPEP-UWM includes sequences, biological activity, chemical mass, IC_{50}, and other important information of peptides [11].

Based on *in vitro* analysis, bioactive peptides were obtained from Tempeh by extraction, separation, purification, and identification [12]. Meanwhile, the offer of *in silico* method in facilitating identification of bioactive peptides is interesting. Therefore, this study aimed to evaluate cleavage of soy protein, especially Tempeh production and to identify bioactive peptides from the in silico from the in silico approach using enzymes derived from the Brenda database (www.brenda-enzymes.org).

Methods

The proteins in soybeans namely glycinin G1, G2, G3, G4, G5, *β*-conglycinin of chain *α* and chain *β* were used as cleavage substrate. Soy protein sequences were obtained from UniProtKB (http://www.uniprot.org) and the ExPASy bioinformatics portal (http://www.expasy.org).

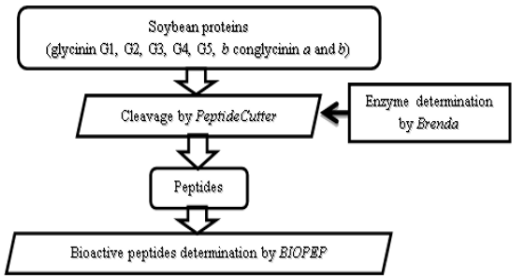

Figure 1.Algorithm of determination of bioactive peptides from soybean proteins using Bioinformatics

In silico approach was used in this study following with several steps. Determining the main soybean proteins, exploring enzymes generated from microorganisms from *Brenda* database, simulating protein cleavage by *PeptideCutter*, and determining the peptides bioactivity by *BIOPEP* (Figure 1). Molecular weights of peptide sequences were calculated using PepDraw application (www.enalut.ude/-biochem/WW/PepDraw/).

Determination of enzymes play an important role in protein cleavage using *Brenda* database (www.brenda-enzymes.org). Enzymes were produced by *Lactobacillus* sp., *Rhizopus oligosporus* (the dominant microorganism in making Tempeh) and *Klebsiella pneumoniae* (contaminant bacteria). Simulations of the protein cleavage in soybean (glycinin G1, G2, G3, G4 and G5 and *β*-conglycinin of chain *α* and chain *β*) using the *PeptideCutter* software (https://web.expasy.org/peptide_cutter/) available on the ExPASy portal were performed in two types of simulations. Simulation that was conducted using enzymes complex produced by *Lactobacillus sp*. and *Rhizopus oligosporus*, while simulation II used complex enzymes produced by *Lactobacillus* sp., *Rhizopus oligosporus*, and *Klebsiella pneumoniae* (Table 1). In addition, determination of peptide biofunctionalities

was performed using BIOPEP database (www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Table 1.Proteolytic enzymes on the *PeptideCutter* application in both simulations

Simulation I (enzymes from *Lactobacillus sp.* and *Rhizopus oligosporus*)	**Simulation II** (enzymes from *Lactobacillus sp., Rhizopus oligosporus,* and *Klebsiella pneumoniae*)
Asp N Endopeptidase	*Asp N Endopeptidase*
Asp N Endopeptidase + N terminal Glu	*Asp N Endopeptidase + N terminal Glu*
Glutamyl endopeptidase	*Glutamyl endopeptidase*
Proline endopeptidase	*Proline endopeptidase*
Trypsin	*Trypsin*
	Chymotrypsin

Result and Discussion

Determination of protein-cutting enzymes

Enzymes had an important role in the process of cutting soy proteins by *PeptideCutter* software. Enzymes produced by *Lactobacillus* sp., *Rhizopus oligosporus* (a dominant microorganism in Tempeh production), and *Klebsiella pneumoniae* (a contaminant microorganism) were referred to the Brenda database (www.brenda-enzymes.org). Proteolytic enzymes found in *Lactobacillus* sp., *Rhizopus oligosporus*, and *Klebsiella pneumoniae* were obtained from the Brenda database.

Determination of soy protein sequences

Soy protein sequences were obtained from the SwissProt database (http://www.uniprot.org)and the ExPASy portal. Soy protein of glycinin G1 (GY1 strain), G2 (GY2 strain), G3 strain GY3), G4 (GY4), G5 (N/A strain), β-conglycinin chains α (N/A strain) and β-conglycinin chain β (Strain CG-4) were main precursors of bioactive peptides from soybeans. The process of searching for soy protein sequences was conducted by entering SwissProt accession number (P04776, P04405, P11828, P02858, P04347, P11827 and P25974 for glycinin G1, G2, G3, G4, G5, β-conglycinin chain α and *β*-conglycinin *β* chain, respectively) on http://www.uniprot.org. The main soy protein sequences were obtained from the https://www.uniprot.org/uniprot/?query=glycinin&sort=score.

Peptides from cleavage using PeptideCutter

Soy protein sequences were obtained from the SwissProt database (http://www.uniprot.org) and cleaved using enzymes available at *PeptideCutter* (https://web.expasy.org/peptide_cutter/). Proteolytic enzymes on *PeptideCutter* involved in simulation I (produced by *Lactobacillus sp*. and *Rhizopus oligosporus)* were *Asp N Endopeptidase, Asp N Endopeptidase + N terminal Glu, Glutamyl endopeptidase, Proline endopeptidase,* and *trypsin.* Meanwhile in simulation II *(*produced by *Lactobacillus* sp., *Rhizopus oligosporus*, and *Klebsiella pneumonia)* were *Asp N endopeptidase, Asp N Endopeptidase + N terminal Glu, Glutamyl*

Table 2. Total number of peptides, bioactive peptides, and percentage of bioactive peptides as a result of PeptideCutter cleavage according to the BIOPEP database

No	Soy protein	Number of amino acid residues	Peptides <1.500Da Simulation		Bioactive peptides Simulation		% Bioactive peptides* Simulation	
			I	II	I	II	I	II
1	Glycinin G1	495	67	93	11	20	16,4	21,5
2	Glycinin G2	485	63	90	13	21	20,6	23,3
3	Glycinin G3	481	58	91	11	24	19,0	26,4
4	Glycinin G4	562	73	103	20	21	27,4	20,4
5	Glycinin G5	516	69	91	13	19	18,8	20,9
6	*β*-conglycinin *α* chains	605	103	125	11	20	10,7	16,0
7	*β*-conglycinin *β* chains	439	69	92	11	17	15,9	18,5
	Average		72	98	13	20	18	21

endopeptidase, Proline endopeptidase, Trypsin and *Chymotrypsin.*

The number of peptides from cleavage simulation I (enzymes of *Asp N endopeptidase, Asp N Endopeptidase+ N terminal Glu, Glutamyl endopeptidase, Proline endopeptidase,* and *Trypsin*), had molecular weights below 1,500 Da. Besides, the glycinin G number of peptides G for 1, G2, G3, G4, G5 ,β-conglycinin chain α ,and β-conglycinin chain β was 67 ;63 ;58 ;73 ;69 ;103 and 69 peptides, respectively. Meanwhile, II simulation II (enzymes of *Asp N Endopeptidase+ N terminal Glu, Glutamyl endopeptidase, Proline endopeptidase, Trypsin,* and *Chymotrypsin*) was 94; 91; 92; 104; 92; 126 and 92 peptides, respectively, as shown on Table 2.

These findings indicated that the more proteolytic enzymes involved in protein cleavage, the more peptides generated. The presence of contaminant microorganisms such as *Klebsiela pneumoniae* produced protease enzymes and contributed in cutting process of soy protein sequences. Therefore, the amount of peptides increased. The highest number of peptides was found rom soy protein *β*-conglycinin chain *α* followed by glycinin G4. Our results were in accordance with the length of amino acid (AA) residue by the soy protein. The lengths of amino acid from *β*-conglycinin α chain and glycinin G4 were 639 AA and 562 AA, respectively. The average of peptides below 1,500 Da resulted from *in silico* cleavage in simulation I and simulation II was 92% and 99%, respectively.

Bioactive peptides resulted from PeptideCutter cleavage

The average number of bioactive peptides resulted from *PeptideCutter* cleavage in simulation I and II were 13 and 20, respectively, as shown in Table 2. Our study demonstrated that the more protease enzymes in the process of protein cleavage, the more peptides and bioactive peptides were produced. The highest percentage of bioactive peptides in simulation I and simulation II was derived from Glycinin G4 and Glycinin G3 (27.4% and 26.4%, respectively). The largest contribution of bioactive peptides by glycinin G4 was due to the second largest number of amino acid residues (562 amino acid residues), following *β*-conglycinin α chains (605 amino acid residues). Interestingly, glycinin G3 had only 481 amino acid residues in simulation II. It indicated that glycinin G3 is the most bioactive peptides compared to other soy proteins.

Various sequence lengths of peptides were found from the cleavage using enzyme complex on *PeptideCutter* application against soy protein (glycinin G1, G2, G3, G4, G5, *β*-conglycinin *α* chain and *β*-conglycinin *β* chain). The results indicated that mostly the number of sequences was below 1,500 Da. β-conglycinin α chains had the largest number of peptides (103 and 125 peptides in simulation 1 and in simulation 2, respectively).

Biofunctionality of bioactive peptides through in silico

Peptides from the cleavage using *PeptideCutter* were confirmed their functional properties through the BIOPEP database (www.uwm.edu.pl/biochemia/index.php/pl/biopep). The bioactive peptides and their activities were found on BIOPEP database as presented in Table 3.

Based on both simulation, antihypertensive property of the peptides was dominant in soybean proteins cleavage followed by antidiabetic and antioxidant properties. Antitumor and ion flow regulator had few numbers. Gibbs *et al*. revealed that Tempeh had ACE inhibition, antithrombotic, surface tension, and antioxidative properties [13]. Additionally, Tamam et al. also found bioactive peptides with antihypertensive, antidiabetic, antioxidant, and antitumor properties in different three Tempeh producers, following extraction and separation with LC/MS [12].

Some of the bioactive peptides are similar, such as *Pro-Leu* (antihypertention); *Pro-Phe* and *His-Phe* (antidiabetics); *Lys-Pro* and *Thr-Tyr* (antioxidant), as highlighted and underlined on Table 3. Our result demonstrated cleavage of proteins by using PeptideCutter provided general characteristics of bioactive peptides through in vitro protein hydrolysis.

Conclusion

PeptideCutter and BioPep could be beneficial to evaluate the presence of bioactive peptides in food or other materials. The *PeptideCutter*, composed with some enzymes, cleaved the sequence of amino acids to generate peptides and bioactive peptides. The more protein enzymes involved in the process of protein cleavage generated the more peptides and bioactive peptides. The BioPep containing bioactive peptide database

Table 3. Bioactivity of bioactive peptides (simulations I and II) identified using the BIOPEP database

Bioactivities	Simulation I		Simulation II	
	Bioactive Peptides	Numbers	Bioactive Peptides	Numbers
Antihipertention	*Arg-Pro; Gly-Lys; **Lys-Pro**; Leu-Arg; Val-Phe; Asp-Gly; Leu-Gln; Gly-Arg; Pro-Gln; Phe-Arg; Leu-Asn; Trp-Phe; Ile-Arg; Asn-Lys; Pro-Arg; Val-Arg; Val-Lys; His-Pro; Ala-Arg; Gly-Ser; Ile-Leu; Asp-Ala; Gln-Lys; **Pro-Leu***	24	*Leu-Leu-Phe; Ala-Phe; Thr-Trp; Arg-Pro; Ser-Tyr; Ile-Thy; Asn-Phe; Gly-Lys; Lys-Pro; Leu-Arg; Val-Phe; Asp-Gly; Leu-Gln; Gly-Arg; Pro-Gln; Ala-Pro; Ser-Phe; Glu-Glu; Ala-Tyr; Leu-Asn; Thr-Phe; Ile-Arg; Tyr-Pro; Asn-Lys; Pro-Arg; Val-Lys; His-Pro; Gln-Phe; Val-Arg; Val-Val-Phe; Ala-Arg; Gly-Ser; Asp-Tyr; Ile-Leu; Asp-Ala; Asp-Phe; Gln-Lys; Pro-Leu*	38
Antidiabetic	*Arg-Pro; Asp-Arg; Phe-Leu; Lys-Pro; Thr-Asn; Pro-Gln; Phe-Arg; Gln-Gln; Leu-Asn; Thr-Phe; Ile-Arg; Ser-Pro; Trp-Gln; Trp-Arg; Ser-Lys; Thr-Arg; Leu-His; Val-Arg; Val-Lys; Val-Ala; Phe-Asn; His-Pro; Ser-Pro; Ile-Leu; Pro-Leu; Tyr-Arg; Gln-Ile*	27	*Ala-Phe; Thr-Trp; Arg-Pro; Ser-Tyr; Ile-Tyr; Asp-Arg; Asn-Phe; Asn-Asn; Gln-Gln; Thy-Leu; Lys-Pro; Thy-Asn; Pro-Gln; Ala-Pro; Ser-Phe; Asp-Arg; **His-Phe**; Ala-Tyr; Val-Val; Leu-Asn; Thr-Phe; Ile-Arg; Thr-Tyr; Ser-Lys; Ser-Pro; Thr-Arg; Leu-His; Val-Lys; Val-Ala; **Thr-Tyr**; His-Pro; Gln-Phe; Val-Arg; Ile-Leu; His-Ser; **Pro-Phe**; Pro-Leu; Gln-Ile*	38
Antioxidant	*Lys-Pro; Leu-Lys; Ile-Arg; Leu-His; Arg-His-Lys*	5	*Leu-Lys; Lys-Pro; Ala-Lys; Ile-Arg; Thr-Tyr; Leu-His; Thr-Tyr; Arg-His-Lys*	8
Antitumor	*Ile-Arg*	1	*Ile-Arg*	1
Ion flow regulator	-		*Asp-Tyr*	1

revealed that the biofunctionalities of bioactive-peptides found in soybean hydrolysis (Tempeh production) were antihypertension, followed by antidiabetics, antioxidant, and antitumor. Bioinformatics approach could contribute in initial screening of the number and potential bioactive peptides in protein cleavage.

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