SQUALEN BULLETIN

Prediction and Mapping of Potential Bioactive Peptides from Traditional Fermented Anchovy (*Rusip*) using Bioinformatics Approaches

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Abstract

Bioactive peptides can be obtained from various sources, including fermented food. *Rusip* is a traditional Indonesian fermented food made from anchovy (*Stolephorus* spp.), which can potentially be a source of bioactive peptides. To date, conventional *in-vivo* and *in-vitro* approaches are still widely used in the analysis of bioactive peptides, although this method is laborious, costly, and time-consuming. The current bioinformatics-guided approach has allowed faster and more efficient analysis. This study aimed to predict, identify, and map the potential of bioactive peptides produced from *rusip* using the bioinformatics approach. Simulations of proteolysis of anchovy parent proteins (actin, myosin, troponin, tropomyosin a-chain, and tropomyosin a-chain) with Peptide Cutter using two different groups of enzymes (simulations I and II) showed that the more enzymes that play a role in proteolysis, the less the number of peptide fragments formed. Identification of the bioactivity of peptide fragments using the BIOPEP-UWM database revealed that the peptide fragment from actin cleavage using simulation 1 has the most significant percentage of bioactive peptides (91.55%). The occurrence frequency analysis also revealed that the bioactivity was anti-diabetic, anti-hypertensive, and antioxidant for each target protein, and the simulation had the highest probability of occurrence. The conclusion of this study indicated that Indonesian fermented anchovy '*rusip*' has high bioactivity potential from actin fragments in anchovy due to several bioactivities such as anti-diabetic, anti-hypertensive, and antioxidant, so it can be part of a healthy diet that potent to prevent non-communicable diseases.

Keywords: bioactive peptides, bioactivity prediction, bioinformatics, fermented anchovy, rusip

Introduction

Bioactive peptides are short-chain protein fragments (2-20 amino acids) that have a small molecular weight (<6 kDa) and have biological activity (Aluko, 2015; Tamam et al., 2018; Kurnianto et al., 2023). The biological activity appeared when the peptide sequences could be separated from the parent protein (Tamam et al., 2021). Bioactive peptides have received wide attention and interest because of their potential to modulate physiological responses and positively impact body health (Darili et al., 2017; Daroit & Brandelli, 2021). Currently, the BIOPEP-UWM Database reports 4,300 bioactive peptide sequences with various biological activities such as anti-diabetic, antibacterial, anti-hypertensive, anti-cholesterol, immunomodulatory and anti-inflammatory (de Castro & Sato, 2015; Minkiewicz et al., 2019). Bioactive peptides can be obtained from various sources, including fermented food (Peredo-Lovillo et al., 2022; Tamam et al., 2021; Kurnianto et al., 2023). Fermented food is a type of food that utilizes microorganisms in its production. In the fermentation process, complex components in food, such as proteins, carbohydrates, and fats, will be hydrolyzed to form simpler components (Rezac et al., 2018). Several studies have reported the potential for bioactive peptides from fermented foods, such as bioactive peptides from tempeh (Tamam et al., 2021), fish sauce (Najafian & Babji, 2019), goat fermented milk (Rubak et al., 2021), fermented sausage (Kononiuk & Karwowska, 2020), and Himalayan cheese (Mushtaq et al., 2019). One of Indonesia's traditional fermented foods that has the potential to be a source of bioactive peptides is *rusip*.

Rusip is a fermented food product derived from anchovy (*Stolephorus* spp.), which is fermented spontaneously under anaerobic conditions for 7-14 days (Susilowati et al., 2014; Kurnianto et al., 2023; Rinto et al., 2019). As an anchovy-based food, *rusip* has a high protein content, reaching 34.86% or ranging from 14.71 to 18.39 mg/mL (Koesoemawardani et al., 2018). This highest protein content is commonly found in fish muscle (Ryu et al., 2021). The proteins in this part consist of sarcoplasmic, myofibrillar, and stromal proteins (Ochiai & Ozawa, 2020). Myofibrillar proteins are the most dominant components (65-75% of the total fish protein) and include contractile proteins (actin and myosin), regulatory proteins (troponin and tropomyosin), and several other small proteins (Ochiai & Ozawa, 2020). In contrast, stromal and sarcoplasmic proteins constitute only a small fraction of fish protein (Ochiai & Ozawa, 2020). During *rusip* fermentation, these proteins are cleaved into fragments by proteolytic enzymes produced by the microbiota in *rusip*, resulting in various peptide fragments (Tamam et al., 2021). *Leuconostoc, Lactobacillus*, and *Streptococcus* are the main groups of lactic acid bacteria that dominate the rusip fermentation process (Kusmarwati et al., 2014). Other suspected contaminants, such as *Saccharomyces cerevisiae* and *Bacillus* sp., are also present during *rusip* fermentation (Sim et al., 2015). The peptide fragments generated from proteolysis can be identified and characterized to determine their potential biological activity.

Identification and characterization of the biological activity of a bioactive peptide generally uses a conventional approach (*in-vitro* or *in-vivo*), which is a very laborious technique, requires high costs, and has a long processing time (FitzGerald et al., 2020; Li-Chan, 2015; Peredo-Lovillo et al., 2022). Currently, along with the rapid development of technology, a new analytical approach, namely in bioinformatics, has been found and can be one of the solutions to the problems of the conventional approach (Peredo-Lovillo et al., 2022; Sitanggang et al., 2018). The bioinformatics approach is a technique that utilizes computational technology to manage, curate and interpret information related to biological systems (Li-Chan, 2015; Kurnianto et al., 2023). Various tools such as databases, web servers and software are used to analyze bioactive peptides using a bioinformatics approach (Tu et al., 2018). Several studies have used bioinformatics methods such as the characterization of the potency of a-lactoglobulin from milk (Tulipano et al., 2015), prediction of bioactive peptides from milk (Sitanggang et al., 2018) and the prediction of bioactive peptides from tempeh (Tamam et al., 2021).

Although the bioinformatics approach has been used in several bioactive peptide studies, most still use conventional methods, especially related to bioactive peptides from fermented fish such as *rusip*. Therefore, the bioinformatics approach is a potential technique to explore the potential of bioactive peptides from *rusip*. This study aims to predict, identify, and map the potential of bioactive peptides produced from fermented anchovy products (*rusip*) using the bioinformatics approach.

Material and Methods

The stages of the bioinformatics approach used in this study are illustrated in Figure 1. This research consists of four stages: identification of the parent protein in anchovy, determination of protein-cutting enzymes and simulation of parent protein cleavages, mapping potential biological activity and occurrence frequency calculation of each bioactive peptide (Tamam et al., 2021; Sitanggang et al., 2018).

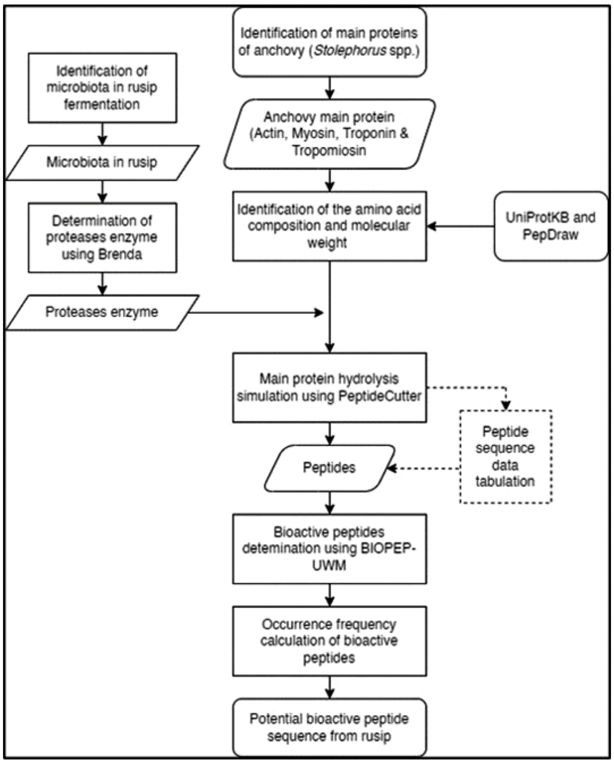


Figure 1. Determination of rusip bioactive peptides using bioinformatics approaches.

Identification of Amino Acid Composition and Protein Sequence in Fish

Parent proteins from anchovies (actin, myosin, troponin, and tropomyosin α and β chain) were used as substrates (Table 1). Amino acid sequences and molecular weight of the anchovy parent proteins were obtained from UniProtKB (http://www.uniprot.org) and PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/), respectively (Sitanggang et al., 2018; Tamam et al., 2021).

Determination of Protein-Cutting Enzymes and Simulation of Substrate Cleavage

Enzyme determination was carried out using the Brenda database (https://www.brenda-enzymes.org). The parent protein cleavage simulation was carried out using the Peptide Cutter (https://web.expasy.org/peptide_cutter/) from the EXPASY portal and consisted of two types of simulations. Simulation type 1 was a combination of enzymes produced by the dominant microbiota of rusip fermentation (*Lactobacillus plantarum, Streptococcus* sp., and *Leuconostoc* sp.). In contrast, simulation type 2 was carried out using a combination of dominant microbiota enzymes and contaminants microbiota of *rusip* fermentation (*Lactobacillus plantarum, Streptococcus* sp., *Leuconostoc* sp., *Saccharomyces cerevisiae*, and *Bacillus* sp). The list of protease enzymes used in simulation type I and II and the specific activities of these enzymes can be seen in Table 1.

Determination of Bioactivity and Molecular Weight of Rusip Peptides

Peptide sequences obtained from substrate cleavage (parent protein of anchovy) simulation results were tabulated manually in Microsoft Excel based on the cleavage number for each simulation. Furthermore, the biological activity of the simulated peptides was obtained by first matching or comparing the AA composition and the simulated peptide sequences with the BIOPEP-UWM Database (www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz et al., 2019). The molecular weight of each bioactive peptide was calculated using PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/).

Table 1. Types of protease enzymes and their specific activities used in the proteolysis of parent proteins in anchovies*

Simulation	Enzymes-Producing Bacteria	Proteases Enzymes	Specific Activity
Simulation type 1	- Lactobacillus plantarum,	AspN endopeptidase	To perform specific cleavage on Asp at the N terminal
	- Streptococcus sp.,	AspN endopeptidase + N-terminal Glu	To perform specific cleavage on Asp or Glu at the N terminal
	- Leuconostoc sp.,	Glutamyl endopeptidase	To perform specific cleavage on Glu at the C terminal
		Chymotrypsin	To perform specific cleavage on Trp, Tyr, and Phe at the C terminal
		Trypsin	To perform specific cleavage on Arg and Lys at the C terminal
Simulation type 2	- Lactobacillus plantarum, - Streptococcus sp.,	AspN endopeptidase	To perform specific cleavage on Asp at the N terminal
	- Leuconostoc sp.,	AspN endopeptidase + N-terminal Glu	To perform specific cleavage on Asp or Glu at the N terminal
	- Saccharomyces cerevisiae	Glutamyl endopeptidase	To perform specific cleavage on Glu at the C terminal
	- Bacillus sp.	Chymotrypsin	To perform specific cleavage on Trp, Tyr, and Phe at the C terminal
		Trypsin	To perform specific cleavage on Arg and Lys at the C terminal
		Pepsin	To perform specific cleavage on Phe, Tyr, Trp, and Leu

Notes* = Simulation 1 (unspontaneous fermentation) and Simulation 2 (spontaneous fermentation). It only differs in the microbes that grow during the fermentation process of the two different methods.

Determination of Occurrence Frequency in Rusip Bioactive Peptides

The occurrence frequency of bioactive peptides with specific activity was calculated using the equation (Montoya-Rodríguez et al., 2015; Darewicz et al., 2016). Determining the occurrence frequency of *rusip* is aimed at screening simulation results such as bioactivity potential in *rusip* quickly and accurately. These results could be used for further steps, *in vitro* or *in vivo* studies.

$$\text{Occurrence frequency}=\frac{\text{the number of peptide fragments with specific activity}}{\text{the number of amino acid of main protein}}$$

Results and Discussion

Anchovy Parent Protein as Bioactive Peptide Precursor

Fish protein, especially in fish muscle, is one of the body parts of fish with high protein content (Ryu et al., 2021). These include sarcoplasmic, myofibrillar, and stromal proteins (Ochiai & Ozawa, 2020; Ryu et al., 2021). Myofibrillar is the predominant protein (65 – 75%), while stroma and sarcoplasm comprise only a small part of the total protein in fish. As the dominant protein, myofibrillar proteins are composed of contractile proteins such as actin and myosin, regulatory proteins such as tropomyosin and troponin, and several other small proteins (Konno 2017; Ge et al., 2020). According to Ochiai & Ozawa (2020), actin and myosin proteins comprise 20% and 60% of the total myofibrillar protein.

Based on the UniProtKB database analysis, several types of myofibrillar protein (actin, myosin, troponin, tropomyosin α-chain and tropomyosin β-chain) sequences are associated with *Stolephorus spp*. The name, code and amino acid composition of the parent protein and the characteristics of the parent protein (the number of amino acids and molecular weight) in anchovy can be seen in Table 2. These parent proteins are precursors of bioactive peptides from fish (Le Gouic et al., 2018). Studies by Borawska-Dziadkiewicz et al. (2021), Huang et al. (2015), Gomez et al. (2019), and Darewicz et al. (2015) reported that actin and myosin proteins from several fish species such as tilapia, salmon, and carp are suitable bioactive peptide precursors with antioxidant, ACE-inhibitor, anti-thrombotic, and anti-amnesic activities.

Peptides Fragment from Proteolysis Simulation using PeptideCutter

As the main precursor of bioactive peptides, the parent proteins of anchovy (actin, myosin, troponin, tropomyosin α-chain and tropomyosin β-chain) will be hydrolyzed by proteolytic enzymes secreted by microbiota. According to Kusmarwati, Sri Heruwati, Utami, & Rahayu (2011) and Nurhikmayani, Daryono, & Retnaningrum (2019), several microbiotas grow dominantly during the *rusip* fermentation process, such as *Streptococcus*, *Leuconostoc*, and *Lactobacillus.* In addition, contaminant microbiota such as *Saccharomyces cerevisiae* SC3 and *Candida glabrata* CG2 were also found (Sim et al., 2015). These microbiota can secrete various types of enzymes, including proteolytic enzymes. These enzymes have a significant role in forming bioactive peptides because the enzymes act to cleave the substrate parent protein into peptides and amino acids during the fermentation process (Kurnianto et al., 2020). Various enzymes secreted by microorganisms, especially proteolytic enzymes, can be known through access to the Brenda database (https://www.brenda-enzymes.org/) (Tamam et al., 2021).

The proteolytic enzymes known from the Brenda database are then used in the proteolysis simulation stage using PeptideCutter from EXPASY to represent the conditions of protein cleavage in rusip fermentation (Sitanggang et al., 2018). There are two proteolysis simulations (simulation type 1 and 2) based on the presence of enzymes produced by contaminant microbiota in rusip fermentation (Table 1; Sitanggang et al., 2018; Tamam et al., 2021). Ten combinations of simulations were obtained based on the number of parent proteins and types of simulation conditions (five types of parent proteins and two types of simulations). To describe the proteolysis simulation using Peptide Cutter, an example of the simulation results of myosin (D6BT34) using a combination of enzymes in simulation type 1 can be seen in Table 3.

Proteolysis using a combination of enzyme simulation type 1 showed that the cleavage processes of actin, myosin, troponin, tropomyosin a-chain and tropomyosin b-chain produced 71, 53, 50, 68, and 62 peptide fragments, respectively. Meanwhile, cleavage processes using simulation 2 produced 70, 46, 44, 61 and 52 peptide fragments, respectively. Based on the analysis results, the more combinations of enzymes

Table 2. Name, code, amino acid composition, and characteristics (number of amino acids and molecular weight) of parent protein in anchovies obtained from the UniprotKB database

Protein Name	Code	AA Composition	The Number of AA	Molecular weight (kDa)
Actin	Q98972	MCDDEETTALVCDNGSGLVKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNELRVAPEEHPTLLTEAPLNPKANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRTTGIVLDAGDGVTHNVPVYEGYALPHAIMRLDLAGRDLTDYLMKILTERGYSFVTTAEREIVRDIKEKLCYVALDFENEMATAASSSSLEKSYELPDGQVITIGNERFRCPETLFQPSFIGMESAGIHETAYNSIMKCDIDIRKDLYANNVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDEAGPSIVHRKCF	377	41,959
Myosin	D6BT34	TKRVIQYFASIAAVGGAKRDESKGTLEDQIIQANPALEAFGNAKTVRNDNSSRFGKFIRIHFGTTGKLSSADIETYLLEKSRVTYQLKAERNYHIFYQIMSNQKPELLDMLLITNNPYDYSYVSQGEITVASINDADELMLTDSAFDVLGFTGEEKLGIYKLTGAIMHYGNMKFKNKQREEQAEPDGTESADKSSYLMGLNSADLVKGLCHPRVKVGNEYVTKGQSVDQVYY	232	25,988
Troponin	A0A3B3I7N7	MKEECISDKNMTGDEEGEAEEGEGDGAKPKFKPFVMPNLIPPKIPDGERVDFDDIHRKRMEKDLMELQTLIEVHFESRKKEEEELINLKDRIEKRRSERAEQQRIRSERDKERQKRLEDERARKEEEEAKKRAEDDAKKKKTLTSLHFGGYMQKLTEKRSGKKQTEREKKKKILNERRKSLDIEKMSQDRLKEKAVELWEWMHQLEAEKFELQYQFARQKYEINVLRNRVSDHQKTTKRTKRGLRK	246	29,756
Tropomyosin α-chain	H2LUJ8	MDAIKKKMQMLKLDKENALDRAEQAESDKKAAEDRSKQLEDDIREMEKKLRTTEDERDKVFEELQTAEEKLLSAEETATKLEDDLVALQKKLKGTEDELDKYSEALKDAQEKLELAEKKATDAESDVASLNRRIQLVEEELDRAQERLATALTKLEEAEKAADESERGMKVIENRAMKDEEKMEIQEIQLKEAKHIAEEADRKYEEVARKLVIIESDLERTEERAELSEGKCSELEEELKTVTNNLKSLEAQAEKYSLKEDKYEEEIKVLTDKLKEAETRAEFAERSVAKLEKTIDDLEEKLSQAKEENIDMHQMLDQTLMELNNL	326	37,786
Tropomyosin β-chain	H2MCV5	MEAIKKKMQMLKLDKENAIDRAEQAEVDKKGAEDKCKQLEEELLGLQKKLKGVEDELDKYSESLKDAQEKLEQAEKKAADAEAEVASLNRRIQLVEEELDRAQERLATALQKLEEAEKAADESERGMKVIENRATKDEEKMEIQEMQLKEAKHIAEEADRKYEEVARKLVILEGDLERSEERAEVAEAKSGDLEEELKNVTNNLKSLEAQAEKYSQKEDKYEEEIKVLTEKLKEAETRAEFAERSVAKLEKTIDDLEDEVYSQKLKGKALSEELDLALNDMTTL	284	32,577

Amino acid nomenclature**:** C (Cys); H (His), I (Ile), M (Met), S (Ser), V (Val), A (Ala), G (Gly), L (Leu), P (Pro), T (Thr), F (Phe), R (Arg), Y (Tyr), W (Trp), D (Asp), N (Asn), E (Glu), Q (gin), Z (Glx), K (Lys); Molecular weight and number of amino acids of protein were analyzed using PepDraw software (http://www.tulane.edu/~biochem/WW/PepDraw/).

Table 3. An example of the simulation results of myosin proteolysis (D6BT34) using a combination of simulated enzyme type 1 (Asp-N endopeptidase, Asp-N endopeptidase + N-Terminal Glu, Chymotrypsin (High Specificity), Glutamyl Endopeptidase, and Trypsin)

Cleavage Position	Cleavage Results (Peptide Sequence)	The Number of AA	Molecular Weight (Da)
2	TK	2	247.29
3	R	1	
7	VIQY	4	521.61
8	F	1	
18	ASIAAVGGAK	10	843.98
19	R	1	
20	D	1	
21	E	1	
23	SK	2	233.27
26	GTL	3	289.33
27	E	1	
37	DQIIQANPAL	10	1,082.22
38	E	1	
40	AF	2	236.27
44	GNAK	4	388.42
47	TVR	3	374.22
48	N	1	
53	DNSSR	5	577.55
54	F	1	
56	GK	2	203.24
57	F	1	
59	IR	2	287.19
62	IHF	3	415.22
67	GTTGK	5	462.24
71	LSSA	4	376.19
73	DI	2	246.12
74	E	1	
76	TY	2	282.12
78	LL	2	244.17
79	E	1	
80	K	1	
82	SR	2	261.14
85	VTY	3	381.18
88	QLK	3	387.24
89	A	1	
90	E	1	
91	R	1	
93	NY	2	295.11
96	HIF	3	415.22
97	Y	1	
105	QIMSNQKP	8	945.1
106	E	1	
108	LL	2	244.17
118	DMLLITNNPY	10	1,193.38
120	DY	2	296.1
122	SY	2	268.1
126	VSQG	4	389.19
127	E	1	
134	ITVASIN	7	716.4
136	DA	2	204.07
137	D	1	
138	E	1	
142	LMLT	4	476.26
146	DSAF	4	438.17
151	DVLGF	5	549.27
153	TG	2	176.07
154	E	1	
155	E	1	
156	K	1	
160	LGIY	4	464.56

161	K	1	
169	LTGAIMHY	8	904.44
173	GNMK	4	448.2
174	F	1	
177	KNK	3	388.47
179	QR	2	302.33
180	E	1	
181	E	1	
183	QA	2	217.22
184	E	1	
185	P	1	
188	DGT	3	291.26
189	E	1	
191	SA	2	176.17
193	DK	2	261.28
196	SSY	3	355.35
203	LMGLNSA	7	704.84
207	DLVK	4	473.57
213	GLCHPR	6	681.81
215	VK	2	245.32
218	VGN	3	288.3
219	E	1	
220	Y	1	
223	VTK	3	346.43
227	GQSV	4	389.41
231	DQVY	4	523.54
232	Y	1	

Amino acid nomenclature**:** C (Cys); H (His), I (Ile), M (Met), S (Ser), V (Val), A (Ala), G (Gly), L (Leu), P (Pro), T (Thr), F (Phe), R (Arg), Y (Tyr), W (Trp), D (Asp), N (Asn), E (Glu), Q (gin), Z (Glx), K (Lys); Molecular weight and number of amino acids of protein were analyzed using PepDraw software (http://www.tulane.edu/~biochem/WW/PepDraw/).

that play a role in a proteolysis process, the less the number of peptide fragments formed. These results are different from the study of Tamam et al. (2021), who reported that cutting soybean parent proteins (G1, G2, G3, G4, G5, a-conglycinin a, and b-conglycinin b) using a combination of enzymes that were more capable of producing a larger number of peptide fragments. Several factors, such as differences in

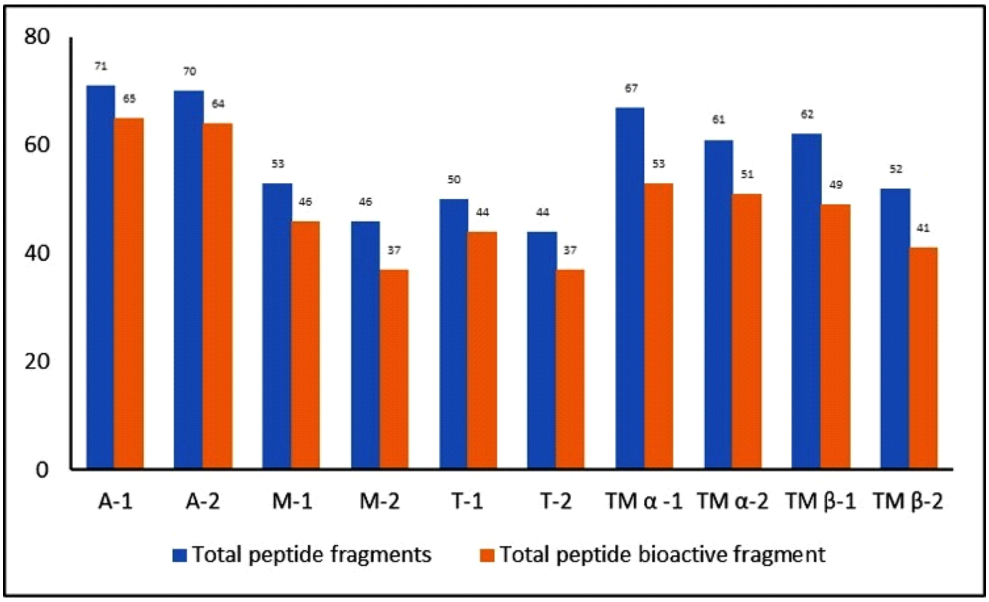


Figure 2. Number of peptide fragments and bioactive peptides produced from each parent protein.

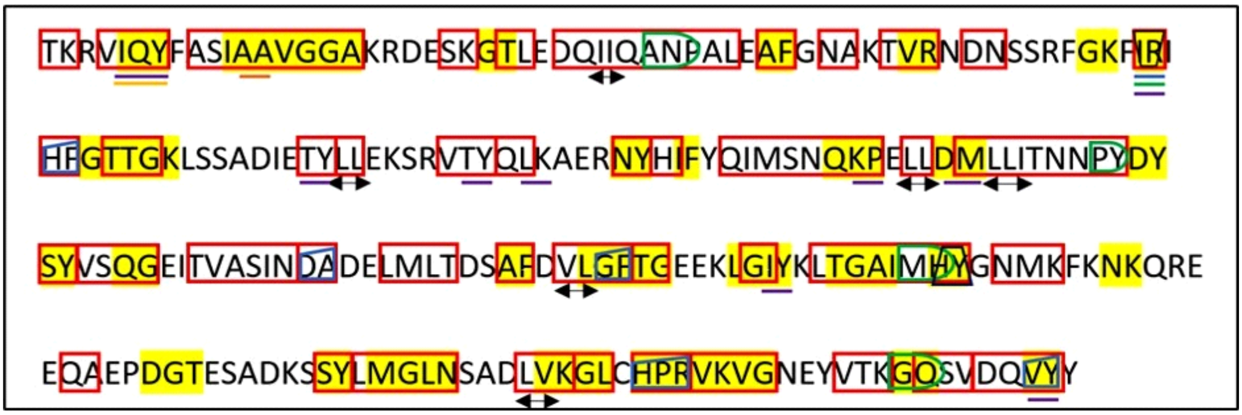
parent proteins, type and specificity of enzymes, and the combination of enzymes used, can cause this difference in results. According to Sitanggang et al. (2018), increasing enzyme combinations did not always give more peptide fragments. The study also explained that the specificity of enzyme action affected the peptide fragments.

Mapping of Potential Bioactive Peptides from Rusip

All peptide fragments generated from the proteolysis simulation process were further analyzed for their amino acid composition and sequence suitability in the bioactive peptide fragment database, BIOPEP-UWM (http://www.uwm.edu.pl/biochemia) (Iwaniak et al., 2020). The analysis revealed that the peptide fragments derived from each parent protein exhibited significant bioactive potential. Actin cleaved using simulation type 1 (actin 1) showed the highest percentage of bioactive peptides at 91.55%, followed by actin 2 (91.43%), troponin 1 (88.0%), myosin 1 (86.79%), troponin 2 (84.09%), tropomyosin a-chain 2 (83.61%), myosin 2 (80.43%), Tropomyosin a-chain 1 (79.10%), tropomyosin b-chain 1 (79.03%), and tropomyosin b-chain 2 (78.85%) (Figure 2). The mapping results of various biological activities of the amino acid sequences of myosin (A) and actin (B) proteins in anchovy, based on the evaluation through BIOPEP-UWM, can be seen in Figure 3. Based on these findings, the bioactive peptide fragments from both parent proteins have dominant anti-diabetic (DPP-IV inhibitor) and anti-hypertensive (ACE-inhibitor) activities. Darewicz et al. (2016) reported that myofibrillar proteins (actin and myosin) from fish are potentially the richest source of bioactive fragments, especially ACE inhibitors and antioxidants. Their study showed that the parent protein myosin heavy chain (Q5NTZ3) from carp could form 933 bioactive peptide fragments, among which 646 had ACE-inhibitor activity and 120 had antioxidant activity. Another study on milk-derived proteins reported that b-casein had the highest percentage of bioactive peptide presentation at 52%, followed by aS1-casein (48%) and b-lactoglobulin (41%). Further identification showed that all these types of milk proteins could be sources of bioactive peptides with anti-hypertensive activity (Sitanggang et al. 2018). Meanwhile, Montoya-Rodriguez et al. (2015) also reported the mapping results of the bioactive peptide capabilities of the parent protein Globulin 11S from Amaranth (*Amaranthus hypochondriacus*), which showed the highest bioactivity potential in inhibiting ACE activity or anti-hypertensive activity.

Occurrence Frequency (OF) of Peptide Fragments with Specific Activity

Occurrence frequency (OF) illustrates the probability of bioactive peptides obtained with a specific target protein activity. Actin protein using simulation type 1 had the highest occurrence frequency of anti-diabetic (OF = 0,472) and antihypertension (OF = 0,366) (Figure 3). Overall, bioactivity as anti-diabetic, antihypertension, and antioxidant for each target protein and simulation had the highest occurrence probability compared to other bioactivities such as anticancer, antibacterial, anti-inflammation, anti-thrombotic, anti-cholesterol, etc. The occurrence frequency result follows research results conducted by Baco et al. (2022), Chaijan et al. (2021), Choksawangkarn et al. (2018), Hamzeh et al. (2020), Kim et al. (2016), Najafian & Babji (2018), Rinto et al., (2019), Rinto et al., (2021), dan Rinto & Hafif (2017) in fermented fish. Rinto et al. (2019) reported that Indonesian fermented anchovy *rusip* extract has antioxidant, anti-cholesterol, and antihypertension activity. In contrast, this research via bioinformatics approach showed that *rusip* also has anti-diabetic activity. Hence, occurrence frequency can screen various peptide fragments with a specific activity with the most potential in each parent



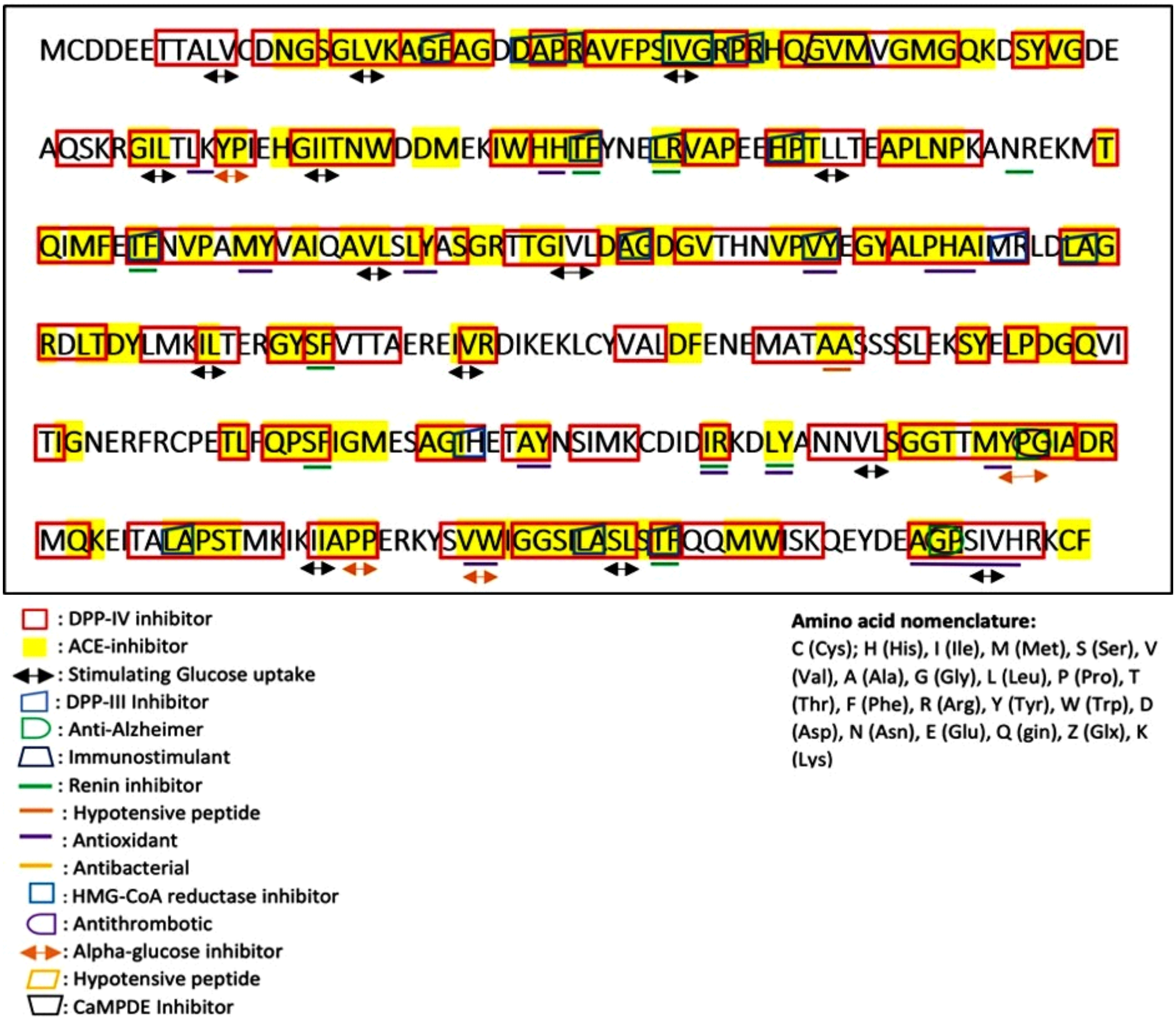


Figure 3. Overview of the bioactive peptide sequences mapping that was cut using enzymes in simulation type 1 found from the myosin (A) and actin (B) parent protein sequences.

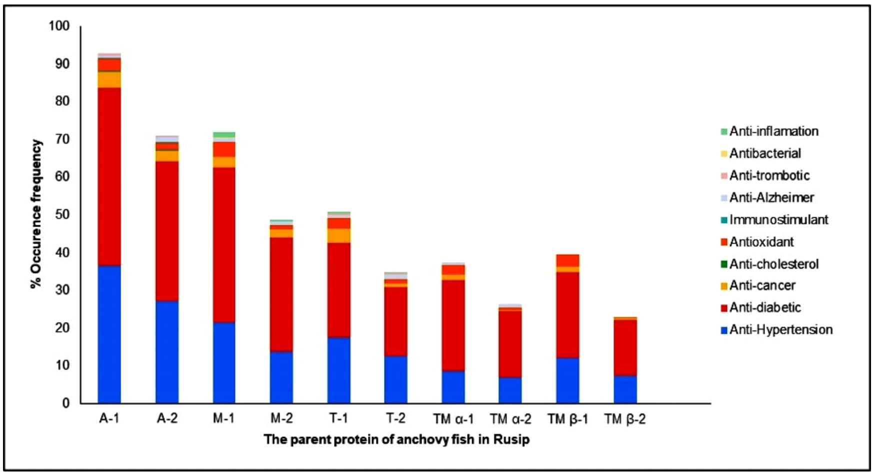


Figure 3. Occurrence frequency for each bioactive peptide of the parent protein in both simulation.

protein. Based on occurrence frequency results, anti-diabetes, antihypertension, and antioxidant activity were selected for further research.

All parent proteins in *rusip* showed high occurrence frequencies of anti-diabetics (OF = 0.148 to 0.472), antihypertension (OF = 0.074 to 0.366), and antioxidants (OF = 0,003 to 0,039). Other parent proteins were detected as anticancer (OF = 0,003 to 0.042) and anti-Alzheimer (OF = 0,006 to 0,012). In addition, of all parent proteins in *rusip,* only actin had anti-cholesterol (OF = 0,003), anti-thrombotic (OF = 0,005), and immunostimulant effect (OF = 0,003), as well as myosin, had antibacterial activity (OF = 0,004). Potential candidates of a bioactive peptide such as anti-diabetes, antihypertension, and antioxidants were obtained in *rusip.* The results of this research promote the basic concept that Indonesian fermented anchovy *rusip* could be part of the “healthy” diet and thereby prevent non-communicable diseases in humans.

The potential bioactive peptide as antihypertension in actin (simulation type 1), namely GP, DG, GV, VG, GY, AA, GF, TG, YP, PR, MG, GR, SG, GM, GY, PT,

Table 4. Predicted bioactivity of peptide fragments from rusip parent proteins with molecular mass <3 kDa.

Parent Protein	Bioactivity	Occurrence Frequency	Potential Bioactive Peptide	References
Actin 1 (Molecular Weight: 41,959 kDa)	Anti-diabetic	0.472	GP, VP, LV, II, IV, VA, MA, LA, PA, LN, HA	(Morifuji et al., 2009; Mojica & Mejia, 2016; Feng et al., 2021)
	Antihypertension	0.366	GP, DG, GV, VG, GY, AA, GF, TG, YP, PR, MG, GR, SG, GM, GY, PT, DA, LA, AG, GS, GG, GL, GT, MG, PP, AG, GV, TQ	(Byun & Kim, 2002; Sentandreu & Toldrá, 2007; Van Platerink et al., 2008; Montoya-Rodriguez et al., 2015; Kleekayai et al., 2015; Bhandari et al., 2020)
	Antioxidant	0.032	LK, HH, IR	(He et al., 2019; Huang et al., 2010; Montoya-Rodriguez et al., 2015; Chai et al., 2020)
Actin 2 (Molecular Weight: 41,959 kDa)	Antidiabetic	0.371	GP, VP, II, IV, VA, MA, LA, PA, LN, HA	(Morifuji et al., 2009; Mojica & Mejia 2016; Feng et al., 2021)
	Antihypertension	0.271	GP, DG, GV, VG, GY, AA, GF, TG, YP, PR, MG, GR, SG, GM, GY, PT, DA, LA, AG, GS, **EI**, GG, GT, MG, PP, AG, GV, TQ	(Byun & Kim, 2002; Sentandreu & Toldrá, 2007; Van Platerink et al., 2008; Montoya-Rodriguez et al. 2015; Kleekayai et al., 2015; Bhandari et al., 2020)
	Antioxidant	0.019	HH, IR	(He et al., 2019; Huang et al., 2010; Montoya-Rodriguez et al., 2015; Chai et al., 2020)
Myosin 1 (Molecular Weight: 25,988 kDa)	Anti-diabetic	0.411	LV, II, VA, LI, PA, LN, VL	(Morifuji et al., 2009; Feng et al., 2021)
	Antihypertension	0.215	MG, GT, IR, AA, VG, GA, GG, VR,, GK, GT, TG, QG, HP, VG, TG, GI,	(Sentandreu & Toldrá, 2007; Bhandari et al., 2020; Chourasia et al., 2023)
	Antioxidant	0.039	IR, TY, KP, LK	(He et al., 2019; Chourasia et al., 2023; Ohata et al., 2016)
Myosin 2 (Molecular Weight: 25,988 kDa)	Antidiabetic	0.302	II, VA, PA	(Morifuji et al., 2009; Mojica & Mejia 2016; Feng et al., 2021)
	Antihypertension	0.138	MG, GT, IR, AA, VG, GA, GG, VR,, GK, GT, TG, QG, HP, VG, TG, GI,	(Sentandreu & Toldrá, 2007; Bhandari et al., 2020; Chourasia et al., 2023)
	Antioxidant	0.013	IR, TY, KP	(Saito et al., 2003; He et al., 2019; Chourasia et al., 2023; Ohata et al., 2016)

DA, LA, AG, GS, GG, GL, GT, MG, PP, AG, GV, TQ (Byun & Kim, 2002; Sentandreu & Toldrá, 2007; Montoya-Rodriguez et al., 2015; Kleekayai et al., 2015; Bhandari et al., 2020). (Table 4). However, EI is the only actin with simulation type 2 with one different potential peptide fragment. Meanwhile, myosin (both of simulation types 1 and 2) had the same potential peptide fragments, namely MG, GT, IR, AA, VG, GA, GG, VR, GK, GT, TG, QG, HP, VG, TG, GI (Byun & Kim, 2002; Sentandreu & Toldrá, 2007; Montoya-Rodriguez et al., 2015; Kleekayai et al., 2015; Chai et al., 2020) (Table 4). Those peptide fragments had molecular mass <3 kDa. For anti-diabetic activity, overall, actin in simulation type 1 (actin 1) and 2 (actin 2) had the same peptide fragments, but only actin 1 had LK fragments. Myosin 1 and 2 had an anti-diabetic candidate of peptide fragments. Myosin 2 had fewer peptide fragments than myosin 1 (Table 4). This might be due to the different enzymes used in the PeptideCutter simulation. Last, in antioxidant activity, both actin and myosin had fewer peptide fragments because their antioxidant activity had a lower occurrence frequency compared to other bioactivities.

Conclusion

The bioinformatics approach, which includes the search for parent proteins and enzymes, simulation of proteolysis, identification of bioactivity and analysis of occurrence frequency, can be used to predict and analyze bioactive peptides derived from traditional Indonesian fermented food, namely *rusip*. Based on the results of this analysis, it is known that the parent protein Actin, which is cleaved using simulation type 1 (unspontaneous fermentation), is capable of producing the largest percentage of bioactive peptides compared to other parent proteins and simulation mode 2 (spontaneous fermentation). In addition, based on the bioactivity potential mapping carried out, bioactive peptide fragments were found to have a high potential for anti-diabetic (DPP-IV inhibitor) and anti-hypertensive (ACE-inhibitor) activity. The results of this study indicate the potential of *rusip* as a functional food that can be part of a healthy diet that effectively prevents NCD (a neurodegenerative disease).

Supplementary Materials

Supplementary materials is not available for this article.

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