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Prospects of bioinformatics approach for exploring and mapping potential bioactive peptide of Rusip (The traditional Indonesian fermented anchovy): A Review

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ABSTRACT

Rusip is a traditional Indonesian side-dishes food that is a fermented anchovy product originating from Bangka Belitung. During the fermentation, various lactic acid bacteria grew in rusip and produced bioactive peptides because of proteolytic enzyme action. Several treatments to obtain bioactive peptides can be conducted (fermentation, *in vivo* digestion, and *in vitro* hydrolysis using enzymes). The *in vivo* and *in vitro* methods are a widely used approach, but these methods are costly and time-consuming. These limitations could be solved by the bioinformatics approach. This method manages and interprets information about biological systems that employ computational methods. This study aimed to review recent studies on rusip and similar fermented fish and peptide bioactive with their bioactivity), steps, advantages, and limitations of bioactive peptide studies using the bioinformatics approach. The review article was written using narrative literature review method, which based on in-depth investigation from scientific literatures by identifing keywords, reviewing the content of articles, and synthesizing the findings. The results showed that using bioinformatics has provided opportunities for the development of bioactive peptides. Through this method, bioactive peptide identification begins with determining the main sample protein and the enzymes in protein hydrolysis. The further steps are protein hydrolysis simulation, determining the potential for bioactivity, and molecular docking. The bioinformatics analyses were performed synergistically to predict the protein or peptide characteristics from the sample and its bioactivity and determine its interaction with their receptor. However, despite the advantages, the bioinformatics approach also has several limitations, such as the lack of certain types of proteins or peptides in the database and hydrolysis simulation tool. Combining conventional and *in silico* methods (hybrid method) is potential to obtain the new and promising bioactive peptides from rusip and other fermented fish (i.e., *budu*, *bekasam*, and *pla duk ra*) and meat products for development product, both functional food and supplements.

Introduction

Rusip is a traditional Indonesian side-dishes food, which is a fermented anchovy product originating from Bangka Belitung (Figure 1). *Rusip* is made from anchovies, salt, and palm sugar, then fermented anaerobically for 7-14 days (Rukmini et al., 2014). *Rusip* has unique organoleptic characteristics, such as light brown to dark gray in color and an aroma and taste that tends to be salty and sour (Kusmarwati et al., 2011). *Rusip* is usually consumed directly as 'sambal' by adding spices such as chili, shallots, and lime juice and eaten with vegetables (Koesoemawardani 2007).

During the *rusip* fermentation processes, various lactic acid bacteria, i.e., *Streptococcus* sp., *Lactococcus* sp., and *Leuconostoc* sp., grow naturally on the product and produce proteolytic enzymes (Koesoemawardani and Yuliana, 2013).

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Figure 1. *Rusip* and the end use of *rusip* product ("sambal rusip")

Those proteolytic microorganisms hydrolyze protein into peptides which possess different amino acid sequences. Many studies reported that peptides exhibit a variety of biological activities, such as antibacterial, antioxidant, anti-inflammatory, antihypertensive, and anti-cholesterol (de Castro and Sato, 2015). Peptides with various bioactivities are commonly known as bioactive peptides (Tamam et al., 2018). The bioactive peptides contained in rusip will have a positive health impact on the people who consume it. This function confirms the role of rusip as a functional food. In addition, the stages of separation and increasing the purity of the rusip bioactive peptide can also be carried out for utilization as a nutraceutical product.

Generally, classical approaches for producing and identifying bioactive peptides involve four steps: identifying suitable protein sources, performing enzymatic hydrolysis (*in vitro*)/fermentation/*in vivo* digestion to obtain peptide fragments, isolating bioactive peptides by chromatographic methods, and validating peptide bioactivity (Li-Chan, 2015). The classical approach to finding these bioactive peptides is laborious, expensive, and long processing time (Peredo-Lovillo et al., 2022). Therefore, the bioinformatics approach was introduced to solve these issues (Ekins et al., 2007). The bioinformatics approach manages, curates, and interprets information about biological systems that employ computational methods (Li-Chan, 2015). Various bioinformatics tools such as databases, web servers, and software have been used to analyze amino acid precursor protein profiles, physicochemical properties, and bioactivity to molecular interactions between compounds (ligands and receptors) (Tu et al., 2018). This approach offers an excellent opportunity for exploratory studies to the discovery of new bioactive peptides since it can identify, characterize, and analyze the functional properties of bioactive peptides at a low cost and even with fewer chemical reagents, resulting in more targeted and faster acquisition/discovery of new bioactive peptides (Holton et al., 2013; Liu et al., 2019).

Moreover, the bioinformatics approach can be carried out synergistically using a classical approach to enable more effective analysis or identification of potential bioactive peptide candidates (Kang et al., 2022). As a result, the use of this method has increased significantly in numerous bioactive peptide assessment studies (Ji et al., 2018). Bioinformatics techniques have been used in several peptide studies, such as studies on the potency of milk's β-lactoglobulin (Tulipano et al., 2015), bioactive peptides from milk (Sitanggang et al., 2018), bioactive peptides from flaxseed (Langyan et al., 2021), bioactive peptide from *Halophila stipulacea* (Kandemir-Cavas et al., 2019), bioactive peptide tempeh (Tamam et al., 2021), and bioactive peptide produced by *Streptomyces* sp. (Kurnianto et al., 2022).

Although the bioinformatics approach has been used in several bioactive peptide studies, most studies on discovering bioactive peptides from *rusip* and other fermented fish products still use the classical approach as their exploration research method. Thus, the bioinformatics approach becomes a potential new approach for exploratory studies of original bioactive peptides from *rusip* or other fermented fish products. This manuscript aims to review recent studies on *rusip* and similar fermented fish (nutritional content,

safety, microorganisms, and peptide bioactive with their bioactivity), the steps, the, advantages, and the limitations of bioactive peptide studies derived from traditional fermented foods using the bioinformatics approach.

Research Methods

The research was conducted using the narrative literature review method. The initial step was to perform a search for scientific literature published from 2012 - 2022 and indexed in the Scopus, Google Scholar, Pubmed, and Sinta (Indonesian indexing journal) databases. The next step is to identify keywords, which keywords used are related to rusip and similar fermented fish products (i.e., *budu, bekasam, pla duk ra*, etc.), fermented food bioactive peptides, bioinformatics, software, and databases used (i.e., expassy, uniprot, BioPEP, autodock, autodock vina, etc.) and implementation of bioinformatics for predicting bioactive peptides of fermented food. The third step is a review of abstracts and articles, in which at this step, articles with appropriate keywords but abstracts and the contents of the articles cannot answer the research objectives will be excluded. In the final step, a synthesis of the findings from the articles is carried out and integrated into the manuscript.

Results and Discussion

Nutrient content and safety of Rusip

Rusip is a traditional Indonesian food fermented anchovy product from Bangka Belitung (Kusmarwati et al., 2014). *Rusip* is made from anchovies, salt, and palm sugar, then fermented anaerobically for 7-14 days (Koesoemawardani and Yuliana, 2013). Generally, *rusip* has sensory characteristics such as the appearance of crumbling fish, a grey-brown color, a hazy and watery liquid, a salty and sour flavor, and the fishy and sour aroma typical of fermented foods (Koesoemawardani and Mahrus, 2016). *Rusip* has a high protein content (34.86%) due to anchovies (*Stolephorus spp*.) being the main ingredients. Its protein has various amino acids, with glutamate and aspartate in the highest concentrations (Koesoemawardani et al., 2018). In addition, *rusip* also contains high minerals (11.49%) and low-fat content (2.60%) (Rinto et al., 2019)

Traditional and spontaneous fermentation has an essential role in the production of *rusip*. In fermentation, salt concentration (13.9 – 15.6%) is the primary selection factor (Rinto and Subarka, 2017). According to Faisal et al. (2015), several groups of bacteria (i.e., lactic acids (LAB), *Bacillus* sp., and *Pseudomonas* sp.) and yeast (i.e., *Saccharomyces cerevisiae*) are present and able to survive till the end of fermentation processes. Various bacteria can provide functional benefits for the body as well as concerns about food safety issues. Several studies reveal that specific metabolites generated by microbial metabolism in fermented fish products, such as purines, histamine, and metabolites associated with uric acid, can pose health risks (Paul and James, 2017). Nevertheless, there has been no reports of allergenicity, pathogenic bacterial infection, or histamine poisoning due to *rusip* (Waisundara et al., 2016).

Microbiota in Rusip and secreted enzymes

Fermentation is one food processing that needs microbiota, such as bacteria, fungi, and yeasts (Narzary et al., 2021). These bacteria transform nutrients and the secretion of enzymes and metabolites that provide benefits such as different organoleptic and physicochemical qualities, extended shelf life, and higher digestibility (Dzikunoo et al., 2021). One of the fermented food products from Indonesia is *rusip.* Several studies have been conducted on the microbiota that plays a role in the fermentation of *rusip*, with the lactic acid bacteria (LAB) group dominating (Nurhikmayani et al., 2019). A study by Kusmarwati et al. (2014) reports that bacteria (such as *Streptococcus, Leuconostoc, Lactobacillus,* and *Micrococcus*) are identified in the final product of *rusip*. Another study by Yuliana et al. (2018) identified the existence of *Streptococcus, Lactococcus* and *Leuconostoc*. *Streptococcus,* and *Lactococcus* are identified from the beginning to the middle of the fermentation, while *Leuconostoc* is always identified from the beginning to the end of the fermentation. Microbiota strains discovered in fermented food, such as *rusip* from Kalimantan (*budu*), including *Micrococcus, Staphylococcus, Pediococcus* sp., and *L. plantarum* LP1 and LP2. Yeast microbiota, such as *Saccharomyces cerevisiae* SC3 and *Candida glabrata* CG2, are also discovered in *budu* (Sim et al., 2014). Several strains of L. *plantarum and P. pentosaceus* are found in Chao, another fermented anchovy product (Nurhikmayani et al., 2019).

Microbiota in fermented products grows and produces various enzymes and metabolites that contribute to the formation of the product's organoleptic properties (Garcia-Cano et al., 2019). One of them is proteolytic enzymes (Giyatmi and Irianto, 2017). The proteolytic enzyme has a role in the degradation of high-protein fermented

substrates such as fish. It is closely related to the development of fermented products' texture, taste, color, and aroma (Garcia-Cano et al., 2019). A study by Lopetcharat and Park (2002) reported that bacteria such as *Micrococcus, Halococcus, Staphylococcus,* and *Pseudomonas* could produce protease enzymes, which have a role in forming fish sauce aroma. Another study showed that *Micrococcus luteus, S. carnosus*, and *S. xylosus* produce peptidase enzymes that form flavor and *budu* texture (Sim, 2009). Sim et al. (2014) also identified that *Bacillus, Micrococcus, Staphylococcus, Streptococcus,* and halophilic LAB has a strong ability to produce proteinase enzymes that play a role in the hydrolysis of fish protein in *budu*. Another further identification also reported that one of the microbiotas originating from *rusip* with the highest proteolytic activity is *Staphylococcus warner*i (Islami et al., 2019). In addition, fermented food microbiota can produce enzymes and other metabolites such as lipolytic and antimicrobial (Koesoemawardani et al., 2018; Kurnianto et al., 2021).

The change of amino acid composition during rusip fermentation

The diversity of microbiota and proteolytic enzymes produced during fermentation causes amino acid composition changes in *rusip*. According to Peralta et al. (2008), peptide/amino acid composition of fermented fish products fluctuated during the fermentation period. This difference in amino acid composition is caused by protein hydrolysis and microbial activity (Jiang et al., 2007). According to Anggo et al. (2015), total amino acids increase from 32.32 g/100 g on the 8th to 37.15 g/100 g on the 32nd day. Moreover, the most abundant amino acids in *Rusip* are glutamic acid (5.52 %), aspartic acid (3.69 %), and arginine (2.49%). Koesoemawardani et al. (2018) and Khairi et al. (2014) also stated that glutamic acid, aspartic acid, alanine, and lysine are the dominant amino acids formed during the *rusip* fermentation process. The results were also similar with Mahamad et al. (2022), who enlighten that the total amino acids in fermented *budu* fish have increased during the fermentation period (6 months). According to the study, the primary amino acids in *budu* include lysine (1,6 %), glutamic acid (1,54 %), and aspartic acid (1,26 %). *Rusip* high glutamic amino acid content contributes significantly to the product's flavor (Jinap et al., 2010). Furthermore, the content of these different amino acids is related to protein digestibility and the functional role of bioactive peptides (Khairi et al., 2014).

Peptide bioactive and peptide bioactivity in Rusip

Specific short-chain protein fragments (2–20 amino acids) with a low molecular weight (<6 kDa) that have biological or functional activity are known as bioactive peptides (Aluko, 2015; Tamam et al., 2018). Bioactive peptides have a variety of functional roles, including antihypertensive, antioxidant, antimicrobial, antithrombotic, anti-obesity, anticancer, and antidiabetic (Abdelhedi et al., 2017; Capriotti et al., 2015; Chakrabarti et al., 2018). These functional roles are strongly related to the peptide's physicochemical characteristics (molecular weight, composition, and amino acid sequences) (Singh et al., 2014). In addition, peptides must be released to carry out their biological action (Sánchez and Vázquez, 2017). According to Wijesekara et al. (2011), the presence of amino acids with aromatic and aliphatic groups on the C atom (i.e., Pro, Phe, and Tyr) and N atoms (i.e., Val and Ile) is associated with ACE inhibitors or antihypertensive. Hydrophobic amino acids, including Tyr, Phe, Trp, Ala, Ile, Val, and Met, and cationic amino acids like Arg and Lys, demonstrate an excellent affinity for ACE (He et al., 2012; Rai et al., 2017). The presence of the amino acids Tyr, Trp, Met, Lys, and Cys can degrade Fe^{3+} ions to Fe^{2+} and chelate Fe^{2+} and Cu^{2+} ions indicating the role of bioactive peptides as antioxidants (Carrasco-Castilla et al., 2012). Other amino acids that contribute to antioxidant activity include Val, Leu, Iso, Phe, and Pro (Najafian and Babji, 2018, 2019). According to Tang et al. (2015), the presence of positively charged (Arg) and amino acids with high hydrophobicity (Gly and Leu) is associated with antimicrobial capabilities because these amino acids are crucial in peptide-bacterial cell membrane interactions.

Several protein hydrolysis processes, including fermentation, can produce bioactive peptides (Daroit and Brandelli, 2021). As a fermented food, *rusip* has great potential as a source of bioactive peptides . Bioactive peptides from *rusip* or fermented anchovies have been proven in numerous studies to offer potential as antioxidants, antihypertensive, antimicrobials, antidiabetics, and anticancer agents. A study by Najafian and Babji (2019) reported that the peptide sequences of Val-Ala-Ala-Gly-Arg-Thr-Asp-Ala-Gly-Val-His and Lue-Asp-Asp-Pro-Val-Phe-Ile-His showed antioxidant activity with an IC_{50} DPPH value of 1.45 and 0.84 mg/mL and IC_{50} ABTS of

0.80 and 0.62 mg/mL, respectively. Choksawangkarn et al. (2018) also investigated the peptide sequences of PQLLLLLL and LLLLLLL possess antioxidant activity with DPPH values of 59.05 % and 63.39 %, respectively. Fish-derived LK peptide exhibits antioxidant activity (Hamzeh et al., 2020). Another study indicates that fermented fish peptides may reduce ACE by 34.40 to 97.52 % (Rinto et al., 2019). Pro-Lys, Gly-Cys-Lys, Asn-His-Pro, and Asp-Gly-Gly-Pro are peptide sequences shown by Kim et al. (2016) to have ACE inhibitory activity with IC_{50} values of 4092, 178, 1175, and 164 M, respectively. Moreover, Hamzeh et al. (2020) identified the potential peptide sequences of the LF, LL, EV, and AVF as ACE inhibitors. Several studies have reported the potential of bioactive peptides from fermented fish as antibacterial and anti-cholesterol agents in addition to their activity as an antioxidant and ACE inhibitor (Kusmarwati et al., 2014; Rinto et al., 2019). The FPIMGHGSRPA peptide sequences with 12 amino acids have been shown to have antibacterial action against Gram-positive and Gram-negative bacteria (Baco et al., 2022).

Rusip bioactive peptide exploration: conventional vs bioinformatics

Protein, as a source of peptides and amino acids, performs the function of nutrition in food, as well as functional and biological roles that have a favorable impact on health through specific peptides, termed bioactive peptides (Daroit and Brandelli, 2021). Currently, conventional methods (*in vitro* and *in vivo*) are widely employed in investigating bioactivity and prospective bioactive peptides produced from specific protein sources on a scientific and industrial scale (Tejano et al., 2019). This approach is extensively used in the lab to isolate and identify bioactive peptides, where peptides are obtained from the parent protein's cleavages through many trial-and-error procedures (Tadesse and Emire, 2020). This process generally entails choosing protein sources, protein preparation, hydrolyzing proteins using various methods to release peptides, assessing biological activity, fractionating active fractions, and identifying peptide sequences with specific bioactivity, followed by *in vivo* validation of bioactivity (Figure 2) (Daroit and Brandelli, 2021; Neves et al., 2017). To facilitate the generation of bioactive peptides and avoid interference during bioactivity analysis, several additional treatments, including high pressure, microwave, ultrasonication, and innovative techniques based on supercritical fluids, are also utilized as alternatives (Olivera-Montenegro et al., 2021; Ulug et al., 2021).

In numerous investigations of its exploration in *rusip*, the usual approach to identifying bioactive peptides is frequently applied. The original bioactive peptide of *rusip* is investigated in a study by Rinto (2019; 2021) studied bioactive peptide of *rusip* using a classical approach including extraction, fractionation, peptide profiling using SDS-PAGE, and bioactivity assessment. These processes result in a *rusip* bioactive peptide with low molecular weight, antioxidant activity, anti-cholesterol, and antihypertensive characteristics. Table 1 highlights further publications exploring the original bioactive peptide of *rusip* or other similar products using a conventional approach. Despite being a typical method, the conventional approach has numerous weaknesses, including being laborious, time-consuming, expensive, and inefficient since the results are frequently off-goal (Peredo-Lovillo et al., 2022). Thus, several research studies are limited to knowing the biological activity of crude extracts. Current computing developments offer a new approach to detecting chemicals and their actions (Sitanggang et al., 2018). Bioinformatics is a new approach to computational modeling or simulation (Ekins et al., 2007). Since they can identify, characterize, and analyze the functional properties of a bioactive peptide, this approach provides a huge opportunity for various exploration studies and even the discovery of new bioactive peptides from alternative sources (Figure 3) (Holton et al., 2013).

Bioinformatics techniques have been used in several peptide studies, such as one that examined the potency of milk's β-lactoglobulin (Tulipano et al., 2015), prediction of bioactive peptides from milk (Sitanggang et al., 2018), prediction of bioactive peptides from flaxseed (Langyan et al., 2021), a study of the bioactive peptide from *Halophila stipulacea* (Kandemir-Cavas et al., 2019), a study of the bioactive peptide from *Glacilaria changii* (Sharmin et al., 2022), prediction of bioactive peptide *tempeh* (Tamam et al., 2021), and prediction of antimicrobial bioactive peptides produced by *Streptomyces* sp. (Kurnianto et al., 2022). Although it has been used in several studies, the literature review results suggest that *rusip* products or similar fermented fish products have not been studied using a computational approach. Exploratory studies of bioactive peptides from *rusip* or similar fermented fish products might thus be conducted using a bioinformatics approach.

Table 1. Studies related to the exploration of bioactive peptides from Rusip or similar products using conventional approaches

No	Type of Food / Source of protein	Exploration Stages	Biological Activity	References
1	*Budu*	Fermentation – extraction – purification – identification of Q-TOF LC/MS – peptide synthesis	Antioxidant	(Najafian and Babji 2018)
2	*Rusip*	Fermentation – extraction – fractionation – SDS-PAGE profiling – bioactivity evaluation	ACE Inhibitor / Antihypertensive	(Rinto et al.,, 2021)
3	Fish sauce	Fermentation and hydrolysis with K proteinase - extraction – SDS-PAGE profiling – fractionation – LC-MS identification – bioactivity evaluation	Antioxidant	(Choksawangkarn et al., 2018)
4	Thai fish sauce	Fermentation – fractionation – bioactivity evaluation – identification of LC-QTOF-MS	ACE Inhibitor / Antihypertensive and antioxidant	(Hamzeh et al., 2020)
5	*Rusip*	Fermentation – extraction – fractionation – bioactivity evaluation	Antioxidant and anti-cholesterol	(Rinto et al., 2019)
6	Fermented anchovy sauce	Fermentation – fractionation – evaluation of bioactivity – identification of Q-TOF-ESI-MS/MS – peptide synthesis	ACE Inhibitor / Antihypertensive	(Kim et al., 2016)
7	*Bekasam*	Fermentation – extraction – fractionation – bioactivity evaluation	Anti-cholesterol	(Rinto and Hafif, 2017)
8	Fish hydrolysate	Hydrolysis with enzymes – evaluation of bioactivity	Antibacterial	Baco et al., (2021)
9	*Pla Duk Ra*	Fermentation – extraction – fractionation – identification of LC-MS/MS – evaluation of bioactivity	Antioxidant	(Chaijan et al., 2021)

Note: *Budu* (traditional fermented anchovy product from Kalimantan, Indonesia); *Rusip* (traditional fermented anchovy product from Bangka, Indonesia); *Bekasam* (traditional fermented anchovy product from South Sumatera, Indonesia); and *Pla Duk Ra* (traditional semi-dried fermented catfish from Thailand).

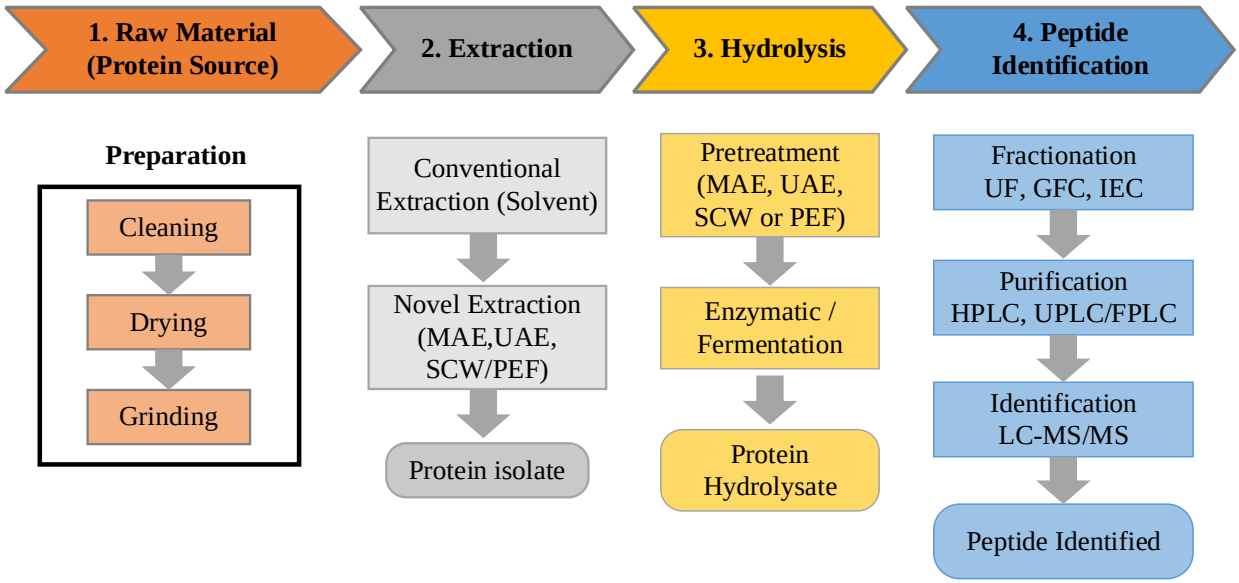

Figure 2. Schematic representation of the exploration of bioactive peptides through conventional approaches

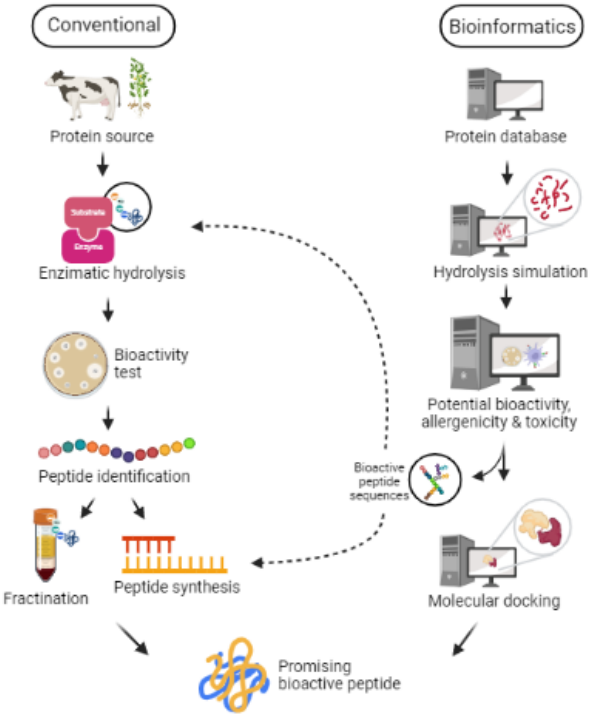

Figure 3. Synergy of conventional and bioinformatics approaches for the generation of the potential bioactive peptides from protein food sources (Figure is made using Biorender.com)

Bioinformatics analysis in the discovery of potential bioactive peptides

The bioinformatics approach is popular since the conventional approach is time-consuming and inefficient. Due to its low cost, use of fewer chemical reagents, and effectiveness of more targeted and efficient discovery/discovery of new bioactive peptides, this approach can be a powerful tool for identifying bioactive peptides (Liu et al., 2019). Furthermore, the bioinformatics approach can be conducted synergistically using a conventional approach to enable more effective analysis or identification of potential bioactive peptide candidates (Figure 3) (Kang et al., 2019). Generally, the bioinformatics approach uses some systems and computational databases such as NCBI, UniProt, BLAST, EXPASY, and BIOPEP-UWM (Tadesse and Emire, 2020). Researchers can use these systems and databases to identify suitable protein and enzyme sources, determine the frequency of occurrence of bioactive peptides, perform protein hydrolysis, predict bioactivity, allergenicity, and toxicity, analyze physicochemical properties and determine the mechanism of action through molecular docking (Peredo-Lovillo et al., 2022; Tadesse and Emire, 2020).

Selection of protein substates and protease enzymes, and simulation of proteolysis

The selection of protein sources and protease enzymes are crucial factors in the bioinformatics study of bioactive peptides (Peredo-Lovillo et al., 2022). The process begins by obtaining the parent protein sequence from a database such as UniProt KB (http://uniprot.org/) or NCBI (https://www.ncbi.nlm.nih.gov/). Some protein databases and bioactive peptides are presented in Table 2. The next stage is a selection of protease enzymes using the BRENDA database (https://www.brenda-enzymes.org/index.php) or even a literature review. The selection of protease enzymes can be based on the presence of enzymes in the human body, such as the digestive process in the GI tract (Chakrabarti et al., 2018; Sitanggang et al., 2018) and enzymes produced by microbes in the fermentation process (Tamam et al., 2018), or addition of enzymes *in vitro* (Han et al., 2019). The Enzyme Action tool on BIOPEP-UWM can be used to simulate the proteolysis process with protease enzymes (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) or the Peptide Cutter tool from EXPASY (https://web.expasy.org/peptide_cutter/) (FitzGerald et al., 2020). These instruments' proteolysis simulation process is based on knowledge about the specificity of cleavage of certain enzymes (Tu et al., 2018).

Table 2. Various databases of bioactive proteins and peptides along with specifications that commonly used in bioinformatics studies

No.	Database	Specification	Website	Ref
1	Uniprot	Protein sequences and universal protein functional information	https://www.uniprot.org/	a
2	Bactibase	Database containing peptide sequences and information on the physicochemical properties of bacteriocin peptides (bacterial production peptides) with antimicrobial activity	https://bactibase.hammamilab.org/	b
3	FermFooDB	Database contains peptide sequences derived from fermented foods	https://webs.iiitd.edu.in/raghava/fermfoodb/	c
4	BioPepDB	Database of food-derived bioactive peptides	http://bis.zju.edu.cn/biopepdbr/	d
5	AHTPDB	Database of experimentally validated Antihypertensive peptides	http://crdd.osdd.net/raghava/ahtpdb/	e
6	MilkAMP	Database that contains valuable information on antimicrobial peptides of dairy origin, including microbiological and physicochemical data.	http://milkamp.hammamilab.org/	f
7	FeptideDB	Database and web application for new bioactive peptide from food protein	http://www4g.biotec.or.th/FeptideDB/	g
8	DBAASP	Database of Antimicrobial Activity and Structure of Peptides	https://dbaasp.org/	h
9	APD3	Database of antimicrobial peptide	https://aps.unmc.edu/	i
10	BIOPEP-UWM	Database of bioactive peptides, especially on these derived from foods and being constituents of diets that prevent development of chronic diseases.	https://biochemia.uwm.edu.pl/en/biopep-uwm-2/	j
11	DFBP	Comprehensive database of food-derived bioactive peptides for peptidomics research	http://www.cqudfbp.net/	k
12	MBPDB	Comprehensive database of milk protein-derived bioactive peptides and novel visualization	https://mbpdb.nws.oregonstate.edu/	l

a(Apweiler et al., 2004); b(Hammami et al., 2010); c(Chaudharyet al., 2021); d(Li et al., 2018); e(Kumar et al., 2015); f(Théolier et al., 2014); g(Panyayai et al., 2019); h(Pirtskhalava et al., 2021); i(Wang et al., 2016); j(Minkiewicz et al., 2019); k(Qin et al., 2022); l(Nielsen et al., 2017).

This stage provides an output in the form of frequency of occurrence and possible peptide sequences that will be formed (Han et al., 2019). Sitanggang et al. (2018) studied the estimation of bioactive peptides from body hydrolyzed milk proteases. The dominant protein sequence of milk is obtained through UniProt, and the hydrolysis stimulation process is conducted through Peptide Cutter EXPASY. Other studies also utilize Uniprot to gain protein sequence from flaxseed, rapeseed, sunflower, sesame, and soybean, and hydrolysis process using the BIOPEP-UWM action tool enzyme as well (Han et al., 2019). The study of bioactive peptides from soybean, the original protein sequence was retrieved from Uniprot, and the enzyme was determined using BRENDA, an enzyme generated by L*actobacillus sp.*, *Rhizopus oligosporus, and Klebsiella pneumoniae.* The hydrolysis process is conducted using Peptide Cutter from EXPASY (Tamam et al., 2021).

Characterization of physicochemical properties of peptides

Peptide sequences can be characterized for their physicochemical properties by using a bioinformatics approach,. This process can be performed using several tools, i.e., PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/) or even ProtParam from EXPASY (https://web.expasy.org/protparam/). Those tools can identify characteristics such as peptide primary structures, sequence length, presentation of the presence of amino acid, molecular weight, isoelectric point, net charge, hydrophobicity, aliphatic index, instability index to estimated half-life (Agyei et al., 2018; Jakubczyk et al., 2020; Syahbanu et al., 2020). Physicochemical

characteristics can be used as a peptide's bioactivity and functional character (Tamam et al., 2018). A study by Wijasekara et al. (2011) informs that hydrophobicity and net charge become the main characteristics of antihypertensive due to their affinity towards ACE (He et al., 2012; Rai et al., 2017).

In another study, high hydrophobicity and positive net charge play crucial roles in antimicrobial peptide due to the interaction of the peptide with the bacterial cell membrane (Kurnianto et al., 2022; Tang et al., 2015), and antioxidant peptides because they are associated with hydrogen donor or acceptor activity (Zhang et al., 2020). Other characteristics correlating with peptide functional properties include sequence length, molecular weight, and isoelectric point. Tamam et al. (2018) reported that antioxidant and antihypertensive peptides have a 7-8 amino acid sequence length, a low molecular weight (800-900 Da), and an isoelectric point between 6-7. Nevertheless, antimicrobial peptides have a 14-15 residue sequence length, a high molecular weight (> 1500 Da), and a greater isoelectric point (9.9). The peptide's structure is also crucial since it relates to its bioactivity. A study carried out by Bhandari et al. (2020) clarifies that most antimicrobial peptide has peptide secondary structure in the form of α-helix. This structure has *a facially amphiphilic* part, which causes the *α-helix* structure easier to interact with the cell membrane and insert its hydrophobic residues into the bacterial cell membrane; thus, it causes damage to the cell membrane (Liang et al., 2020).

Prediction of potential biology activity profile

Based on the relationship between structure, sequence, and function, bioinformatics can also predict biological characteristics and activities from the sequence of peptides or amino acids obtained from the results of computational hydrolysis (Peredo-Lovillo et al., 2022). Several prediction methods for biological activities have been developed. Han et al. (2019) study identified the peptide activities as an ACE inhibitor and dipeptidyl peptidase-IV inhibitor from the oilseed protein using PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/).

Moreover, a study by Ding et al. (2015) found three original peptides of pea protein hydrolysate with a PeptideRanker score greater than 0.5, namely YSSPIHIW, ADLYNPR, and HYDSEAILF. PeptideRanker is one of the tools to predict the probability of peptide bioactivity that works based on a neural network (Garg et al., 2018). Another method developed is usingd 'profile of potential biological activity' tools from BIOPEP-UMW (https://biochemia.uwm.edu.pl/en/biopep-uwm-2/). BIOPEP-UWM has collected 4485 bioactive peptides (accessed July 2022), which are grouped into 44 activity clusters (Dziuba and Dziuba, 2010; Minkiewicz et al., 2019). Currently, BIOPEP-UWM has become a popular tool, particularly for its relation to foods with functional characteristics (Minkiewicz et al., 2019). A study by Devita et al. (2021) reports that according to BIOPEP-UWM, a peptide fraction from tuna skin collagen possesses anti-diabetic, anti-hypertensive, and antioxidant properties. Another study found that tempeh-derived peptides exhibit significant bioactivities such as ACE inhibitors, antioxidants, and antithrombotic (Sitanggang et al., 2020), and the majority of peptides from Giant Grouper have dipeptidyl peptidase-IV and ACE inhibitor activities (Panjaitan et al., 2018).

There are other methods, namely QSAR (Quantitative structure-activity relationship) (Mahmoodi-Reihani et al., 2020). According to Agyei et al. (2018), the prediction of peptide physicochemical parameters and chemometric analyses such as principal component analysis, artificial neural network, and least squares regression are used in this method. In their study, Chen et al. (2018) perform that peptides predicted by the QSAR method are proven to have good antioxidant activity after *in vitro* validation. Similar study has discovered that several hydrophobic, steric, and amino acid positions near the C terminus of a peptide contribute to its ACE inhibitor efficacy (Deng et al., 2017).

Toxicity and allergenicity prediction

Aside from biological activity, the bioinformatics approach may predict the toxicity and allergenicity of a bioactive peptide. This is crucial since these two factors can impede the application of a prospective peptide (Agyei et al., 2018; Peredo-Lovillo et al., 2022). Several bioinformatics tools have been developed to evaluate those parameters, such as ToxinPred (http://crdd.osdd.net/raghava//toxinpred/) and DRAMP (https://zhenghcpu123.pythonanywhere.com/) to predict the toxicity, and AlgPred (https://webs.iiitd.edu. in/raghava/algpred/submission.html), AllergenFP (https://ddg-pharmfac.net/AllergenFP/), and AllerTop (https://www.ddg-pharmfac.net/AllerTOP/) to predict allergenicity (Table 3). Some studies have utilized those tools to analyze allergenicity and toxicity. In a study to predict the toxicity of iron-

chelating peptides from tilapia (*Oreochromis niloticus*) skin collagen conducted using ToxinPred, it is shown that four peptides have potential toxicity properties (Lin et al., 2021). Another study using Toxinpred also reports that peptides from potato, yam, sweet potato, and taro are not toxic (Ibrahim et al., 2019). In allergenicity prediction studies, Wong et al. (2021) claim that 1 of 9 peptides from quinoa seeds shows the possible allergenicity based on the analysis results using the Aller TOP tool. Another study using Allergen FP has evaluated the allergic potential of flaxseed protein, of which 21 of the 23 peptides are likely to be allergic.

Molecular docking

Molecular docking is one of the methods in bioinformatics study, which is crucial to discovering various biological molecules. This method predicts binding mode and ligand affinity in the target receptor active site (Khalesi et al., 2016). Implementing molecular docking offers the possibility of higher output in the selection of bioactive peptides, reducing costs, and offering fast results (FitzGerald et al., 2020). Generally, molecular docking consists of four stages: selection and preparation of protein structures, preparation of ligands, docking, and analysis of results (Figure 4) (Tu et al., 2018). Several softwares can be used to analyze molecular docking, such as AutoDock Vina, AutoDock 4.0, GRIDock, MdockPep, Tag-Dock, Schrodinger, GOLD, DOT, and so on (FitzGerald et al., 2020, Syahbanu et al., 2022). Various studies have used molecular docking in selecting the bioactive peptide from foods to illustrate the biological mechanism, such as the study of antithrombotic peptides from milk proteins (Tu et al., 2018), antimicrobial peptides from milk (Liu et al., 2015), DPP-IV inhibitor peptide from spinach seed protein, ACE inhibitor peptides from kefir and edible rhizomes (Sompinit et al., 2020), and antimicrobial peptides produced by *Streptomyces* (Kurnianto et al., 2021). Although this method has several advantages, *in vitro or in vivo* studies are still required to verify a small number of selected peptides.

Limitations of bioinformatics approaches for bioactive peptide study

The bioinformatics study can be a solution for various shortcomings of study with *in vitro or in vivo* approaches that tend to be laborious, time-consuming, and expensive, especially studies related to the exploration of potential bioactive peptides from *rusip* or similarly fermented fish products (Agyei et al., 2018). Nevertheless, despite the advantages, the bioinformatics approach also has several limitations (Peredo-Lovillo et al., 2022). The first limitation is related to the database, which plays an important role in the analysis process by providing a collection of protein/peptide data ranging from sequences to physicochemical and bioactivity properties (Han et al., 2019). The lack of certain types of proteins or peptides in the database makes bioinformatics analysis difficult or impossible. The second limitation is the hydrolysis simulation tool, which does not include all types of enzymes and does not account for operational parameters such as the effect of pH, total solids of the substrate, temperature, incubation duration, and the effect of ions/salts on enzyme activity (Cheison and Kulozik, 2017). The third limitation is in the molecular docking stage, where the results are sometimes more difficult to comprehend than the experiment itself. This relates to several parameters, including algorithms, flexibility calculations, molecular conformational changes, peptide instability under actual conditions affected by temperature, pH, concentration, crystal structure, protein source properties, and interactions with inhibitory peptides (FitzGerald et al., 2020). The exploration, discovery, and identification of bioactive peptides can benefit significantly from using bioinformatics, despite the approach's numerous limitations (Nongonierma et al., 2017).

Future prospect of bioinformatics approaches for bioactive peptide study

The continuous discovery of bioactive peptides research has proved that these peptides' bioactivity is closely related to their constituent amino acids. Therefore, the selection of appropriate protein sources is a great beginning to discover new bioactive peptides. Discovering bioactive peptides through bioinformatics approaches is very promising because it can save costs, reduce implementation time, and eliminate unexpected factors from conventional methods (Dziuba and Dziuba, 2010; Minkiewicz et al., 2019). Bioinformatics has been used and applied in the biological sciences, especially genomics and proteomics, making it easier to understand the biological basis of disease. The relationship between disease, health, and food is getting more and more attention. Therefore, the application of bioinformatics technology in *foodomics* is currently being researched extensively and involves the analysis of the food and nutrition field through the application and integration of proteomics, transcriptomics, and metabolomics using bioinformatics analysis techniques (Sánchez and Vázquez, 2017).

Table 3. Databases and tools commonly used in bioactive peptide research using a bioinformatics approach

Clustering	Nama database / tools	Function	Website
Proteolysis tools	Peptide Cutter by EXPASY	Identifying potential cleavage sites for certain protease enzymes.	https://web.expasy.org/ peptide_cutter/
	Enzyme Action by BIOPEP-UWM	Identifying potential cleavage sites for certain protease enzymes.	https://biochemia.um.edu.pl/ en/biopep-uwm-2/
Physicochemical characteristics prediction	PepDraw	Predicting peptide properties such as molecular weight, isoelectric point (pI), net charge, and hydrophobicity	http://www2.tulane.edu/~biochem/WW/PepDraw/
	ProtParam by EXPASY		https://web.expasy.org/protparam/
	DBAASP		https://dbaasp.org/tools?page=property-calculation
Bioactivity prediction	BIOPEP-UMW	Predicting proteins/peptides as precursors of bioactive peptides	https://biochemia.uwm.edu.pl/en/biopep-uwm-2/
	PeptideRanker		http://distilldeep.ucd.ie/PeptideRanker/
	APD3	Predicting the probability of bioactivity of a bioactive peptide	http://milkamp.hammamilab.org/ http://zhenghcpu123.pythonanywhere.com/ https://dbaasp.org/tools?page=general-prediction
	MilkAMP	Predicting the bioactivity of proteins/peptides as antimicrobials and their probability of bioactivity	https://aps.unmc.edu/ https://dbaasp.org/tools?page=special-prediction
	DRAMP		
	DBAASP		

Clustering	Nama database / tools	Function	Website
Allergenicity/toxicity prediction	BIOPEP-UWM	Predicting the probability of protein/peptide bioactivity as antimicrobial Predicting the probability of antibacterial activity in general, and specific antibacterial activity against some bacteria Predicting protein allergenicity with its epitope	https://biochemia.uwm.edu.pl/en/biopep-uwm-2/ http://crdd.osdd.net/raghava//algpred/ http://crdd.osdd.net/raghava//toxinpred/ http://zhenghcpu123.pythonanywhere.com/
	AlgPred	Predicting the probability of protein or peptide allergenicity	
	ToxinPred		
	DRAMP	Predicting toxic and non-toxic peptides Predicting hemolytic properties of peptides	
Protein / peptide structure prediction	PEP-FOLD 3	De novo approach aims to estimate the peptide structures from amino acid sequences	https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/ https://zhanggroup.org/I-TASSER/
	I-TASSER	Protein structure prediction and annotation of structure-based function	

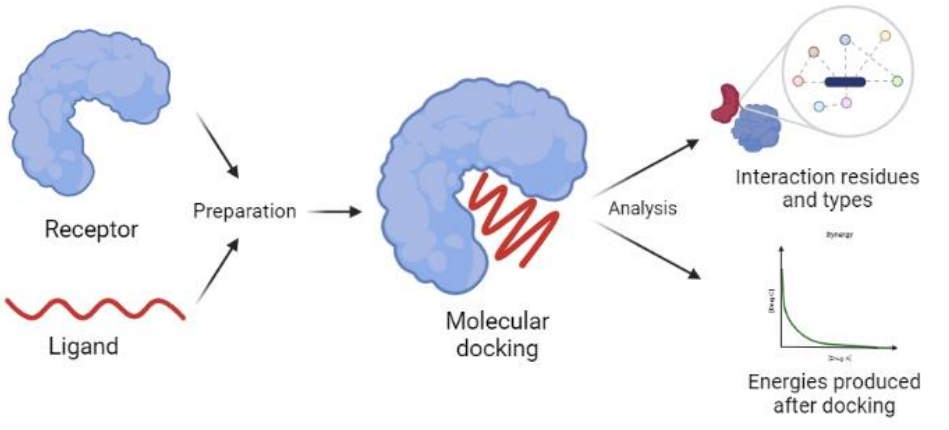

Figure 4. General procedure of molecular docking method (Figure is created using Biorender.com)

Bioinformatics technology has been widely applied to select suitable protein and protease sources from foodstuffs, thus helping researchers to obtain information related to the sequence of peptides after hydrolysis and structural conformation of these peptides, predict their potential activity, obtain an overview of the molecular interaction mechanism (molecular docking), as well as obtain more information related to the characteristics of peptides (Cheison and Kulozik, 2017). In addition, this approach can predict undesirable features, such as peptide bitterness and allergy epitopes, as well as rank various dietary proteins by facilitating the selection of top candidates for bioactive peptides. Further studies are needed to address the challenges discussed in this manuscript to strengthen the use of bioinformatics in the research of *rusip*-derived bioactive peptides (Tadesse and Emire, 2020).

The study method of bioactive peptides integrated with bioinformatics techniques has solved some limitations of conventional research methods. Today, more and more researchers are applying bioinformatics approaches to efficiently produce bioactive peptides that are beneficial to health. In addition, it is important to point out that the biological effects predicted by bioinformatics should be supported based on *in vitro* and *in vivo* studies (Sánchez and Vázquez, 2017). Therefore, combining conventional and bioinformatics approaches, known as hybrid or integrated methods, can quickly obtain new and promising bioactive peptides without wasting time and resources (Peredo-Lovillo et al., 2022).

The bioinformatics approach has never been made in exploratory studies of *rusip* bioactive peptides. The application of this bioinformatics approach accelerated the tie between finding an active hydrolysate of *Rusip* and discovering the peptides responsible for this activity. Several computational systems and databases used in the bioinformatics approach include NCBI, UniProt, BLAST, EXPASY, and BIOPEP-UWM. These systems and databases enable researchers to (1) determine suitable protein and enzyme sources, (2) determine the frequency of occurrence of bioactive peptides, (3) perform protein hydrolysis, (4) predict bioactivity, allergenicity, and toxicity, (5) analyze physicochemical properties, and (6) determine the mechanism of action through molecular docking.

Conclusion

In summary, various conventional approaches (*in vitro*) have been widely carried out in exploring and analyzing the potential and bioactivity of peptides from rusip, such as antioxidants, antihypertensives, and antibacterial. However, this conventional approach has several weaknesses, such as being laborious, time-consuming, expensive, and inefficient because the results are often not on target. Recently, the bioinformatics approach has become one of the promising methods to identify bioactive peptide compounds of fermented fish (such as *rusip* (Indonesian fermented anchovy)) and its activity because it utilizes computational simulation. Several applications in bioinformatic tools are used to explore and analyze the potential of the bioactive peptide from rusip, such as Uniprot, Brenda, ExPASy, PepDraw, BIOPEP, and Autodock. Nevertheless, it is critical to prove the biological effects of peptides predicted by bioinformatics using *in vitro* and *in vivo* studies because bioinformatic methods have several limitations. Therefore, combining conventional and *in silico* methods (hybrid method) is potential to obtain new and promising bioactive peptides from *rusip*.

Declarations

Conflict of interests The authors declare no competing interests.

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