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Research

Novel Bioactive Peptides From Red Bigeye (*Priacanthus macracanthus*) Flesh Protein

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ABSTRACT

Red bigeye (*Priacanthus macracanthus*) is a common fish species in Malaysia. This study reported an in silico assessment of the main proteins in red bigeye flesh as precursors for bioactive peptides. Six major proteins were chosen as precursors from the proteomic profiles of red bigeye proteins. Analyses using the BIOPEP-UWM database found that Protein number 4 gave the highest total number of bioactive peptides (5052 peptides), with dominant bioactivity in angiotensin-I-converting enzyme (ACE) inhibition (1571 peptides) and dipeptidyl peptidase-IV (DPP-IV) inhibition (2238 peptides). The ACE inhibitors had a frequency of bioactive fragment occurrences (A) of 0.4098, while the DPP-IV inhibitors gave a frequency of 0.5805. In silico proteolysis using BIOPEP-UWM found that pepsin (pH > 2) was the most promising proteinase in releasing a high number of DPP-IV and ACE inhibitory peptides. A novel peptide with significant potential was identified as QYKF. This study shows that red bigeye is a potential source of antihypertensive and antidiabetic peptides.

Key words: Bioactive peptides, red bigeye, angiotensin-I-converting enzyme (ACE), dipeptidyl peptidase-IV (DPP-IV), *in silico*

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INTRODUCTION

Red bigeye (*Priacanthus macracanthus*) is an economically important fish species that is intensively exploited in Malaysia. The landing of red bigeye in Malaysia was 15,708 tons in 2021, with a price of RM 5.41/kg (Department of Fisheries Malaysia, 2021). The Priacanthidae family is used in a variety of Asiatic culinary foods, including fish balls and surimi products. The length of red bigeye ranges between 5-34 cm, with an average length of 21-22 cm. Red bigeye between 20-34 cm in length is preferable for surimi production, while smaller sizes (below 15 cm) are categorized as low-value fish. This work aimed to identify the potential bioactivity of small-sized red bigeye fish.

Bioactive peptides derived from seafood protein hydrolysates have emerged as valuable components with significant health benefits and functional properties. These peptides, which consist of short chains of amino acids, are produced through the enzymatic hydrolysis of proteins found in seafood. The biological activities of these peptides, such as antioxidant, antihypertensive, antimicrobial, and anti-inflammatory effects, are attributed to their unique sequences and compositions (Nasri *et al*., 2019). According to Ochiai and Ozawa (2020), fish proteins constitute approximately 50% to 70% of salt-soluble myofibrillar proteins, 20% to 50% of sarcoplasmic proteins, and 3% of insoluble stromal proteins. The proximate composition of flesh from red bigeye

is 17.2% protein, 0.7% fat, 79.8% moisture, 1% carbohydrate, and 1.3% ash.

Recent research has shown that enzymatic hydrolysis, as reported by Malagón-Rojas *et al*. (2020), remains a widely employed technique for releasing these bioactive peptides. The antihypertensive properties of these peptides are particularly noteworthy, as they have been shown to inhibit angiotensin-converting enzyme (ACE), which plays a crucial role in regulating blood pressure (Erdmann *et al*., 2018). Additionally, seafood-derived peptides have exhibited antimicrobial activity against a broad spectrum of pathogens, making them potential candidates for use as natural preservatives in food products (Kim & Wijesekara, 2021).

In this study, the researchers investigated the potential of red bigeye flesh proteins as precursors of bioactive peptides using an *in silico* approach. Various bioactive peptides including ACE inhibitory activity, DPP-IV inhibitors, opioids, immunostimulants, antimicrobials, antithrombotic, hypercholesterolemic, and antioxidative peptides, have been explored using BIOPEP-UWM as reported by Marciniak *et al*. (2018). While *in silico* analyses of bioactive peptides from other sources like tilapia and salmon fin have been conducted, no such study has been reported for red bigeye flesh peptides, making this research a significant contribution to understanding potential bioactive compounds with natural, mild, and safe effects on human health.

MATERIALS AND METHODS

Materials

Small-sized red bigeye (*Priacanthus macracanthus*) was purchased from the Fisheries Development Authority of Malaysia (LKIM) jetty, located at Pulau Kambing, Terengganu. The whole fresh fish was carefully transported in an ice-filled box to the laboratory. Upon arrival, the fish samples were thoroughly cleaned by rinsing with tap water. Subsequently, the fish underwent immediate degutting and further cleansing with ample cool water, maintaining the fish temperature below 5°C throughout the process using ice. After cleaning, the fish were filleted, homogenized, and then stored at -20°C for future use. In this study, only chemicals and reagents of analytical grades were used. To facilitate subsequent analysis, the frozen red bigeye samples were thawed and pre-minced into smaller pieces.

Protein extraction

Protein extraction from fish flesh was performed as described by Shaviklo (2013). Initially, the fish flesh was minced and combined with an equal volume of deionized water at 23°C. The mixture was homogenized in a high-speed blender (Kenwood, Malaysia) for 2 min until the flesh was thoroughly mashed. Next, the homogenized sample was transferred into a beaker containing 30 mL of deionized water and heated on a hot plate stirrer (IKA, Malaysia) at 85°C while continuously stirred for 60 min. After heating, the mixture was poured into a 50 mL Falcon tube and centrifuged at 2560 × *g* for 15 min at 4°C using a swing bucket centrifuge (Thermo Fisher, USA). The resulting supernatant was carefully collected and stored at -80°C, before undergoing lyophilization (Labconco, USA).

Protein quantification

The Pierce™ 660 nm Protein Assay (Thermo Scientific, USA) was utilized to determine the total protein quantification of fish hydrolysate samples. Samples were prepared in a deionized water buffer at a concentration of 2.0 mg/mL. A standard curve was generated by diluting bovine serum albumin (BSA) to various concentrations ranging from 0 to 2 mg/mL, and each standard and sample (5 µL) were triplicated and injected into the wells of a microplate. To these wells, 150 µL of Pierce reagent was added, and the contents were thoroughly mixed. The microplate was then incubated at 25°C for 10 min using a SpectraMax® M5 instrument, and subsequently, absorbance readings were taken at a wavelength of 660 nm using SoftMax Pro software V5 (Molecular Devices, USA).

In-solution trypsin digestion

The *in-solution* trypsin digestion was performed as outlined by Kinter and Sherman (2005). Initially, a 100 µL portion of the protein sample solution containing 1 mg of total protein was combined with 6 M urea and 100 mM Tris buffer. After adding 5 µL of the reducing reagent and 125 µL of Tris stock, resulting in final concentrations of 200 mM Dithiothreitol and 100 mM Tris, the protein mixture was reduced for 1 hr at room temperature. Subsequently, 20 µL of the alkylating reagent and 125 µL of the Tris stock were added and mixed gently using a vortex, and the protein mixture was subjected to alkylation for 1 hr at room temperature. The urea concentration was reduced by diluting the reaction mixture with 775 µL of water, followed by gentle vortex mixing. To initiate the digestion, trypsin stock solution (200 mg/µL in

Tris solution) was added to achieve a sample-to-trypsin ratio of 50 to 1. The sample was gently mixed with a vortex and left for overnight digestion at 37°C. After completion, 2 drops of concentrated acetic acid were mixed gently using a vortex mixer, and the mixture was finally concentrated using a vacuum concentrator.

LC-MS/MS analysis

LC-MS/MS analysis was carried out as outlined by Kinter & Sherman (2005). The peptides were reconstituted in 50 µL of 0.1% formic acid in deionized water and then filtered using 250 µL polypropylene LC vials (Agilent, USA) with a 0.2 µm RC-membrane filter (Sartorius, Germany) syringe filter. For the LC-QTOF-MS analysis, 25 µL of the sample was introduced via an auto-sampler. The separation was carried out on a Hypersil Gold C18 column (C18, 2.1 x 150 mm, 3 µm particles) (Thermo Scientific, USA) using two different mobile phase buffers, and the flow rate was set to 15 µL/min. The mass spectrometry acquired a mass range of m/z 100-2000 for both the MS and MS/MS studies. During fragmentation, a maximum of 4 most abundant precursors were selected per cycle.

Protein identification and data analysis using PEAKS Studio

In this study, PEAKS Studio Version 7.5 (Bioinformatics Solution, Waterloo, Canada) was utilized for both de novo sequencing and database matching. The UniProt database from March 2016 served as the reference for the database matching process. The LCMS/MS raw data was uploaded into the PEAKS software to generate de novo peptide sequences. The analysis parameters were set according to the guidelines outlined by Kwan *et al*. (2016). Specifically, the following post-translational modification parameters were fixed: Oxidation, Carbomethylation, and Acetylation, with a maximum of 2 allowed missed cleavages. The database for Bony fish (Osteichthyes) was used, and the precursor mass tolerance was set at 50 ppm, while the monoisotopic mass for fragment ions was set at 0.8 Da. For protein acceptance, the study employed a False Detection Rate (FDR) of at least 1% and a De-novo confidence rate of at least 20%. The PEAKS PTM feature was employed to search for available PTMs. Additionally, to ensure accurate protein identification, the average local confidence (ALC) threshold was set at >15%.

Protein sequence of red bigeye flesh

Protein identification via LC-MS/MS analysis and subsequent evaluation with PEAKS software involved matching the acquired sequences against entries in the Universal Protein Knowledgebase (UniProtKB) library database (http://www.uniprot.org). The protein sequences in FASTA format, along with the general characteristics of the proteins were collected from the UniProt database.

Profile of potential bioactive peptide from red bigeye

In the investigation of potential bioactive peptides from red bigeye, the BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) (Minkiewicz *et al*., 2011) was employed. To accomplish this, the chosen protein from UniProt underwent an examination using the "profiles of potential biological activity" tool provided by BIOPEP-UWM. This option enables the retrieval of vital information, including BIOPEP ID, peptide names, their potential activities, the number of peptides, and their respective positions in the protein sequences. Furthermore, the frequency of occurrence of fragments with a specific activity (A) within the selected protein was calculated using the specified equation.

$$A = a/ N$$

Where a = number of bioactive peptides and N = total number of amino acid residues in the protein chain.

The BIOPEP-UWM website not only offers information on the frequency of bioactive fragment occurrences (A) in the protein sequence of red bigeye but also provides the total frequency of fragments displaying all biological activities present in food sequences available in the database (∑A). Furthermore, the potential biological activity (B) of the protein is also described on the BIOPEP-UWM website.

***In silico* proteolysis to release ACE and DPP-IV inhibitory peptides**

BIOPEP-UWM can predict the most effective enzyme to generate the bioactive peptides via *in silico* proteolysis by using the 'enzymes action' tool. To determine the most effective proteases to generate ACE and DPP-IV inhibitory peptides from red bigeye protein, the *in silico* proteolysis application in the

BIOPEP-UWM database was employed. Six proteases were selected for evaluation. The single enzyme and combinations of 2 or 3 enzymes were applied to compare the production of ACE inhibitory peptide fragments. The efficiency of the released bioactive fragments was assessed based on the frequency of release of peptides with a given activity by the chosen enzymes (AE) and the relative frequency of release of peptides with a given activity by the selected enzyme (W).

$A_E = d/N$
$W = A_E/A$

In the context of evaluating the efficiency of released bioactive fragments, "d" represents the number of fragments with a given activity in the protein sequence that may be released by enzymes, while "N" stands for the total number of amino acid residues present in the protein chains.

Prediction of toxicity and allergenicity

To evaluate the potential toxicity of peptides derived from red bigeye protein, the ToxinPred tool (http://crdd.osdd.net/raghava/toxinpred) was employed. ToxinPred identifies toxic peptides by analyzing various motifs present in the sequences and utilizes this motif information for prediction purposes, as described by Gupta *et al*. (2013). Additionally, for the analysis of allergenicity in the red bigeye protein, the bioinformatic tool AllerTop (https://www.ddg-pharmfac.net/AllerTOP) was utilized.

Characterization of novel tripeptide and tetrapeptide

The bioactive fragments released from the red bigeye protein, along with their known activities, were manually counted with the help of BIOPEP-UWM, which provided fragments with known bioactivities available in its database. The majority of the reported sequences in BIOPEP-UWM were di- or tripeptides, known for their ACE and DPP-IV inhibitory activities. To explore potential novel peptides, tripeptides, and tetrapeptides were screened using the BIOPEP-UWM database to identify any previously unreported sequences. Peptide Ranker (http://distilldeep.ucd.ie/PeptideRanker/) was then used to rank the fragments with three and four amino acids for their potential activity, with peptides having a peptide rank higher than 0.7 being selected.

The resulting bioactive peptides with tripeptide and tetrapeptide sequences from the red bigeye protein were listed, and two enzymes with higher degrees of hydrolysis were selected for further analysis. *In silico* analysis was conducted to determine the potential characteristics of the peptides, including water solubility, resistance to digestion, toxicity, allergenicity, and IC50. PepCalc (http://pepcalc.com) was used to predict water solubility, while PeptideCutter (https://web.expasy.org/peptide_cutter) was employed to predict gastrointestinal digestion. Toxicity and allergenicity assessments were performed using bioinformatics tools ToxinPred (http://crdd.osdd.net/raghava/toxinpred) and AllerTOP (https://www.ddg-pharmfac.net/AllerTOP), respectively. For toxicity prediction, the SVM-based method with a threshold value of 0.0 was chosen, following Lafarga *et al*. (2014). The predicted IC50 was estimated using AHTpin (http://crdd.osdd.net/raghava/ahtpin), an *in silico* tool specialized in predicting, screening, and designing antihypertensive peptides.

RESULTS AND DISCUSSION

Total soluble protein concentrations of red bigeye sample

The red bigeye sample gave a total soluble protein concentration of 23.72±0.015 mg/mL.

Analysis of sequence and amino acid of red bigeye

LC-MS/MS analysis provided the protein sequence of red bigeye (*Priacanthus macracanthus*) flesh. Among the identified proteins, six of them were selected for *in silico* evaluation, and their details are presented in Table 1. The proteins that gave the highest confidence level were chosen. Notably, protein number 1 (myosin heavy chain) exhibited the highest confidence level among the other proteins The protein sequences were retrieved from the red bigeye library in the UniprotKB database. The results from ToxinPred and AllerTop bioinformatic tools, as shown in Table 1 indicate that all peptides were determined to be nontoxic and non-allergenic, relevant information such as the name of the protein, Uniprot accession number, amino acid residues, and protein functional group of the selected proteins is provided.

Table 1. The proteins that were chosen for analysis were identified through LC-MS/MS using the PEAKS Studio database

Protein Number	Proteins ID	Accession number	Proteins name	-10l gP Confidence level	Coverage (%)	Protein functional group	Molecular mass (kDa)	Species	Toxicity	Allergenicity
1	290065	A0A671XP17_SPAAU	Myosin heavy chain, fast skeletal muscle-like	233.29	26	Regulatory	221.74	*Sparus aurata* (Gilthead sea bream)	Non-Toxin	Non-allergen
2	292855	A0A1A8MMV6_9TELE	Myosin heavy chain 13, skeletal muscle	230.47	32	Regulatory	181.85	*Nothobranchius pienaari*	Non Toxin	Non allergen
3	290054	G0YU49_SINCH	Fast skeletal muscle, myosin heavy chain isoform 2	229.48	24	Regulatory	221.56	*Siniperca chuatsi* (Mandarin fish)	Non-Toxin	Non-allergen
4	290084	A0A5C6NSV1_9TELE	Myosin heavy chain, fast skeletal muscle	227.25	13	Regulatory	448.59	*Takifugu flavidus* (sansaifugu)	Non Toxin	Non-allergen
5	290133	A0A665XCQ9_ECHNA	Myosin heavy chain, fast skeletal muscle	224.03	25	Regulatory	223.55	*Echeneis naucrates* (Live sharksucker)	Non-Toxin	Non-allergen
6	290108	A0A3Q1AK85_AMPOC	Myosin heavy chain, fast skeletal muscle	223.33	26	Regulatory	220.63	*Amphiprion ocellaris* (Clown anemonefish)	Non-Toxin	Non-allergen

The frequency of peptide fragments (A) and potential biological activity (B)

The BIOPEP database provides essential information, including values of A, ∑A, and B, as well as lists of potential bioactive peptides identified from the protein sequences of red bigeye. The analysis of potential biological activity for each selected protein served as a valuable parameter for quantitatively predicting protein sequences that could act as precursors to bioactive peptides. Moreover, the BIOPEP database enabled the determination of the number of bioactive peptides that might be released from proteins, and the biological activities of 23 subclasses were presented in Table 2(a) and 2(b) for the red bigeye proteins. The frequency of bioactive peptides (A) played a significant role, being closely linked to the biological activity of each peptide fragment. Higher A values were indicative of a greater likelihood of bioactivity occurrence within the protein.

Based on the A values, Table 2 displays the bioactivities of red bigeye flesh protein. Notably, among the proteins, protein no. 5 (myosin heavy chain) showed the highest bioactivity (∑A = 1.3358) with the highest A value attributed to ACE inhibitor activity (A = 0.4208). Additionally, for DPP-IV, protein no. 3 had the highest A value (A = 0.5875). Moreover, BIOPEP-UWM encompasses 48 major classes of peptide bioactivity, and an analysis of the red bigeye protein sequences using BIOPEP-UWM revealed 23 subclasses of potential bioactivities (B) as presented in Table 3. Interestingly, among these 23 subclasses, five were found in all six red bigeye protein sequences, including ACE inhibitor, dipeptidyl peptidase IV (DPP IV) inhibitor, dipeptidyl peptidase III (DPP-III) inhibitor, antioxidative, and stimulating activities.

Notably, protein no. 4 exhibited the highest number of peptides, with 5052 peptides showing ACE inhibitory activity (1571 fragments) and DPP-IV inhibitory activity (2238 fragments), surpassing the other proteins (Table 2(b)). This suggests that the red bigeye protein contains a considerable amount of DPP-IV and ACE inhibitory bioactive peptides. A previous investigation conducted by Borawska-Dziadkiewicz *et al*. (2021) found that salmon collagen and myofibrillar proteins of carp are the best potential sources of ACE inhibitors and antioxidant peptides. According to Table 3, the most potent bioactivity in red bigeye proteins, based on the B value, was ACE inhibitor (ranging from 0.0626 to 0.0749), followed by DPP-IV inhibitor (ranging from 0.0002 to 0.0003), while DPP-III inhibitor and stimulating activities had no assigned values.

Table 2. The frequency of bioactive fragment occurrence in the red bigeye protein chain (A) was examined

Proteins number	∑A	The frequency of occurrence of a bioactive fragment in the protein chain (A)				
		ACE inhibitor	Dipeptidyl peptidase IV Inhibitor	Dipeptidyl peptidase III Inhibitor	Antioxidative	Stimulating
1	1.3172	0.4104	0.5839	0.0759	0.0656	0.0501
2	1.2817	0.4126	0.5621	0.0662	0.0631	0.0524
3	1.3348	0.4177	0.5875	0.0774	0.0697	0.0485
4	1.3147	0.4098	0.5805	0.0735	0.0701	0.0477
5	1.3358	0.4208	0.5771	0.0774	0.0743	0.0477
6	1.3310	0.4197	0.5777	0.0767	0.0710	0.0508

The study assessed both the frequency of bioactive fragment occurrence in the red bigeye protein sequence (A) and the total frequency of fragments exhibiting all biological activities in food sequences available in the BIOPEP database (∑A).

***In silico* proteolysis to release ACE and DPP-IV inhibitory peptides**

The *in silico* proteolysis revealed that pepsin (pH > 2), bromelain, and papain could release relatively larger quantities of bioactive peptides (Tejano *et al*., 2019). The red bigeye proteins generated ACE and DPP-IV inhibitory peptides with a degree of hydrolysis (DHt%) ranging from 29.91% to 77.99% through *in silico proteolysis* using the enzymes (Table 4). Figure 1 presents the range of degree hydrolysis (DHt%) for releasing bioactive peptides from red bigeye proteins using single and combinations of enzymes, showing values ranging from 29.86% to 92.30%. The highest degree of hydrolysis value

(92.3%) was achieved with the combination of pepsin (pH > 2) and papain. In Figure 1(b), the predicted frequency of releasing ACE and DPP-IV inhibitory peptides from selected red bigeye proteins using single and combinations of enzymes is displayed. For ACE inhibitory peptides, the combination of papain with chymotrypsin C showed the highest A_E value (0.0712), while for DPP-IV inhibitory peptides, the combination of papain with proteinase K had the highest AE value (0.1156).

The frequency of releasing ACE inhibitory peptides showed that for single enzyme proteolysis, pepsin (pH > 2) gave the highest AE value (0.0609), followed by stem bromelain and papain (0.0594 & 0.0470, respectively). For the combination of two enzymes, the combination of papain with chymotrypsin C showed the highest AE ACE inhibitory value (0.0712), while the combination of papain with thermolysin gave the lowest value (0.0465). Among the three-enzyme combinations, papain, pepsin, and chymotrypsin C gave the highest AE ACE inhibitory value (0.0485) compared to the other combinations.

Table 3. BIOPEP-Profiles, the total number of potential bioactive peptides identified from red bigeye, along with their corresponding potential biological activity (B)

Proteins Number	Number of activities	Number of peptides and potential bioactivity value (B)						
		ACE inhibitor	Dipeptidyl Peptidase IV Inhibitor	Dipeptidyl peptidase III Inhibitor	Antioxidative	Stimulating	Other activities	Total Activities
1	22	781	1113	145	120	94	254	2507
		(0.0641)	(0.0002)	(0.0000)	(0.0000)	(0.0000)		
2	22	642	879	105	96	81	196	1999
		(0.0749)	(0.0002)	(0.0000)	(0.0000)	(0.0000)		
3	22	802	1119	150	128	93	259	2551
		(0.0632)	(0.0002)	(0.0000)	(0.0000)	(0.0000)		
4	22	1571	2238	284	262	181	516	5052
		(0.0626)	(0.0003)	(0.0000)	(0.0000)	(0.0000)		
5	22	808	1108	149	139	92	266	2562
		(0.0638)	(0.0003)	(0.0000)	(0.0000)	(0.0000)		
6	23	802	1094	146	132	96	254	2524
		(0.0641)	(0.0003)	(0.0000)	(0.0000)	(0.0000)		

The potentials of biological activity values (B) for specific activities were rounded off to four decimal places.
*Other activities: activating ubiquitin-mediated proteolysis, alpha-glucosidase inhibitor, anti-inflammatory, antiamnestic, antibacterial, anticancer, antithrombotic, bacterial permease ligand binding, CaMPDE inhibitor, HMG-CoA reductase inhibitor, hypolipidemic, hypotensive, immunomodulating, immunostimulating, neuropeptide, regulating, renin inhibitor.

Table 4. The frequency of peptide release with a specific activity by selected enzymes (AE) and the relative frequency of peptide release with a given activity by the selected enzyme (W) from the chosen red bigeye proteins was determined using *in silico* hydrolysis

Protein number	Enzymes	DH_t (%)	ACE inhibitory		Dipeptidyl peptidase IV Inhibitory	
			A_E	W	A_E	W
1	Papain	38.220	0.0470	0.1147	0.0831	0.1423
	Proteinase K	29.855	0.0181	0.0442	0.0351	0.0601
	Thermolysin	32.954	0.0305	0.0744	0.0387	0.0663
	Stem bromelain	45.868	0.0594	0.1449	0.0811	0.1389
	Chymotrypsin C	40.806	0.0428	0.1044	0.0609	0.1043
	Pepsin (pH > 2)	77.996	0.0609	0.1486	0.0960	0.1644
3	Papain	37.706	0.0501	0.1199	0.0883	0.1503
	Proteinase K	29.907	0.0201	0.0481	0.0367	0.0625
	Thermolysin	33.264	0.0315	0.0754	0.0423	0.0720
	Stem bromelain	45.868	0.0594	0.1422	0.0790	0.1345
	Chymotrypsin C	40.909	0.0444	0.1063	0.0573	0.0975
	Pepsin (pH > 2)	78.203	0.0594	0.1422	0.0965	0.1643
4	Papain	38.291	0.0477	0.1164	0.0814	0.1402
	Proteinase K	29.592	0.0191	0.0466	0.0342	0.0589
	Thermolysin	33.214	0.0303	0.0739	0.0423	0.0729
	Stem bromelain	46.097	0.0599	0.1462	0.0788	0.1357
	Chymotrypsin C	40.332	0.0390	0.0952	0.0538	0.0927
	Pepsin (pH > 2)	78.036	0.0604	0.1474	0.0979	0.1686

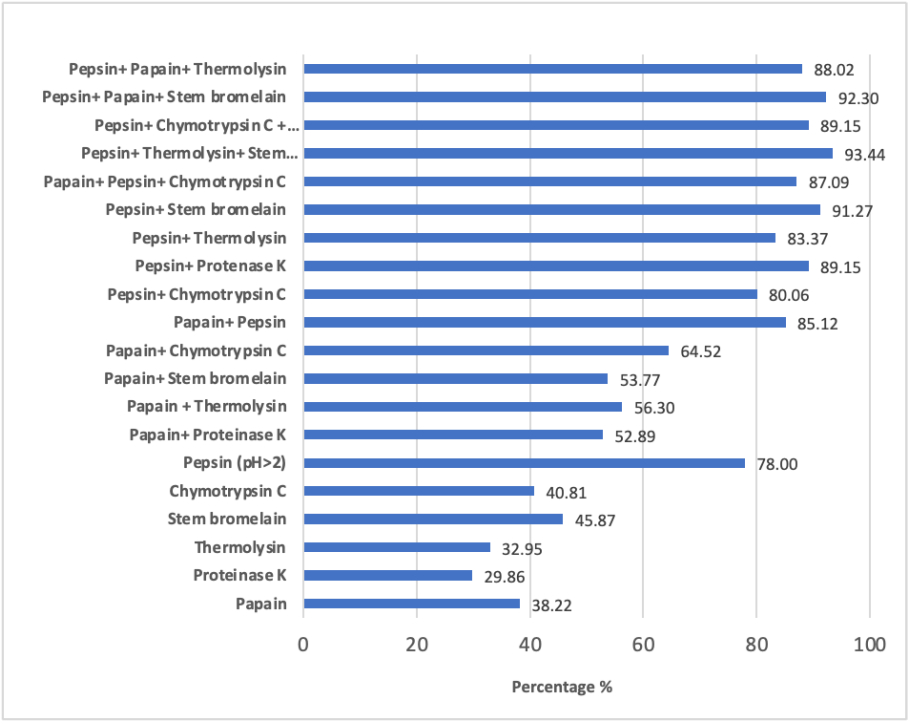

Fig. 1. Degree hydrolysis to release bioactive peptides from selected red bigeye proteins (1) with selected single and combination of enzymes by *in silico* hydrolysis.

For DPP-IV inhibitory peptides, pepsin (pH > 2) had the highest A_E value (0.0960) in single enzyme proteolysis, followed by papain (0.0831), stem bromelain (0.0811), chymotrypsin C (0.0609), thermolysin (0.0387), and proteinase K (0.0351). In the combination of two enzymes, the combination of papain with proteinase K had the highest AE DPP-IV inhibitory value (0.1156). Among the three-enzyme combinations, the combination of papain, pepsin, and chymotrypsin C showed the highest AE DPP-IV inhibitory activity (0.0650) (Figure 2).

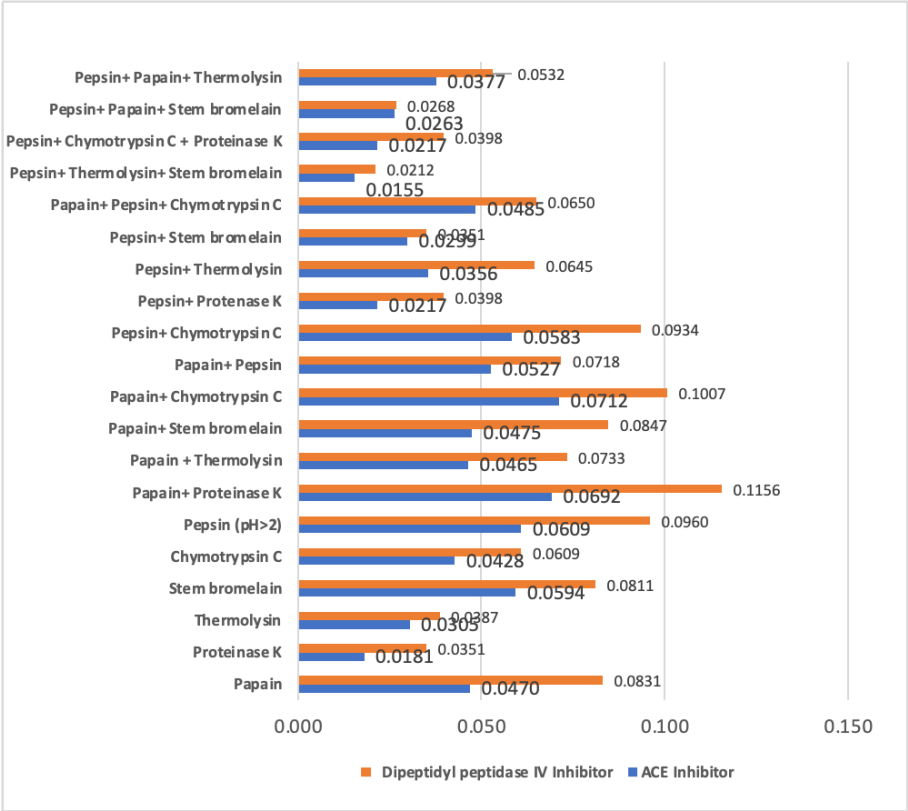

Fig. 2. ACE inhibitory and DPP-IV inhibitory frequency (AE) in red big eye protein (1) sequence using single and combination of enzyme

Dipeptidyl peptidase IV (DPP-IV) inhibitors are generally characterized by short peptide sequences, often comprising fewer than five amino acids, according to Bao *et al*. (2020). The combination of enzymes proved to be highly effective in releasing ACE inhibitory peptides from the red bigeye protein, resulting in the highest AE ACE inhibition. Interestingly, enzyme combinations have shown the potential to yield more ACE inhibitory peptides compared to using a single enzyme, as reported by Lin *et al*. (2018). However, it is worth noting that Tu *et al*. (2018) reported a lower number of ACE inhibitory peptides when employing a combination of three enzymes compared to using just two enzymes (papain and pepsin). Additionally, extensive proteolysis may lead to the release of peptides with no bioactive properties, as observed by Sousa *et al*. (2019).

Table 5 presents the results of in-silico proteolysis, displaying the predicted ACE and DPP-IV inhibitory peptides released from red bigeye proteins. The majority of the reported peptides with these activities were found to be dipeptides and tripeptides. In the *in silico* analysis, pepsin (pH > 2) and stem bromelain were identified as enzymes capable of releasing bioactive peptides (Table 5). Among these enzymes, pepsin (pH > 2) yielded the highest number of peptide sequences for DPP-IV inhibitors, a total of 186 peptides with a degree of hydrolysis of 77.99% and displaying a total of 400 activities. On the other hand, stem bromelain produced the highest number of peptide sequences for ACE inhibitors, resulting in 112 fragments. As part of the screening process, novel peptides with tripeptide and tetrapeptide potential were also investigated. Pepsin (pH > 2) yielded a total of 48 fragments, while stem bromelain produced 179 fragments for tripeptides and tetrapeptides (Table 6).

Table 5. *In-silico* proteolysis, the release of bioactive peptides with ACE inhibitor and DPP-IV inhibitor activities from the red bigeye protein was predicted

Protein	Enzyme	DHt (%)	ACE inhibitors	DPP-IV inhibitors	Other Activities	Total Fragments
Myosin heavy chain.	Pepsin (pH >2)	77.99	107	186	107	400
			RY (2) IY (4) VF (1) VY (2) PR (1) PT (1) PL (3) VK (15) PQ (1) IA (5) RA (7) HK (1) IF (4) VG (2) VM (2)IG (3) HL (4) IL (3) SG (3) PG (3) WM (1) PPK (1) SY (2) WL (2) SF (1) RR (1) RG (4) IE (12) VE (12) ST (4)	VA (9) HA (2) IA (5) RA (7) HL (4) SL (6) WRT (1) PL (3) WK (1) WL (2) WM (1) HD (1) HE (3) HF (1) HT (3) IL (4) IM (1) IN (4) IQ (6) IR (3) PF (1) PG (3) PK (2) PM (2) PQ (1) PT (1) PY (2) RG (4) RH (1) RK (8) RL (8) RM (2) RN (3) RR (1) SF (1) SK (7) SY (2) VD (7) VE (12) VF (1) VG (2) VK (15) VL (7) VM (1) VN (8) VQ (10) VT (4) VY(2) PA (1)		
	Stem bromelain	45.86	112	157	97	366
			MF (1) YL (3) YG (2) IA (3) DF (2) KR (3) YA (2) IF (1) IG (2) HL (3) KG (6) DA (9) QG (4) EG (5) EA (8) NG (2) PG (1) MKG (1) NKL (2)DG (1) KL (10) NF (1) EV (8)KF (1) YV (1) KA (11) IL (2) DF (2) EQR (1) WL (1) ER (10) EF (2) DR (2) YNL (1)	KA (11)PA (1) HA (2) IA (3) HL (3) WL (1) DR (2) EG (5) ES (6) ET (6) EV (8) HF (1) HS (1) HT (1) HV (1) IL (2) IR (3) KF (1) KG (6) KR (3) KS (5) KT (7) KV (8) MF (1) MV (2) ML (1) NA (5) MR (1) NG (2) NF (1) NL (3) NR (2) NT (3) NV (1) QA (7) PF (1) QF (2) PG (1) QG (4) PS (1) QL (12) PV (1) QS (1) QT (4) QV (3) YA (2) YF (1) YG (2) YL (3) YR (1) YS (1) YV (1)		
	Chymotrypsin C	40.80	76	118	50	244
			RY (2) IY (1) KW (1) VY (1) FP (1) GY (1) AY (2) GP (1) GW (1) IP (1) AP (1) GL (4) HL (2) GQ (3) GE (5) SY (1) KY (2) KL (11) KP (3) IE (5) VE (3) TE (8) TQ (1) KE (10) DY (1) DM (2) FQ (1) VM (1)	GP (1) AP (1) KW (1) TY (3) IP (1) FP (1) KY (2) VE (3) KP (3) FL (3) RL (7) VM (1) HL (2) AL (7) RM (2) VN (3) SL (2) GL (4) RN (3) VQ (4) AE (16) AY (2) SY (1) VY (1) DN (1) DQ (3) TE (8) FQ (1) GE (5) TL (4) GW (1) GY (1) TM (1) HE (3) IN (2) TN (1) IQ (2) KE (10) TQ (1)		

Other activities: The red bigeye protein sequence was found to contain other bioactive peptides with various activities, including peptide regulating the stomach mucosal membrane activity, beta-lactokinin, antithrombotic peptide, prolyl endopeptidase inhibitor, peptides from soybean protein isolates: beta-conglycinin and glycinin, synthetic peptides, antioxidative peptides, CaMPDE inhibitor, renin inhibitor, glucose uptake stimulating peptide, stimulating vasoactive substance release, DPP-III inhibitor, alpha-glucosidase inhibitor, anti-inflammatory peptide, regulator of phosphoglycerate kinase activity, immunostimulating peptide, anxiolytic peptide, calcium binding peptide, kyotorphin, hypolipidemic peptide, and wheylin-2.

Table 6. Tripeptide and tetrapeptide sequence released using pepsin (pH > 2) and stem bromelain

Protein	Enzyme	DHt (%)	Tripeptide	Tetrapeptide	Total Fragments
Myosin heavy chain.	Pepsin (pH > 2)	77.99	PPK, SWM, PPH, ISD, VRN, IHF, RSY, VPQ, VSA, IWE, RST, IPN, IHQ, RCN, PSR, IPD, CRG, RRE, RCE, CSE, SHQ, SRE, ISE, RRD, SSN, SRQ, VSQ, RHD, RSL, ISD, VRG, RRN, HSQ, SRN, HRL, VRE, RRG, VRK, SRL, SRD, VCL	SSRF, IHHG, HPHF, VRCL, ICRK, IRSF, SSHL	48
	Stem bromelain	45.86	EPT, DCG, MMT, YNL, KER, YDT, KKR, IEA, DNQ, YQF, KKS, NPL, IHF, EKS, YQL, EIT, EEF, MKF, EEA, DML, PQV, NNS, CKS, KQA, DIA, EIF, EQL, QQF, NHT, IDF, MDL (2), PKA, HYA, NDS, NKL, MPG, KKG, ENL, CNG, ICR, KQR, YKV, EKL, IDV, DHT, KEF, IYS, DNL, EER, CEG, IKS, KET, KKR, KNL, QES (3), DDL (2), EEA (3), KKR, QKL, EES, NDV, QKA, ENG, KQA, KNA, EQF, QWR, IQR, EEL, EES (2), QDA, EES, KCA, EKT, KQR, EDL, DKV, DHL, MKR, EKA, KKT, EKS, EEA, KIL, IDS, MQS, NDA, KDA, DDA (2), DQT, QNT, MNT, KKA, MMA, EEL, KDL, QHR, DEA, ENL, MKG, EQR, KEL, YQT, QDL, DKL, YKR, HMS, QER, DIA, NKL,	EMYL, KKEG, ENQS, IQYF, KIKG, NDNS, DIET, YHIF, IDDV, IDIL, EEKL, IYKL, IHHG, EEQA, EPDG, DKIS, CYPR, NEMV, CINF, NEKL, CIEL, KNKL, KKGG, HPHF, MENF, IHQL, IPDG, QYKF, EEMR, DEKL, EKEL, KIQL, EEMA, QDES, HQQT, EQQV, IMDL, EKQR, EEIS, QHEA, KKQA, KQKL, NMEA, EDQL, EEEV, KYET, KKKL, MIDV, EKEA, YEEA, EQIG, IHEL, EIQS, QKQL, EQEL, KKKL, EEKA, KKQL, KYER, EEQA, QHEF,	179

Characterization of novel peptides

Through the *in silico* analyses conducted in this study, it was revealed that the sequences of ACE inhibitors and DPP-IV inhibitors generated by the BIOPEP-UWM database primarily consisted of only two amino acids. To explore potential novel peptides, tripeptides and tetrapeptides were specifically selected based on a peptide ranker score >0.7 (Table 7). Among the screened peptides, SSRF and HPHF (from pepsin pH > 2) and IQYF, IHF, MMA, YHIF, WMV, CINF, and QYKF (from stem bromelain) were identified as novel peptides and were predicted to be released by the proteolysis of specific enzymes according to their Peptide Ranker scores. Notably, MMA (0.825), WMV (0.893), and YHIF (0.770) stood out as the most promising novel peptides, as they exhibited non-toxic and non-allergenic activities. However, the scores for the other peptides were <0.7. Both MMA and WMV peptides displayed poor solubility in water due to their hydrophobic residues. Interestingly, MMA demonstrated resistance to digestion, indicating that enzymes like chymotrypsin, pepsin, and trypsin did not cleave this sequence. On the other hand, WMV and YHIF were not resistant to digestion. For peptides that are not resistant to digestion, this issue can be addressed through encapsulation, as suggested by Zhao *et al*. (2020). The IC50 values for MMA (5.32 µM), WMV (5.23 µM), and YHIF (0.47) indicated their potency as novel peptides. Additionally, the novel peptide QYKF displayed good solubility in water, was non-toxic and non-allergenic, but was not resistant to digestion, with a peptide ranker score of 0.702 and an IC50 value of 0.89 µM.

In a virtual screening study of a novel *Larimichthys crocea* protein, researchers discovered that the peptide HGR (His-Gly-Arg) exhibited ACE inhibitory activity, with an IC50 value of 106 ± 1.35 µM (Yu *et al*., 2019). Additionally, in a recent study by Paee *et al*. (2021), four potent ACE-inhibitory peptides were identified from the α-1 subunit of tilapia skin, namely VW, YW, LPG f(1661–1663), and LPG f(556–558), with IC50 values of 1.4 (Peptide score, 0.80), 10.5 (Peptide score, 0.97), 5.73 (Peptide score, 0.80), and 5.73 (Peptide score, 0.80) µM, respectively.

The study also revealed that most ACE-inhibitory peptides derived from fish proteins were tripeptides. As the demand for natural bioactive compounds increases, the exploration of novel seafood sources and innovative processing methods continues to expand the potential applications of bioactive peptides. These peptides offer promising opportunities for the development of functional foods, nutraceuticals, and therapeutic agents aimed at improving human health and well-being (Sampath Kumar *et al*., 2020).

Table 7. The in-silico analysis predicted the potential release of novel peptides from the red bigeye protein

Peptide**S**	SSRF (Ser-Ser-Arg-Phe)	HPHF (His-Pro-His-Phe)	IQYF (Iso-Glu-Tyr-Phe)	IHF (Iso-His-Phe)	MMA (Met-Met-Ala)	YHIF (Tyr-His-Iso-Phe)	WMV (Try-Met-Val)	CINF (Cys-Iso-Asp-Phe)	QYKF (Glu-Tyr-Lys-Phe)
Activity	ACE inhibitor, DPP III inhibitor	ACE inhibitor, Antioxidative, DPP III inhibitor, DPP IV inhibitor	ACE inhibitor, DPP IV inhibitor, Antioxidative	ACE inhibitor, Antioxidative, DPP III inhibitor, DPP IV inhibitor, Antibacterial	DPP III inhibitor, DPP IV inhibitor	ACE inhibitor, DPP III inhibitor, DPP IV inhibitor	ACE inhibitor, DPP III inhibitor, DPP IV inhibitor	ACE inhibitor, DPP IV inhibitor	ACE inhibitor, DPP III, DPP IV inhibitor, CaMPDE inhibitor, Renin inhibitor
Predicted bioactivity	0.785	0.880	0.779	0.765	0.825	0.770	0.893	0.849	0.702
Enzymes	Pepsin	Pepsin, stem bromelain	Stem bromelain	Stem bromelain	Stem bromelain	Stem bromelain	Stem bromelain	Stem bromelain	Stem bromelain
Molecular weight (g/mol)	495.53	536.58	569.65	415.49	351.49	578.66	434.55	495.59	584.66
Solubility in water	Good	Poor	Poor	Poor	Poor	Poor	Poor	Poor	Good
Net charge at pH 7	1	0.2	0	0.1	0	0.1	0	-0.1	1
Iso-electric point pH	10.57	7.72	3.65	7.82	3.37	7.52	3.54	2.98	9.47
*Resistance to digestion	No	No	No	No	Yes	No	No	No	No
Toxicity	Non-toxin	Non-toxin	Non-toxin	Non-toxin	Non-toxin	Non-toxin	Non-toxin	Non-toxin	Non-toxin
Allergenicity probability	Probably allergen	Probably allergen	Probably allergen	Probably allergen	Non-allergen	Non-allergen	Non-allergen	Probably allergen	Non-allergen
Predicted IC 50 (μM)	Non- AHT (-1)	1	0.77	3.71	5.32	0.47	5.23	Non AHT (-0.97)	0.89

*Resistance to digestion using enzymes: chymotrypsin, pepsin (pH1.3), pepsin (pH > 2), trypsin

CONCLUSION

The results of this study demonstrated that selected red bigeye proteins hold the potential to act as precursors for angiotensin-I-converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides. Pepsin (pH > 2) was identified as the most suitable enzyme for releasing these peptides from red bigeye protein, and a combination of papain and proteinase K also exhibited promising outcomes. Among the novel peptides discovered, QYKF emerged as the most potent, displaying good solubility, a low IC_{50} value of 0.89, and being non-toxic and non-allergenic. These findings strongly suggest that red bigeye proteins are promising sources of bioactive peptides with antihypertensive and antidiabetic properties.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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