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COX-2 Inhibition by Bioactive Peptides from Peanut Worm (*Siphonosoma australe*) Collagen Through *In Vitro* Digestion Simulation

Running title: COX-2 Inhibition by *Siphonosoma australe* Peptides

Suwarjoyowirayatno[1,2], Chusnul Hidayat[1], Tutik Dwi Wahyuningsih[3] and Retno Indrati[1*]

[1]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Flora Street, Bulaksumur, Yogyakarta 55281, Indonesia
[2]Department of Fish Product Technology, Faculty of Fisheries and Marine Science, Universitas Halu Oleo, H.E.A. Mokodompit Street, Kendari 93231, Indonesia
[3]Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Gadjah Mada, Sekip Utara, Bulaksumur, Yogyakarta 55281, Indonesia

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SUMMARY

Research background. Chronic, unregulated inflammation is a crucial factor in developing numerous diseases and is closely linked to the increased expression of cyclooxygenase-2 (COX-2). While various bioactive peptides from marine organisms have exhibited COX-2 inhibitory effects, peptides derived from the collagen of the peanut worm (*Siphonosoma australe*) have not yet been demonstrated. This study aimed to evaluate peanut worm collagen's potential COX-2 inhibitory activity through in vitro simulated digestion using pepsin-pancreatin followed by molecular docking.

Experimental approach. During simulated *in vitro* digestion, commercial pepsin (at pH 3) and pancreatin (at pH 7.5) were applied for 240 min at 37 °C to evaluate the degree of hydrolysis, peptide

*Corresponding author:
Phone: +62811267259
Fax: +62274589797
E-mail: indrati@ugm.ac.id

concentration, and COX-2 inhibitory activity. Samples showing the most significant COX-2 inhibitory activity were subsequently separated into fractions and identified.

Results and conclusions. The 210 min *in vitro* simulated digestion showed the highest COX-2 inhibitory activity (64.31 %). This finding was confirmed by the elevated degree of hydrolysis (DH) and peptide concentrations observed during the in vitro simulated digestion. The peptide fraction of <1 kDa exhibited the highest inhibitory activity (89.05 %), followed by peptide sequencing. Three novel peptides, ADIAGQAAQVLR, LNNEITTLR, and VGTVEK, were identified and contain crucial amino acids, confirming them as COX-2 inhibitors. VGTVEK has the most potent interaction, as shown by the lowest binding energy (−4.41 kcal/mol). The molecular docking revealed that VGTVEK (631.35 Da) binds to the active side of COX-2, forming hydrogen bonds with Gln178, Leu338, Ser339, Tyr371, Ile503, Phe504, Val509, and Ser516 and hydrophobic interactions with Met99, Val102, Val330, Ile331, Tyr334, Val335, Leu345, Trp373, Leu517, and Leu520. Other biological activities of the produced peptides included ACE inhibitors, DPP-IV inhibitors, and α-glucosidase inhibitors. According to the toxicity prediction, peptides have been classified as non-toxic.

Novelty and scientific contribution. The study found that peptides generated from peanut worm collagen exhibit potential as novel, natural agents for anti-inflammatory therapy. Their broader application in functional foods, nutraceuticals, and pharmaceuticals could offer new options for individual sufferers of inflammation, supporting both treatment and overall health maintenance.

Keywords: bioactive peptide; COX-2 inhibition; digestion simulation; molecular docking; peanut worm collagen; *Siphonosoma australe*

INTRODUCTION

Cyclooxygenase-2 (COX-2) is crucial in the conversion of arachidonic acid to prostaglandins (PG), which are key regulators in inflammation. The activation of pro-inflammatory mediators triggers the expression of COX-2. The upregulation and overexpression of COX-2 are primarily linked to inflammatory processes (*1*). Inflammation is the host's defense mechanism, responding adaptively to injury, tissue damage, and infections. Although inflammation generally provides protective benefits to the body, prolonged and unregulated inflammation can play a role in developing numerous diseases (*2*). It is the starting point for multiple chronic conditions, such as asthma, cancer, skin disorders, cardiovascular disease, neurological ailments, and arthritis. Treating inflammatory conditions typically involves using non-specific small-molecule drugs (*3*). Although various anti-inflammatory medications are commercially available, all are associated with potential side effects (*4*). These medications are

frequently linked to gastrointestinal (GIT) side effects, including peptic ulcers, GIT bleeding, intestinal obstruction, and GIT erosion (*5*).

Concerns regarding the adverse effects of synthetic substances have heightened interest in using natural compounds and their derivatives as safer alternatives for therapeutic purposes, including functional foods and nutraceuticals (*4*). Numerous efforts are focused on developing alternative and more selective anti-inflammatory treatments, with several involving peptides. Peptides have proven to be the preferred lead compounds for various targets due to their high specificity and the application of recent and innovative synthetic methods (*3*). Anti-inflammatory peptides are present in many living organisms, with numerous peptides derived from plants, mammals, bacteria, and marine organisms demonstrating notable anti-inflammatory properties (*6*). Anti-inflammatory peptides have recently been identified and extracted from specific species of peanut worms. Specifically, peptides derived from *Sipunculus nudus* demonstrate anti-inflammatory activity through multiple mechanisms, including reducing pro-inflammatory mediator expression and inhibiting COX-2 (*7,8*).

Bioactive peptides, typically short chains of amino acids derived from proteins, exhibit a range of biological activities that extend beyond their nutritional value. These peptides are normally inactive within the context of their parent proteins and require proteolytic cleavage to be released and exert their specific bioactive functions (*4*). Proteolytic processes encompass food processing, microbial fermentation, germination, and the activity of various protease enzymes (*9*). Anti-inflammatory peptides have been identified in peanut worms using proteolytic enzymes for hydrolysis (*2*). Digestive enzymes have the ability to degrade peptides, resulting in alterations to their bioactivity within the gastrointestinal tract. Enzymatic hydrolysis during food processing can generate peptides with shorter amino acid chains (*10*). Hydrophobic amino acids in short-chain peptides are associated with biological activities that confer health benefits (*9*).

Anti-inflammatory peptides derived from food generally contain between 2 to 20 amino acids. Beyond their length, the composition and sequence of these peptides are essential in influencing their anti-inflammatory effects (*11*). Recently, Sangtanoo *et al.* (*8*) isolated two peptides, LSPLLAAH (821.48 Da) and TVNLAYY (843.42 Da), were isolated from the peanut worm *S. nudus* after hydrolysis using alcalase, neutrase, and flavorsome enzymes. These peptides exhibited strong anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages, significantly lowering the expression of pro-inflammatory mediators (iNOS, IL-6, TNF-α, and COX-2) following a 12-hour treatment. Conversely, Lin *et al.* (*7*) extracted peptides from *S. nudus* collagen using hydrolytic proteases derived from animals and flavor proteases. The study revealed that these peptides alleviated inflammation in mice skin wounds by reducing the mRNA expression levels of TNF-α, TGF-β1, and IL-1β.

The amino acid sequence of low-molecular-weight peptides produced during the simulated digestion of peanut worm collagen could represent a promising source of bioactive peptides with COX-2 inhibitory properties. In Indonesia, *S. australe* is infrequently consumed as a food source, while in China, it is primarily employed as a raw material in traditional medicine (*12*). *S. australe* remains underexplored in research, particularly in anti-inflammatory potential, compared to other marine organisms such as fish, molluscs, and crustaceans. Found exclusively in the waters of eastern Indonesia and Australia (*13*), *S. australe* possesses a distinctive protein and amino acid composition. Consequently, there is a significant opportunity to identify novel bioactive peptides that could contribute to the advancement of alternative natural compounds and nutraceuticals in developing anti-inflammatory medications. The amino acid composition of *S. australe*, which contains both positively charged amino acids and hydrophobic, contributes to its anti-inflammatory properties (*14*). *Siphonosoma australe* collagen was found in our previous research to inhibit COX-2 by 41.12 % with an IC_{50} value of 59.9 μg/mL (*15*). The anti-inflammatory activity of *S. australe* can be further enhanced through enzymatic hydrolysis, facilitating the production of more potent COX-2 inhibitory peptides. However, there are no published studies on using *S. australe* as a collagen source for producing COX-2 inhibitory peptides. Therefore, this study aimed to collect thorough information about the profile of COX-2 inhibitory peptides in *S. australe* by conducting *in vitro* simulated digestion using pepsin and pancreatin. The hydrolysate with the most significant COX-2 inhibitory potency was fractionated using a molecular weight cut-off (MWCO) membrane, assessed for its inhibitory activity, and subjected to molecular docking for characterization.

MATERIALS AND METHODS

Materials

Peanut worms (*S. australe*) were sourced from Rombo Village in Southeast Sulawesi Province, Indonesia, in their dried form. Merck, New Jersey, USA, supplied the o-phthaldialdehyde (OPA). Pepsin (EC.3.4.23.1 from porcine gastric mucosa P7012), pancreatin (EC.232-468-9 from porcine pancreas P7545), and the COX-2 inhibitor screening kit (Fluorometric) (MAK399) was obtained from Sigma-Aldrich, St. Louis, Missouri, USA. Regenerated cellulose membranes (Membra-Cel) with molecular mass of 1 kDa, 3.5 kDa, and 14 kDa (MD44) were acquired from Viskase Co., Lombard, Illinois, USA. All other chemicals utilized were of analytical grade.

Preparation of peanut worm collagen

The collagen extraction procedure was modified by Chuaychan *et al.* (*16*). In order to remove non-collagen proteins, peanut worms were immersed in 0.1 M NaOH at 4 °C for 6 hours at a 1:10

ratio (10 %), with the solution being changed every 3 hours. The demineralization process involved treating the worms with 0.5 M of disodium EDTA at a neutral pH level (7.4) for 48 hours at the same 1:10 ratio, with the solution replaced every 24 hours. Following demineralization, the worms were incubated in 0.5 M CH_3COOH (1:10) for 48 to 72 hours at 4 °C with agitation. The solution was filtered using a double layer of cheesecloth. Subsequently, NaCl was added to the collagen solution, reaching a final concentration of 2.6 M, resulting in collagen precipitation. The precipitate was collected after centrifugation at 15,000×*g* for 30 minutes at 4 °C and subjected to dialysis using a 14 kDa MWCO membrane against 30 volumes of 0.1 M CH_3COOH and deionized water for 48 hours. The resulting solution was freeze-dried to yield acid-soluble collagen from the body wall of peanut worm (ASC-PW).

In vitro simulated digestion

This study employed an *in vitro* digestion method, following the procedure previously outlined by Puspitojati *et al.* (*17*), which was modified slightly for this study. Each sample was prepared to a pH of 3 using phosphate-buffered saline (BR0014G, Oxoid, Basingstoke, England) and 1 M HCl, achieving a protein concentration of 5 mg/mL. Pepsin (EC.3.4.23.1, 2,000 U/mL) was added, maintaining an enzyme-to-substrate ratio of 1:10. The reactions were carried out at 37 °C for 120 minutes, with samples taken at 30-minute intervals for analysis. After the 120-minute pepsin digestion, the hydrolysates were subjected to a duodenal digestion simulation. The pH was increased to 7.5 by adding 2 N sodium hydroxide solution, followed by the addition of pancreatin (EC.232-468-9, 100 U/mL) at an enzyme-to-substrate ratio of 1:25. The reactions were incubated at 37 °C for another 120 minutes, with samples collected every 30 minutes for analysis. The hydrolysis process was terminated by heating the solution to 100 °C for 10 minutes and adjusting the pH to 7 with 2 N sodium hydroxide. The hydrolysate was centrifuged at 8,000×*g* at 4 °C for 10 minutes, and the supernatant was stored at −20 °C for future use.

Peptide fractionation using MWCO

The fractionation procedure was carried out using a membrane with an MWCO of 1, 3.5, and 14 kDa, following the protocol outlined by Agustia *et al.* (*18*). Before use, the MWCO membranes were activated with (10 mM) $NaHCO_3$ and (10 mM) Na2EDTA solutions. To rehydrate the membranes, they were immersed in $NaHCO_3$ solution for 10 minutes and then transferred to Na_2EDTA solution for an additional 10 minutes. The membranes were subsequently placed back into a fresh Na_2EDTA solution and boiled for another 10 minutes. After the activation process, the membranes were thoroughly washed with sterile distilled water and were deemed ready for use.

Peptide solutions, with a protein concentration of 10 mg/mL, were filtered using a 1 kDa membrane, ensuring both sides were securely sealed with a membrane clip. The membranes were then placed in an Aqua Pro injection beaker with a magnetized stirring rod and maintained at an 80-100 rpm stirring speed. The fractionation process was carried out at 4 °C for 12 hours. After this period, the 1 kDa peptide fraction was separated using Aqua Pro Injection. The remaining solution was sequentially filtered through membranes with MWCOs of 3.5 kDa and 14 kDa, following the same procedure. The resulting fractions contained peptides with molecular masses (MM) of less than 1 kDa, between 1 and 3.5 kDa, 3.5 and 14 kDa, and greater than 14 kDa. These peptide fractions were stored at -20 °C until further analysis.

Assay of peptide content

In this study, the peptide concentration was determined using the method adapted from the research conducted by Church *et al.* (*19*). The OPA reagent was prepared by mixing 25 mL of 100 mM $Na_2B_4O_7$, 2.5 mL of 20 % $NaC_{12}H_{25}SO_4$, and 1.1 mL of OPA (40 mg of OPA dissolved in 1 mL of $CH_3OH(l)$ and 100 mL of $C_2H_6OS(l)$), followed by the addition of 21.4 mL of distilled water. The sample (20 µL) was added to 1 mL of the prepared OPA reagent, incubated at room temperature for 2 minutes, and then the absorbance was measured at 340 nm. Peptide content was quantified using Tripton as the reference standard, and a standard linear regression curve was constructed with a range of concentration from 0 to 1.50 mg/mL (0, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.50 mg/mL).

Assay of degree of hydrolysis (DH)

The degree of hydrolysis (DH) was assessed using a modified method based on Lin *et al*. (*20*). Collagen from peanut worms was hydrolyzed using an 8 M hydrochloric acid solution (1:10 ratio) and incubated for 24 hours at 110 °C. After neutralization with 8 M sodium hydroxide solution, the mixture was adjusted to a final volume of 10 mL with distilled water and filtered. The peptide concentration was then measured using the OPA reagent. The degree of hydrolysis (%) was determined using the formula below:

$$DH=((NH_2)_{tx}-(NH_2)_{t0})/((NH_2)_{total}-(NH_2)_{t0})\cdot 100 \qquad /1/$$

where $(NH_2)_{tx}$ represents the amount of free amino acids at x minutes, $(NH_2)_{t0}$ indicates the quantity of free amino acids at 0 min, and $(NH_2)_{total}$ denotes the total number of free amino acids present.

Assay of COX-2 inhibition

The inhibition of COX-2 enzymes by collagen *in vitro* was assessed through Merck's Fluorometric COX-2 inhibitor screening kit. The assay was conducted in a 96-well plate, with a mixture

comprising 10 µL of arachidonic acid/NaOH solution, 1 µL of recombinant COX-2, 76 µL of COX assay buffer, 2 µL of COX-2 cofactor working solution, 10 µL of the test sample, and 1 µL of COX probe solution. Fluorescence kinetics were measured over 10 minutes at 25 °C using a multimode microplate reader (Spark, Tecan, Männedorf, Switzerland), with excitation and emission wavelengths set to 535 nm and 587 nm, respectively. Celecoxib was a positive control, while the negative control consisted of the enzyme without any drugs or peptides. All peptide and control samples were tested in triplicate, and the reaction conditions adhered to the standard protocol provided with Merck's COX-2 inhibitor assay kit.

To obtain the corresponding fluorescence values (RFU1 and RFU2), two points (T1 and T2) were selected within the linear portion of the graph. The slope (S) for each sample was calculated by dividing the net fluorescence change (∆RFU) by the associated time interval (∆T), following this formula:

$$\text{Relative inhibition (\%)} = ((SEC - SS)/SEC) \cdot 100 \quad /2/$$

where SEC is the slope of enzyme control and SS is the slope of the sample.

Characterization and identification of peptide sequence

The peptide fraction displaying the highest COX-2 inhibitory activity after digestion simulation was analyzed and identified using liquid chromatography (Thermo Scientific™ Vanquish™ UHPLC Binary Pump) combined with Orbitrap high-resolution mass spectrometry (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High-Resolution Mass Spectrometer) (Waltham, Massachusetts, USA) (*21*). Separation was performed through gradient elution with a mobile phase comprising MS-grade water with 0.1 % formic acid (A) and MS-grade acetonitrile with 0.1 % formic acid (B). The gradient was set as follows: 0–1 minute at 5 % B, a linear increase from 5 % to 50 % B over 1–31 minutes, followed by 2 minutes at 50 % B. The initial conditions were restored and held for 47 minutes. The flow rate was maintained at 0.075 mL/min, and a C18 HPLC column (150 mm length, 1 mm ID, 3 µm particle size, Thermo Scientific™ Acclaim™ PepMap™, Waltham, Massachusetts, USA) was used with a 3 µL injection volume. Mass spectrometry was analyzed using positive ionization in full MS/dd-MS2 mode. Conditions included a sheath gas flow rate of 15 AU, an auxiliary gas flow rate of 5 AU, a capillary temperature of 300 °C, and a spray voltage of 4.00 kV. The scan range spanned 150–2000 m/z with resolutions of 140,000 for full MS and 17,500 for dd-MS2. Peptide sequences were identified using Proteome Discoverer 2.2 (*22,23*) and MaxQuant v2.4.14.0 software (*23*). The identified peptide sequence was compared for similarity using https://www.uniprot.org/, accessed on February 28, 2024 (*16*).

Biological activity of peptide sequence evaluation

Following the method outlined by Minkiewicz *et al.* (*24*), BIOPEP-UWM was utilized to identify the potential biological activities of the fractionated peptides (https://biochemia.uwm.edu.pl/biopep-uwm/, accessed on March 6, 2024. This database provides A and B values, which represent the frequency of bioactive fragments and the biological activity of protein fragments, respectively, allowing for the characterization of the sequence's biological properties. Additionally, the toxicity of the peptide sequences was evaluated using the ToxinPred database (*25*) (https://webs.iiitd.edu.in/raghava/toxinpred/, accessed on March 1, 2024).

Molecule preparation for computational modelling

The peptide's three-dimensional (3D) structure was created using novoprolab.com. Initially, the peptide sequence was transformed into SMILES string format (*26*) (https://www.novoprolabs.com/tools/convert-peptide-to-smiles-string, accessed on March 6, 2024). Then, the SMILES string was used to create a 3D structure (https://www.novoprolabs.com/tools/smiles2pdb, accessed on March 6, 2024) (*26*). The 3D structure was optimized by minimizing its energy with Yasara-structure software (*27*). Moreover, the three-dimensional configuration of the COX-2 enzyme was acquired from the protein data bank (https://www.rcsb.org/) under PDB ID 3LN1 (*28*). The enzyme's structure was refined by eliminating water and irrelevant ligands through Yasara-structure software (*27*).

Molecular docking of the peptide and COX-2

The docking procedure was performed using the Yasara structure application, employing a force field scoring functional approach to compute the binding energy values (*73*). This process utilized the docking macro command (dock_run.mrc) in Yasara-structure. A cubical box with a 5.0 Å radius was centered around the native ligand to direct the docking. After the docking process was completed, the results were stored in PDB (.pdb) file format. Subsequently, the post-docking data were analyzed and visualized using Discovery Studio Visualisation software (*18*).

Statistical analysis

The experimental data are reported as mean values with standard deviations from three replicates. Before analysis, the data were tested for normality using the Shapiro-Wilk test. Subsequently, a one-way analysis of variance (ANOVA) was conducted, followed by Duncan's multiple range test (DMRT) for comparative analysis. A t-test was employed to assess significant differences between the means of the two hydrolysate peptide groups. All statistical analyses were

performed using SPSS IBM 25 (IBM, Armonk, New York, USA), with a significance threshold set at 5 % (*18*).

RESULTS AND DISCUSSION

Degree of hydrolysis

The hydrolysis of peanut worm (*S. australe*) collagen using pepsin and pancreatin mirrors two critical stages in the human digestive process. Pepsin operates in the gastric phase, while pancreatin functions in the small intestine phase (*29*). The concentrations of these enzymes are consistent with their reported activity levels in the stomach and intestine (*30*), ensuring effective collagen hydrolysis to generate bioactive peptides with potential anti-inflammatory effects. Sampling at 30-minute intervals reflects the typical duration of gastric and intestinal digestion (*29*), enabling the tracking of peptide formation at various stages (early, middle, and late stages of digestion). The peptide sampling will generate an in-depth profile of peptide release and degradation over time, enabling the identification of optimal hydrolysis stages at which the peptides display maximum bioactivity.

The hydrolysis patterns observed in peanut worm collagen extracts at 48 h (C48), and 72 h (C72) were similar (Fig. 1a). The degree of hydrolysis (DH) values for both samples increased from 0 to 120 minutes with pepsin, followed by an increase in hydrolysis from 120 to 240 minutes after adding pancreatin. The DH peaked at 210 minutes, with C48 exhibiting a value of 92.71 % and C72 showing 84.03 % ($p<0.05$). The higher DH observed in the 48-hour collagen extraction may be attributed to the production of longer collagen polypeptide chains than in the 72-hour extraction. Acetic acid truncation of collagen chains is more pronounced at 72 hours than 48 hours. As a result, the collagen polypeptide chains remain longer at 48 hours, providing more substrates for cleavage by the pepsin-pancreatin enzymes. Due to the distinct substrate specificities, pepsin and pancreatin cutting patterns enhance the hydrolysis process's efficiency (*10*). Each enzyme’s specificity results in the production of smaller peptides more effectively and efficiently. The study found that pancreatin produced more hydrolysis (DH) when applied to peanut worm collagen than pepsin. This finding aligns with Agustia *et al.* (*18*), who reported lower hydrolysis with pepsin compared to pancreatin in the hydrolysis of jackbean protein. Additionally, pancreatin resulted in a more substantial increase in hydrolysis than pepsin (*31*).

Peptide concentration

The peptide concentration values followed a pattern similar to the hydrolysis degree (DH). After 240 minutes of hydrolysis with pepsin-pancreatin, the higher DH resulted in an increased

production of peptides, with the peak concentrations reaching 1.07 mg/mL for C48 and 1.12 mg/mL for C72 (Fig. 1b). Applying pepsin for 0-120 minutes effectively increases peptide concentration, followed by adding pancreatin from 120-150 minutes, significantly increasing peptide concentration ($p<0.05$). Pepsin specifically cleaves the telopeptide bonds in collagen, producing smaller peptides with a strong specificity for hydrophobic amino acids (*32*). Both endoprotease and exoprotease enzymes in pancreatin significantly enhance protein hydrolysis (*33*). The combination of pepsin and pancreatin effectively increases the yield of collagen peptides. These results suggest that pepsin is pivotal in breaking down the native, well-folded protein, while pancreatic enzymes generate shorter peptides (*34*).

However, between 150 and 240 minutes, the DMRT test for C48 and C72 revealed no significant difference ($p>0.05$). This can be attributed to a reduced peptide concentration at 240 minutes, suggesting a diminished availability of collagen telopeptide substrate for further hydrolysis (Fig. 1b). The observed decrease in value at 240 minutes may be due to the continued hydrolysis of collagen peptides by the pancreatin enzyme, which generates free amino acids. As a result, the peptide chains shorten, and the MM distribution decreases with increasing degrees of hydrolysis. This ultimately causes the cleavage of peptide bonds and a subsequent rise in free amino acid concentration. This study aligns with the findings of Khushairay *et al.* (*35*), who reported that the peptide content of pancreatin-treated swallow nest hydrolysate increased during the first 90 minutes of hydrolysis but declined after that. Following an initial phase of rapid hydrolysis, the rate of hydrolysis typically slows down, eventually reaching a stationary phase. Although there are more cleavage sites available, the rate of hydrolysis is influenced by the enzyme's cleavage specificity and the accessibility of peptide bonds to the enzymes involved.

COX-2 Inhibitory activity

DH and peptide concentration increases are closely associated with the efficacy of the resulting COX-2 inhibitory activity. The COX-2 inhibition capacity of collagen hydrolysate was found to significantly enhance as the hydrolysis time progressed ($p<0.05$) (Fig. 1c). Collagen samples C48 and C72 exhibited peak COX-2 inhibition at 210 minutes, achieving 64.31 % and 59.27 %, respectively. This effect is attributed to the acetic acid collagen extraction process followed by pepsin-pancreatin enzymatic digestion, which generates small collagen peptides, as reflected in the peptide concentration and DH values. These peptides, which consist of hydrophobic and positively charged amino acids, can bind to the active site of COX-2, inhibiting its function. Anti-inflammatory peptides with COX-2 inhibitory effects commonly contain hydrophobic amino acid residues, such as alanine (Ala), glycine (Gly), tryptophan (Trp), valine (Val), tyrosine (Tyr), phenylalanine (Phe), isoleucine (Ile),

and methionine (Met), alongside positively charged residues like arginine (Arg) and lysine (Lys) (*11*). The findings of this study indicate a COX-2 inhibition value for hydrolyzed *S. australe* collagen that exceeds that of its non-hydrolyzed form, which demonstrated an inhibition level of 41.12 % (*15*), Additionally, the novel COX-2 inhibitor peptides YCS, YAD, WCD, and GYW, with inhibition percentages of approximately ≥27 %, ≥33 %, ≥35 %, and ≥45 %, respectively (*36*). The results align with peptides WGD, WYS, and WAY, which showed inhibition levels of ≥63 %, ≥67 %, and ≥68 %, respectively (*36*), and are similar to the short peptide compound 30, which achieved 60 % inhibition (*37*).

Moreover, the COX-2 inhibitory activity in collagen samples C48 and C72 declined after 240 minutes. This reduction is attributed to continuous hydrolysis over the 240-minute period, which degrades peptides into free amino acids, potentially altering amino acid sequences and diminishing or even abolishing the peptides' anti-inflammatory activity. These findings are consistent with those of Agustia *et al.* (*18*), who reported a decline in DPP-IV inhibitory activity in jack bean peptides at 210 and 240 minutes of digestion. This decrease in activity is attributed to these peptides' specific sequence and composition, which impair their ability to effectively bind to the enzyme, thereby reducing their inhibitory potential.

Peptide fractionation with MWCO

Peptides derived from simulated *in vitro* digestion were separated through membrane dialysis to assess their MM distribution and its impact on COX-2 inhibitory activity. Using varied MWCO membrane sizes aims to optimize the identification of peptides with suitable sizes for bioactivity and therapeutic applications. Larger peptides tend to be more stable, while smaller peptides exhibit higher biological activity and can better permeate cell membranes (*38*); consequently, peptide molecular mass significantly influences functional activity. Numerous studies have demonstrated that diverse MWCO membrane sizes can yield peptides with distinct anti-inflammatory properties (*8*).

After a 210-minute digestion, samples C48 and C72 were fractionated into four molecular weight categories based on MWCO: <1, 1–3.5, 3.5–14, and >14 kDa (Table 1). Among these fractions, peptides with molecular mass under 1 kDa exhibited the most substantial COX-2 inhibitory activity ($p<0.05$), suggesting that smaller collagen peptides possess a higher binding affinity for the COX-2 active site. The MWCO membrane facilitates the selective separation of low MM peptides, enhancing anti-inflammatory activity (*8*). These findings suggest collagen peptides with low MM inhibiting COX-2 can be effectively generated using pepsin and pancreatin hydrolysis. The obtained results were higher than those of the peptides WAY, WCY, and FCS, which exhibited inhibition rates of ≥68 %, ≥70 %, and ≥78 %, respectively (*37*). Additionally, it is similar to WCS peptide ≥88 % (*37*)

and new selective COX-2 inhibitors (compounds 50 and 51) at 9.5 % (*39*). The findings of this study align with those of Rizkaprilisa *et al.* (*31*), who reported that tempe peptides subjected to pepsin-pancreatin hydrolysis throughout digestion yielded an increased proportion of low MM peptides. Previous studies have indicated that small peptide fractions, including those with MM of <5, <3, 1, 0.8, 0.6, and 0.3 kDa, exhibit significant anti-inflammatory effects by inhibiting the COX-2 enzyme (*7,8*). Additionally, the hydrolysis process under simulated *in vitro* gastrointestinal conditions promotes the formation of low MM peptides, which may further enhance COX-2 inhibitory activity.

Small MM peptides exhibit limited enzymatic recognition and fewer cleavage sites. This reduced enzymatic degradation enables these peptides to remain intact as they enter the bloodstream, allowing them to reach their target organs effectively (*40*). In contrast to larger protein molecules and even smaller amino acid molecules, these peptides are efficiently absorbed by the body through specialized transport mechanisms in the intestines. Subsequently, they are delivered to the target area to exhibit anti-inflammatory effects (*41*). Their transit mechanism minimizes energy expenditure and reduces strain on the gastrointestinal system, making it particularly beneficial for individuals with chronic conditions (*14*). Once digested in the gut, small MM peptides have been shown to benefit conditions such as arthritis (*42*) and breast cancer (*43*). Further concerns regarding small peptides include the quantity of peptides produced and their purity levels. Additional research is needed to evaluate the stability of these peptides against proteolytic enzymes and environmental factors. Additionally, the absorption process through the digestive tract and peptide-related parameters should be examined through *in vivo* testing.

Identification of peptide sequence of peanut worm collagen and its potential inhibitory effect

The peptide fractions exhibiting the strongest COX-2 inhibitory activity (<1 kDa) from MWCO dialysis were sequenced. Three novel peptides with COX-2 inhibitory potential were identified: ADIAGQAAQVLR, LNNEITTLR, and VGTVEK. The 3D structures of these peptides are presented in Fig. 2. The peptides exhibited MM ranging from 631.35 to 1212.37 Da. The presence of peptides larger than the membrane pore size can be attributed to variations in pore stability during the dialysis process and the peptides' molecular characteristics. Additionally, peptides with molecular weights similar to the MWCO pore size may still pass through the membrane, as their dialysis rates are influenced by factors such as molecular shape and solubility (*44*). Furthermore, the transport of peptides through membrane filtration is influenced by their charge, with peptide separation primarily resulting from the combined effects of electrodialysis charge selectivity and the size exclusion capabilities of the filtration membranes (*45*). Each peptide molecule possesses distinct characteristics, and factors such as concentration, interactions, and hydrophobicity significantly

influence its ability to diffuse through a dialysis membrane. It is crucial to account for variables such as temperature, volume, agitation, and the frequency of external buffer exchange during the process (*44*). Despite exceeding the filter size, the peptide derived from the peanut worm falls within the inhibitory COX-2 peptide range as specified in the BIOPEP-UWM database (955–1400 Da) (*46*).

The sequencing lengths of the COX-2 inhibitor peptide fragments varied from 6 to 12 amino acids. The N-terminal region of the peptide contains hydrophobic amino acids such as alanine (A), leucine (L), and valine (V). In contrast, the C-terminal region comprises positively charged amino acids, including arginine (R) and lysine (K). The presence of hydrophobic residues at the N terminus and positively charged residues at the C terminus enhances the peptide's ability to inhibit COX-2, with IC_{50} values ranging from 4.31±0.99 to 15.53±1.78 μg/mL (*47*). Singh *et al.* (*37*) noted that the hydrophobic nature of the COX-2 active site led to the selection of Gly, Ala, Val, Leu, and Phe for synthesizing the COX-2 inhibitor peptide. Additionally, Hong *et al.* (*48*) demonstrated that tetrapeptides derived from walnut dregs, which contain hydrophobic amino acid residues at the N terminus (LFPD, FPGA, AGFP, and VGFP), formed strong hydrogen bonds and hydrophobic interactions with residues within the COX-2 active site.

The values for A and B (Table 2) were derived through peptide sequence simulations using the BIOPEP-UWM database and ToxinPred to assess the potential toxicity of the peptides. According to the BIOPEP-UWM simulation, the peptides identified in this study have not been previously documented in the database, presenting an opportunity to enrich the BIOPEP-UWM library with new data on COX-2 enzyme inhibitors. The simulation results from BIOPEP-UWM also indicated that the peptides exhibit various biological activities, including ACE, DPP-IV, and α-glucosidase inhibition, suggesting their potential for diverse future applications. However, these findings require further validation. The peptide toxicity predictions indicated that none of the sequences were toxic, suggesting that the peptides generated during the gastrointestinal simulation of peanut worm collagen are safe for use.

Computational modelling for the binding structure of the peptide and COX-2

The peptide sequences (ADIAGQAAQVLR, LNNEITTLR, and VGTVEK) demonstrated the most favorable binding conformations with COX-2, as depicted in Fig. 3. Based on the binding energy values, VGTVEK (-4.41 kcal/mol) exhibited the strongest interaction, followed by ADIAGQAAQVLR (0.96 kcal/mol) and LNNEITTLR (1.28 kcal/mol). Binding energy values indicate the strength of the interaction between peptides (ligands) and COX-2 (receptors). The lower binding energy value means less energy is required for a ligand to bind or interact with its receptor.

The molecular docking revealed that VGTVEK binds to the active side of COX-2, forming hydrogen bonds with Gln178, Leu338, Ser339, Tyr371, Ile503, Phe504, Val509, and Ser516 and hydrophobic interactions with Met99, Val102, Val330, Ile331, Tyr334, Val335, Leu345, Trp373, Leu517, and Leu520. While ADIAGQAAQVLR shows interactions with Pro71, His75, Arg106, Gln178, Gln336, His337, Leu338, Ser339, Gly340, Tyr341, Tyr371, Met508, Val509, and Ala513 forming hydrogen bonds, and interactions with Ile331, Val335, Ala502, Phe504, and Leu520 forming hydrophobic interactions. Whereas LNNEITTLR shows interactions with His75, Leu338, Arg499, Pro500, Asp501, Phe504, Gly512, Ala513, and Leu517, forming hydrogen bonds, and interactions with Val102, Val335, Tyr341, Leu345, Tyr371, Trp373, Met506, and Leu520, forming hydrophobic interactions. Additionally, all peptides display several van der Waals forces with amino acid residues at the COX-2 active site and act competitively to inhibit COX-2. Their hydrogen bonding interactions with residues such as Leu338, Ser339, Arg499, and Phe504 in the active site mirror those of celecoxib, suggesting their potential to inhibit COX-2 selectively (*49*).

The VGTVEK peptide emerges as a candidate with the highest COX-2 inhibitory activity. Celecoxib and VGTVEK peptide interactions are similar, as evidenced by hydrophobic interactions between amino acid residues Val335 and Trp373, hydrogen bonds with Leu338, Ser339, and Phe504, and van der Waals contacts involving Gly340, Phe367, Ala502, and Met508. As a result, it demonstrates a more effective inhibition of the COX-2 enzyme. Chakrabarti and Wu (*50*) suggested that the Val residue at the N-terminal of the peptide contributes to its anti-inflammatory properties. Similarly, the VG segment of this peptide forms hydrogen bonds with Tyr371 and Ser516 within the enzyme's active site, thereby inhibiting COX-2 activity, similar to the VGFP peptide derived from walnut hydrolysate (*48*). Several researchers also reported that the Lys position at the C-terminal of this peptide has good anti-inflammatory ability (*51*). The position of amino acids is crucial in facilitating peptide with enzyme interactions. Enzymes recognize and position different peptides in distinct ways, thereby influencing their binding and activity (*52*).

This peptide's inhibition of COX-2 may contribute to alleviating chronic inflammation, which is often linked to a range of degenerative diseases and autoimmune disorders (*53*). Derived from food sources, specifically collagen hydrolysate, this peptide holds significant potential for developing health-oriented functional diets and food additives. Moreover, this peptide shows the potential for usage in cancer-prevention food products, owing to its ability to suppress COX-2, a known carcinogen (*53*). In addition, these peptides offer a safer alternative to nonsteroidal anti-inflammatory drugs (NSAIDs), which are commonly associated with gastrointestinal and cardiovascular adverse effects (*54*). These peptides represent a safer long-term therapeutic alternative for chronic inflammatory conditions, such as arthritis and autoimmune diseases, as their incorporation into natural anti-

inflammatory treatments may minimize the risk of adverse effects (*54*). While additional research is necessary, the findings suggest that peptides derived from the collagen of *S. australe* may serve as a valuable natural source for functional foods, nutraceuticals, and pharmaceuticals with potent COX-2 inhibitory activity.

CONCLUSIONS

This study provides peanut worm collagen-derived peptides as novel and promising natural inhibitors of COX-2, a key enzyme in inflammation pathways. These peptides contribute to the growing array of marine-based bioactive compounds with anti-inflammatory properties. Through in vitro digestion and molecular docking, three peptides (ADIAGQAAQVLR, LNNEITTLR, and VGTVEK) were identified, with VGTVEK exhibiting the strongest COX-2 inhibition. These findings underscore the therapeutic potential of these peptides for use in functional foods, nutraceuticals, and pharmaceuticals, providing new options for managing inflammatory conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Suwarjoyowirayatno contributed to the conception, data collection, data analysis and interpretation, performing the analysis, and drafting the article. C. Hidayat and T.D. Wahyuningsih

provided constructive discussion and critical revision, and they served as co-supervisors. R. Indrati contributed as a supervisor, critical revision, and final approval of the version for publication.

ORCID ID

Suwarjoyowirayatno https://orcid.org/0009-0000-7801-7978
C. Hidayat https://orcid.org/0000-0001-6540-5306
T.D. Wahyuningsih https://orcid.org/0000-0001-5741-2848
R. Indrati https://orcid.org/0000-0002-2809-5887

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Table 1. Percentage of COX-2 inhibitory activity of peanut worm collagen hydrolysate fractions

Fraction/kDa	COX-2 inhibition/%	
	C48	C72
<1	$(85.64\pm1.16)^{dA}$	$(89.05\pm1.49)^{dB}$
1–3.5	$(75.84\pm0.59)^{cA}$	$(80.44\pm1.88)^{cB}$
3.5–14	$(71.69\pm0.85)^{bA}$	$(73.04\pm1.54)^{bA}$
>14	$(61.95\pm2.47)^{aA}$	$(63.31\pm1.78)^{aA}$

Mean values±S.D. of three replications. Superscripts with small alphabets within the same samples are significantly different (Duncan's test, $p<0.05$), and those with capital alphabet superscripts in the same variable but different samples are significantly different (t-test, $p<0.05$). C48 is peanut worm collagen hydrolysate with 48 h extraction times; C72 is peanut worm collagen hydrolysate with 72 h extraction times

Table 2. Peptide sequence of peanut worm collagen and its potential inhibitory effect

No.	Peptide sequence	MM/ Da	Toxicity prediction	Activity	Frequency of bioactive fragments (A)	Possible biological activity of protein fragments (B)	Fragments peptide	Accession number
1	ADIAGQAAQVLR	1212.37	Non-toxin	ACE inhibitor	2.89	0.00125173	IA, AA, AG, GQ,LR	A0A117DPD2
				DPP-IV inhibitor	4.05	0.00113499	IA, AA, AD, AG,QA, QV, VL	
				α-glucosidase inhibitor	1.16	0.00000324	AD, LR	
2	LNNEITTLR	1073.21	Non-toxin	ACE inhibitor	3.09	0.00086699	ITT, EI, LN, LR	A0A976XJT2
3	VGTVEK	631.35	Non-toxin	ACE inhibitor	4.63	0.00018075	VG, GT, VE, EK	A0A1A8HCI8
				DPP-IV inhibitor	4.63	0.00005181	EK, TV, VE, VG	
				α-glucosidase inhibitor	1.16	0.00000751	VE	

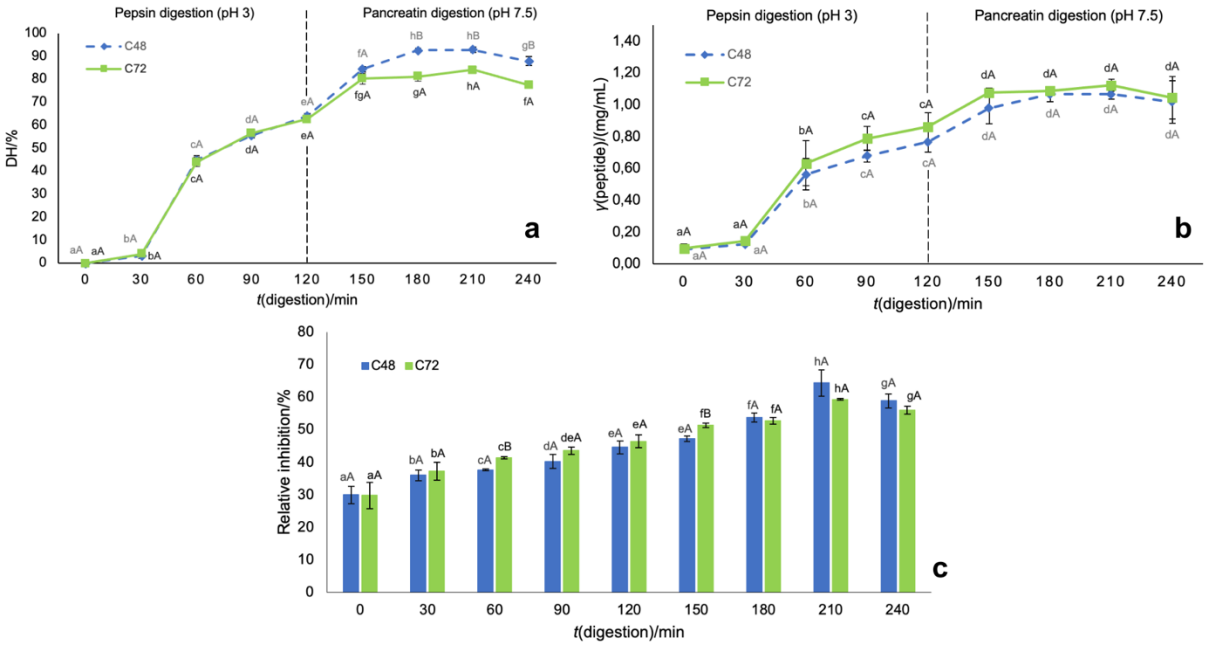

Fig. 1. Degree of hydrolysis (a), peptide concentration (b), and COX-2 inhibitory activity (c) of peanut worm collagen during 240 min of digestion simulation with pepsin-pancreatin. Mean values±S.D. of three replications. Superscripts with small alphabets within the same samples of digestion time are significantly different (Duncan's test, $p<0.05$), and those with capital alphabet superscripts in the same digestion time but different samples are significantly different (t-test, $p<0.05$). C48 is peanut worm collagen with 48 h extraction times; C72 is peanut worm collagen with 72 h extraction times

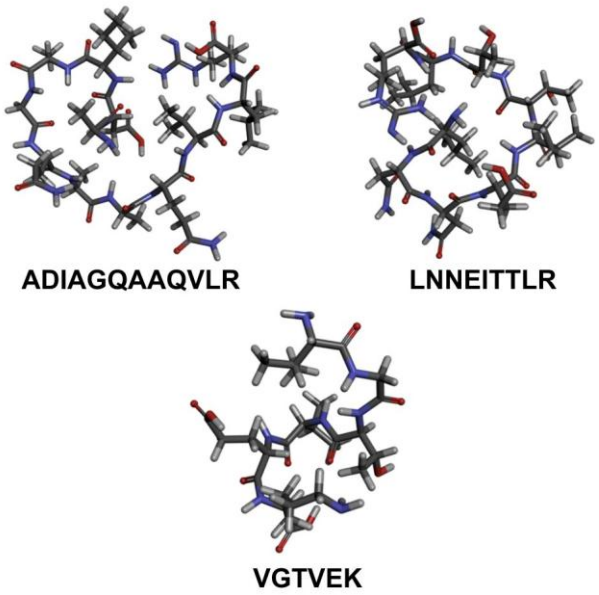

Fig. 2. The 3D structures of peanut worm peptide

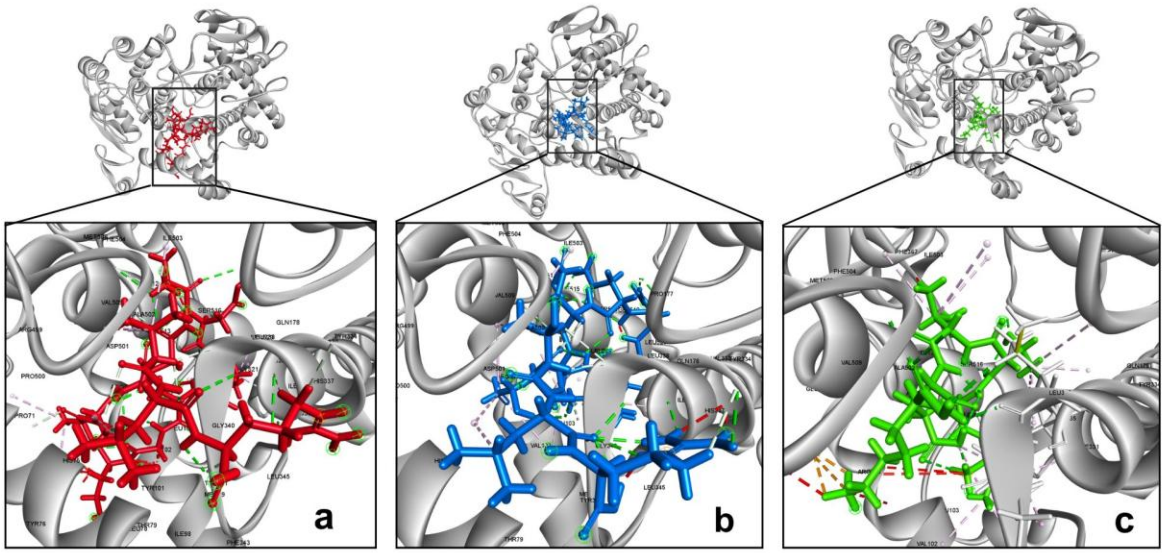

Fig. 3. The interaction of peanut worm peptide and COX-2; a) ADIAGQAAQVLR, b) LNNEITTLR, and c) VGTVEK. The grey ribbon was the visualization of COX-2, while red, blue, and green were ADIAGQAAQVLR, LNNEITTLR, and VGTVEK, respectively