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Characteristics and bioinformatics of peptides from natural and cultured sandfish (*Holothuria scabra*)

Pawitporn Daopa [a,b], Chakkapat Aenglong [b], Sittiruk Roytrakul [c], Teerasak E-kobon [d], Xue Zhao [a,*], Wanwimol Klaypradit [b,*]

[a] College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China
[b] Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Chatuchak, Bangkok 10900, Thailand
[c] Functional Proteomics Technology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathum Thani, Thailand
[d] Department of Genetics, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

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ABSTRACT

This study aimed to investigate the characteristics and bioinformatics identification of peptides in sandfish (*Holothuria scabra*) protein hydrolysate from natural and cultured sources, with the hypothesis that different sources of sandfish and enzymatic treatments would result in distinct peptide profiles. Sandfish from both sources were subjected to double enzymatic hydrolysis using neutral protease–alcalase (NA), papain–neutral protease (PN), and papain–alcalase (PA) to obtain protein hydrolysates. The hydrolysates were analyzed for amino acid composition, protein molecular weight distribution, functional groups using FTIR spectroscopy, LC-MSMS analysis coupled with bioinformatics for protein and peptide identification, and mineral profile. The resulting hydrolysates exhibited similar amino acid profiles, characterized by high levels of glycine, alanine, glutamic acid, and aspartic acid. The functional groups of the sandfish protein hydrolysates were primarily found at wavenumbers 1658 cm^{-1} (amide I), 1541 cm^{-1} (amide II), and 1246 cm^{-1} (amide III). Peptides with molecular weights below 500 Da predominated in every sample, highlighting their small molecular size. LC-MSMS analysis, coupled with bioinformatics database comparisons, indicated that sandfish from natural and cultured sources, as well as different enzymes used in the hydrolysis process, significantly affect protein and peptide pattern. Of the 2132 identified proteins, 1258 exhibited significant differences ($p < 0.05$, FDR < 0.05). This study provides insights into how source and enzymatic treatment influence peptide profiles, which could be applied in the development of functional ingredients or bioactive peptides from sandfish.

1. Introduction

Sea cucumber has been consumed for its nutritional and health benefits, including its use in traditional medicine and culinary practices (Doungapai et al., 2022; Li et al., 2021). It contains valuable compounds such as peptides, collagen, saponins, phenolics, glycoprotein, and chondroitin sulfates (Bordbar et al., 2011). These bioactive compounds exhibit various functional properties including anti-inflammatory (Kareh et al., 2018), anti-cancer (Janakiram et al., 2015), antioxidant (Althunibat et al., 2009), antifungal (Kumar et al., 2007), antidiabetic (Wang et al., 2020), anti-obesity (Guo et al., 2015), and anticoagulant effects (Popov et al., 2013), while also contributing to immune system support (Janakiram et al., 2015). Given its multifunctionality, sea cucumbers are promising candidates for applications in functional foods and nutraceuticals, owing to their bioactive compounds and nutritional value.

Holothuria scabra (Jaeger, 1833), commonly known as the sandfish, is a species of sea cucumber that is recognized as one of the most valuable marine invertebrates animal (Munprasit, 2009). Sandfish command high commercial value among tropical sea cucumbers used in food processing and consumption (Agudo, 2006). Sandfish is renowned for its high-quality protein content, rich in amino acids like glycine, proline, and glutamic acid (Sroyraya et al., 2017), while being relatively low in calories and fat. Currently, sandfish can be sourced from both natural and cultured environments, which may lead to differences in their characteristics due to various external factors, such as

* Corresponding authors.
E-mail addresses: zhaoxue@ouc.edu.cn (X. Zhao), ffiswak@ku.ac.th (W. Klaypradit).

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environmental conditions and diet. Cultured sandfish can be raised in specific locations with controlled farming practices, including dietary management (Agudo, 2006). In contrast, natural sandfish are typically found benthically along coastlines (Junus et al., 2018) or at depths not exceeding 20–30 m, where external factors cannot be controlled. To ensure comparability in this study, we selected sandfish samples that were matched based on specific criteria, such as age, size, and origin (natural or cultured). The cultured samples were sourced from a controlled aquaculture farm known for consistent farming practices, while the natural samples were collected from a coastal environment with minimal human influence. These criteria were chosen to minimize the variability introduced by environmental factors, ensuring that the observed differences in bioactive properties are primarily due to the differences between natural and cultured origins.

Protein or peptides constitute the crucial components in sandfish responsible for its functional properties, such as antioxidant (Doungapai et al., 2022), immune properties (Gowda et al., 2008), and anti-inflammatory effects (Rod-In et al., 2020). However, there remains limited information regarding the differences in these properties, particularly in the protein and peptides, between sandfish obtained from cultured and natural sources. Enzymatic hydrolysis is a widely employed process to enhance the biological or functional properties of proteins from various sources. Double enzymatic hydrolysis involves using two different enzymes, each targeting distinct bonds within the substrate. This dual approach leads to a more efficient and specific breakdown of the molecule, resulting in a more thorough and controlled hydrolysis process. By enhancing peptide bond cleavage and improving bioactivity, double enzymatic hydrolysis offers advantages over the use of a single enzyme (Schlegel et al., 2019). Schlegel et al. (2019) demonstrated that this method not only increases the effectiveness of the hydrolysis but also enhances antioxidant and antihypertensive activities compared to single enzyme hydrolysis.

Bioinformatics is a highly efficient tool for analyzing protein and peptide identification, characterization, and predicting bioactivities. According to Doungapai et al. (2022), their study used LC-MS/MS combined with bioinformatics to identify and characterize antioxidant and UV-B protective peptides from sea cucumber hydrolysate fractions obtained through gel filtration chromatography. Phetchthumrongchai et al. (2022) investigated the potential bioactivities of the identified peptides in protein hydrolysates of skipjack tuna roe using bioinformatics tools.

Currently, information on the differences in peptide characteristics between natural and cultured sources of sandfish is limited. Therefore, this research aims to study the effect conditions of double enzymatic hydrolysis for the production of sandfish peptide obtained from natural and cultured sources. Three commercial proteases (papain, alcalase, and neutrase) were used. The properties including amino acid composition, protein molecular weight distribution, FTIR spectroscopy, LC-MSMS analysis coupled with bioinformatics for protein and peptide identification, and elemental composition were investigated.

2. Materials and methods

2.1. Materials

Sandfish (*Holothuria scabra*) from both natural and cultured sources were treated in accordance with standard ethical procedures, with approval for animal care and use for scientific research obtained from Kasetsart University (ID no. ACKU66-FIS-001). Natural sandfish (*Holothuria scabra*) were collected from Yao Noi Island, Phang Nga Province, in the coastal regions of the Andaman Sea, Thailand. The natural sandfish population typically resides in coastal areas where environmental conditions such as water quality, temperature, salinity, and natural food sources are subject to natural variation. Cultured sandfish were obtained from the Coastal Fisheries Research and Development Center, Prachuap Khiri Khan Province, Thailand. These sandfish were raised in a controlled environment, where factors such as stocking density, water temperature, salinity, and artificial diet were carefully regulated to ensure optimal growth conditions. The sandfish were dissected, and the viscera were removed. The bodies were scrubbed to eliminate spicules, then cut into 1 × 1 cm pieces, dried. These pieces were dried and ground into powder using a grinding machine (CF-500 K, Jiangsu Yongli Machinery Co., Ltd., Yangzhou, China). The sandfish powder from each source were subjected into three treatments: NA, PN, and PA. For each treatment, the powder (30 g) was used for the enzymatic hydrolysis experiments.

2.2. Preparation of peptide from sandfish

The dried sandfish powder was hydrolyzed using double enzymatic hydrolysis. The powder was mixed with distilled water at a ratio of 1:20 (*w/v*), which facilitated the hydrolysis of protein molecules through the enzymatic treatment and subjected to further hydrolysis with double enzymatic treatment. The enzymes used were papain (Sigma-Aldrich, St. Louis, Missouri, USA), alcalase (Novozymes, Bagsværd, Denmark), and neutral protease (Labogene, Lynge, Denmark). Three conditions were tested: 1) NA (neutral protease, pH 6.0 - alcalase, pH 8.0), 2) PN (papain, pH 5.0 - neutral protease, pH 6.0), and 3) PA (papain®, pH 5.0 – alcalase, pH 8.0). For each treatment, the first enzyme was applied at 55 °C for 6 h, followed by the second enzyme at 55 °C for an additional 6 h. HCl was used to adjust the pH, while NaCl was used to maintain the ionic strength of the solution. The hydrolyzed solution was centrifuged using a centrifuge (SUPREMA 21, Tommy Seiko, Tokyo, Japan) at 4 °C, 10,000 ×g for 15 min. The resulting supernatants (peptide solutions) were then lyophilized using a freeze dryer (Coolsafe 95–15, Labogene, Lynge, Denmark). The hydrolysates were pre-frozen at −20 ± 1 °C overnight and subsequently freeze-dried under vacuum at −40 ± 1 °C for 24 h. The resulting peptide powder samples were labeled as N-NA, N-PN, N-PA, C-NA, C-PN, and C-PA, where 'N-' and 'C-' represent natural and cultured sandfish, respectively, followed by the specific enzyme combinations used. The dried peptide powders were transferred to glass bottles and stored in a desiccator for further analysis. The dried peptide powders were analyzed for peptide yield: N-NA (67.72 %), N-PN (75.58 %), N-PA (77.20 %); C-NA (68.49 %), C-PN (63.00 %), C-PA (71.24 %).

They were also analyzed for protein content, resulting in the following values: N-NA (94.35 g/100 g), N-PN (91.24 g/100 g), and N-PA (90.97 g/100 g); C-NA (94.29 g/100 g), C-PN (94.07 g/100 g), and C-PA (92.94 g/100 g).

2.3. Analysis of properties of the peptide powder

2.3.1. Amino acid profile

The amino acid composition of peptide measured by HPLC (1260 infinity series, Agilent, California, USA), equipped with TSKgel G3000SWXL column (5 μm, 7.8 mm ID × 30 cm) at 35 °C and detected for excitation at 230 nm and emission at 450 nm using Evaporative light scattering macromolecular characterization instrument (DAWN® HELEOS® II) detection.

Briefly, sample (30 mg) was hydrolyzed using 5 mL of 6 M HCl with 5 % 2-Mercaptoethanol (2-ME). The extract was added with solvent 0.02 M of HCl. The final volume of extracted samples was 50 mL. The extracted sample (1 mL) was transferred into a new tube and nitrogen was blown over it. The sample was diluted with 0.02 M HCl to a final volume of 2 mL. All samples were filtered through 0.22 μm syringe filter membrane. Mobile Phase A consisted of 10 mM di-sodium hydrogen phosphate (Na_2HPO_4), 10 mM sodium tetra-borate ($Na_2B_4O_7 \bullet 10H_2O$), and 5 mM sodium azide (NaN_3). Mobile Phase B consisted of acetonitrile, methanol, and water in the ratio (45:45:10 [*v*/v/v]). The flow rate was 1.0 mL/min. The content of amino acid was reported as g/100 g protein.

2.3.2. Molecular weight

The peptide sample (1 mg) was mixed with 1 mL of mobile phase consisted of acetonitrile, trifluoroacetic acid, and water in a ratio of 30/0.1/69.9 (*v*/v/v). The solution was then filtered through a 0.22-μm nylon membrane filter. For calibration of the HPSEC - High Pressure Size Exclusion Chromatography (1260 infinity series, Agilent, California, USA), five molecular mass markers were utilized: bovine serum albumin (66,000 Da), Cytochrome C (12,384 Da), bovine insulin (5733 Da), Bacitracin (1422 Da), and Glutathione (307 Da).

The peptide solution was injected with a volume of 20 μL into an HPLC system equipped with a UV detector set at 225-nm wavelength. Separation was achieved using a TSKgel 9200SWXL column (300 × 7.8 mm; AIT France, Paris, France) at 35 °C, employing the same mobile phase conditions as in sample preparation, at a flow rate of 0.5 mL/min. Peak areas were calculated using a standard curve equation to determine the average molecular weight.

2.3.3. FTIR

Fourier transform infrared (FTIR) spectroscopy was used to determine the functional groups and investigate the molecular vibrations of the peptide. Each functional group is associated with specific infrared absorption bands that correspond to its fundamental vibrational frequencies (Berthomieu & Hienerwadel, 2009). The FTIR spectra were obtained from KBr-discs that contained 1 mg of peptide sample in approximately 200 mg potassium bromide (KBr). A mixture of the sample and KBr was ground and well blended, then placed in a palletizer hydraulic press (RIIC-Beckmann, Glenrothes, Scotland) to form a miniature thin disc. The disc was then inserted in the FTIR Spectrometer (Invenio S, Bruker, Billerica, Massachusetts, USA). Spectra from 4000 to 400 cm-1(mid-IR region). The signals were automatically gathered and background deduction was accomplished with Opus software

2.3.4. LC-MSMS

Ultra-high performance liquid chromatography coupled with multiple mass spectrometry (UPLC-MS/MS) was used to analyze the peptide fingerprints of the peptide samples. The peptide (1 mg/mL dissolved in dH_2O) was added to acetonitrile (200 μL) and all samples were filtered through a 0.22 μm syringe filter membrane. Peptide solution was loaded onto a manually packed reverse phase C18 column (1.0 m × 150 mm 1.7 μm) coupled to ULTIMATE 3000 UPLC system chromatography (Thermo Fisher Scientific, Massachusetts, USA). The column temperature was set to 40 °C; the injection volume was 10 μL; and the injection time was 50 min. Mobile Phase A consisted of 0.1 % formic acid-acetonitrile and mobile Phase B consisted of 0.1 % formic acid-water. Peptide were eluted from 5 % - 80 % solvent B in solvent A at flow rate 0.15 mL/min. Mobile phase for gradient 0–2 min (Phase A: 2–27 min; Phase A eluted from 5 % to 80 %: 5 %-27–37 min; 10 %- 37–39 min; 20 %- 39–42 min; 80 % 42–43 min; 5 % 43–50 min). The eluted peptide was analyzed by Q-Exactive quadrupole-orbitrap ultra-high resolution mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA). Parameters were as follows: For full MS spectra, the scan range was *m/z* 200–1500 Da with a resolution of 70,000. MS/MS acquisition was performed in top speed mode with cycle 20, the resolution was 17,500; collision energy 20 eV, 35 eV, 45 eV; sheath gas (nitrogen) flow rate was 40 Arb; auxiliary gas flow rate was 10 Arb; The auxiliary gas heating temperature was 300 °C. Extracted ion current mass spectrometry was used to analyze the peptide content. Each sample underwent 3 replicates, and the average intensity was used as the final measurement to detect differences in protein pattern between groups using the web-based tool MetaboAnalyst (version 5.0; RRID: SCR_015539). Protein quantities obtained from LC-MSMS data in each sample were analyzed using MaxQuant (version 1.6.6.0; RRID: SCR_014485). MS/MS spectra were submitted to a database search against the UniProt *Holothuria* familiaris database for protein identification and using the Andromeda search engine with a significance threshold of $p < 0.05$. The ANOVA test with Tukey's post hoc analysis was used to compare different groups. The database was downloaded from UniProt on May 06, 2024. The MS proteomics data from this study have been submitted to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via jPOSTrepo partner (https://repository.jpostdb.org/entry/JPST003277) and can be found under the dataset identifier PXD054884.

2.3.5. Mineral profile (ICP-MS)

The peptide sample (0.5 g) was digested and mineralized by adding 65–68 % concentrated HNO_3 (10 mL). The mixture was heated in a water bath at 60 °C for 2 h. The digest was diluted with ultrapure water (100 mL) and filtered by 0.22 μm syringe filter membrane before the ICP-MS analysis.

The selected elements were measured using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, 8800 Triple Quadrupole ICP-MS, Agilent, Santa Clara, California, USA). Calibration curves were created using five different concentrations to perform a quantitative analysis of each peptide sample.

2.4. Statistical analysis

Sandfish powder from both natural (N) and cultured (C) sandfish was digested with three enzymes: NA, PA, and PN, resulting in six treatment groups: N-NA, N-PA, N-PN, C-NA, C-PA, and C-PN. Each sample was analyzed for properties in triplicate as technical replicates. Data are presented as the mean ± SD. Statistical analyses were performed using one-way ANOVA ($p < 0.05$), followed by Duncan's New Multiple Range Test (Duncan, 1955) to compare the means of the treatment groups.

3. Results and discussion

3.1. Amino acid composition

The amino acid composition of sandfish protein hydrolysate, peptides, is shown in Table 1. Among the sixteen identified amino acids, there are eight essential amino acids (EAAs) and eight non-essential amino acids (NEAAs). The ratio of EAAs to NEAAs is approximately 1:3. The peptide samples are rich in glycine, alanine, glutamic acid, and aspartic acid (Gly, Ala, Glu, and Asp, respectively). Glycine was identified as a major amino acid in all samples, ranging from 32.39 to 34.62 g/100 g protein, and is particularly abundant in structural collagen (González-Ortiz et al., 2001), which has been recognized as the primary protein source in many sea cucumber species, including those from the genera *Holothuria*, *Parastichopus*, *Thelenota*, *Actinopyga*, and *Bohadschia* (Bordbar et al., 2011; Roggatz et al., 2018; Wen et al., 2010). This finding is consistent with our experimental results, which also showed high protein content. Alanine is one of the most abundant free amino acids found in aquatic animals and contributes significantly to their flavor and taste. Glutamic acid, together with ribonucleotides, contributes to the umami flavor, which is one of the five basic tastes. Umami is often described as a savory or meaty taste, and it plays a key role in enhancing the overall flavor profile of foods (Prescott, 2001). Aspartic acid can also contribute to the flavor profile of foods, although its impact is generally less pronounced than other amino acids like glutamic acid. It may enhance the overall taste experience in protein-rich foods.

Our study aligns with previous research by Doungapai et al. (2022) who reported that the *H. scabra* hydrolysate was rich in Glutamic acid + glutamine and cysteine that showed 8.32 and 8.22 g of amino acids per 100 g of hydrolysate, respectively. García et al. (2019) investigated the amino acid profile of a two-step hydrolysate (Protamex/Pepsin) derived from whole *H. forskali* organisms. The most abundant amino acids identified were glycine, glutamic acid, and aspartic acid. Mildenberger et al. (2021) studied the amino acid composition of *P. tremulus* hydrolysate, glutamic acid was present in the highest concentration, with glycine and followed by lysine. Our study is also related to reports on the amino acid content in dried sandfish without hydrolysis. Sroyraya et al. (2017) identified glutamic acid, arginine, and aspartic acid as the

Table 1
Amino acid profile of peptides from natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using two-step enzymatic hydrolysis.

Amino acid		Amino acid content (g/100 g protein)					
		N-NA	N-PN	N-PA	C-NA	C-PN	C-PA
Essential amino acids (EAA)							
Histidine	(HIS)	0.48 ± 0.00^{c}	0.65 ± 0.00^{a}	0.51 ± 0.01^{b}	0.52 ± 0.01^{b}	0.52 ± 0.01^{b}	0.48 ± 0.00^{c}
Isoleucine*	(ILE)	1.55 ± 0.00^{a}	1.45 ± 0.01^{c}	1.47 ± 0.00^{b}	1.48 ± 0.0.1^{b}	1.44 ± 0.00^{c}	1.42 ± 0.00^{c}
Leucine*	(LEU)	2.34 ± 0.00^{f}	2.78 ± 0.02^{d}	2.86 ± 0.00^{c}	2.96 ± 0.00^{a}	2.76 ± 0.01^{e}	2.92 ± 0.00^{b}
Lysine	(LYS)	1.35 ± 0.01^{f}	1.94 ± 0.00^{a}	1.68 ± 0.01^{d}	1.72 ± 0.01^{c}	1.77 ± 0.01^{b}	1.62 ± 0.00^{e}
Methionine*	(MET)	1.52 ± 0.01^{a}	1.05 ± 0.01^{b}	0.98 ± 0.02^{c}	0.97 ± 0.01^{c}	0.76 ± 0.00^{e}	0.94 ± 0.01^{d}
Phenylalanine*	(PHE)	1.58 ± 0.02^{b}	1.61 ± 0.02^{a}	1.40 ± 0.00^{d}	1.39 ± 0.01^{d}	1.46 ± 0.01^{c}	1.34 ± 0.00^{e}
Threonine	(THR)	5.09 ± 0.00^{a}	4.94 ± 0.00^{d}	5.02 ± 0.01^{b}	4.86 ± 0.00^{e}	4.98 ± 0.01^{c}	4.83 ± 0.01^{f}
Valine*	(VAL)	5.83 ± 0.04^{a}	4.22 ± 0.01^{b}	3.92 ± 0.01^{d}	3.81 ± 0.00^{e}	4.16 ± 0.02^{c}	3.76 ± 0.00^{f}
Non-essential amino acids (NEEA)							
Arginine*	(ARG)	5.45 ± 0.01^{f}	5.65 ± 0.00^{d}	5.74 ± 0.02^{b}	5.80 ± 0.02^{a}	5.69 ± 0.01^{c}	5.80 ± 0.01^{a}
Alanine	(ALA)	13.16 ± 0.00^{f}	13.38 ± 0.02^{e}	13.50 ± 0.01^{c}	13.47 ± 0.01^{d}	13.52 ± 0.02^{b}	13.57 ± 0.00^{a}
Aspartic acid	(ASP)	7.79 ± 0.01^{e}	7.86 ± 0.01^{c}	8.00 ± 0.01^{a}	7.87 ± 0.01^{c}	7.97 ± 0.01^{b}	7.84 ± 0.01^{d}
Glutamic acid	(GLU)	12.81 ± 0.02^{a}	12.44 ± 0.00^{d}	12.58 ± 0.01^{c}	12.75 ± 0.01^{b}	12.82 ± 0.00^{a}	12.76 ± 0.00^{b}
Glycine	(GLY)	32.39 ± 0.01^{e}	33.87 ± 0.00^{d}	34.24 ± 0.01^{b}	34.24 ± 0.01^{b}	33.98 ± 0.02^{c}	34.62 ± 0.01^{a}
Serine	(SER)	5.09 ± 0.03^{a}	4.69 ± 0.01^{e}	4.75 ± 0.01^{c}	4.72 ± 0.02^{d}	4.84 ± 0.00^{b}	4.63 ± 0.00^{f}
Tyrosine*	(TYR)	1.90 ± 0.00^{a}	1.63 ± 0.04^{b}	1.49 ± 0.02^{d}	1.47 ± 0.01^{e}	1.56 ± 0.02^{c}	1.43 ± 0.00^{f}
Proline*	(PRO)	1.83 ± 0.00^{d}	2.00 ± 0.01^{b}	2.00 ± 0.01^{b}	2.10 ± 0.02^{a}	1.93 ± 0.01^{c}	2.10 ± 0.00^{a}
EAA (%)		25.20	24.29	23.60	23.54	23.54	23.11
NEEA (%)		74.99	75.89	76.58	76.64	76.64	76.96
HPB* (%)		32.04	30.90	30.50	30.63	30.34	30.41
HPL (%)		70.94	72.68	73.03	73.01	73.10	73.04

Values are presented as mean ± SD (n = 3). Means in the same row with different superscripts are significantly different ($p < 0.05$). EEA = Essential amino acid, NEEA = non-essential amino acid, HPB = hydrophobic amino acids, HPL = hydrophilic amino acids.

predominant free amino acids in both the whole body and body wall of dried sandfish, with glutamic acid present in the highest concentrations. The high glutamic acid content likely enhances the taste of sea cucumber, contributing to its umami flavor, which is produced by glutamic acid and ribonucleotides and is recognized as a basic taste (Prescott, 2001). Rahael et al. (2019) found that glycine, glutamate, aspartic acid, alanine, and arginine had the highest amino acid content among the amino acids analyzed in dried sandfish. Additionally, Ardiansyah et al. (2020) reported that glycine, proline, and glutamic acid were the most abundant amino acids in dried sandfish from various coastal areas.

In the present study, the hydrolyzed sandfish samples contained approximately 70.94 % to 73.10 % hydrophilic amino acids and 30.34 % to 32.04 % hydrophobic amino acids. This results in a hydrophilic to hydrophobic amino acid ratio of 7:3, indicating a higher proportion of hydrophilic amino acids. Typically, the hydrophilic amino acids increase charge density, which enhances solubility (Berti et al., 2007). In addition, polar and non-polar amino acids affect antioxidant activity, with greater hydrophobicity leading to increased antioxidant activity (Zou et al., 2016).

Overall, the main types of amino acids did not differ significantly, though there were slight variations in their quantities. The types of enzymes and sources of sea cucumbers generally maintained a

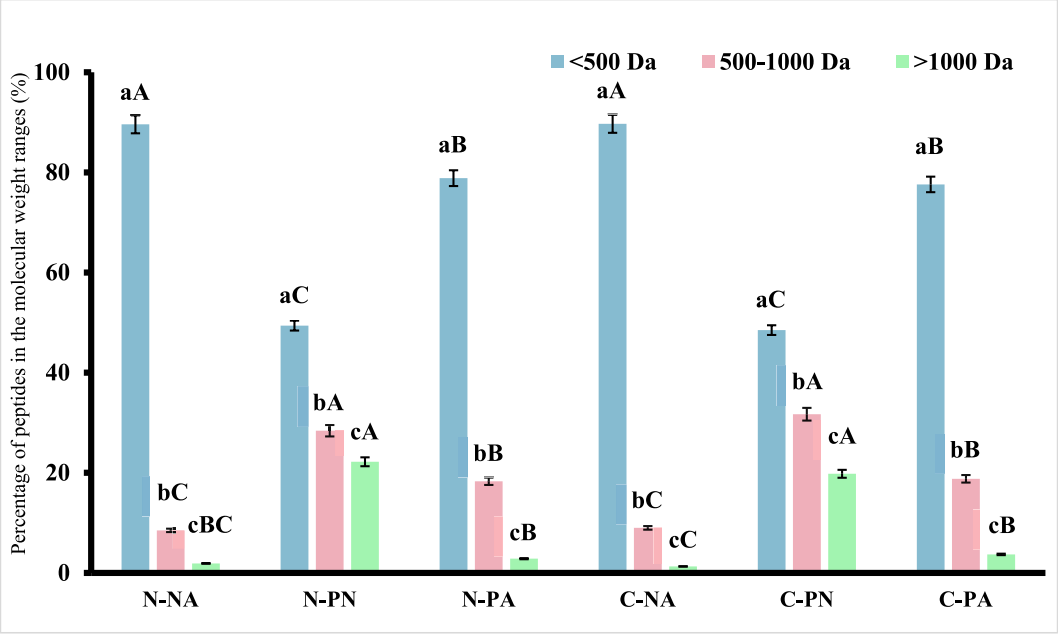

Fig. 1. Percentage of peptide in the molecular weight ranges from natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using two-step enzymatic hydrolysis; NA: neutral protease-alcalase, PA: papain-alcalase, and PN: papain-neutral protease. The standard deviation of the mean ($n = 3$) is represented by the error bars. Different lowercase letters indicate significant differences in the range of protein molecular weight within the same sample ($p < 0.05$). Different uppercase letters indicate significant differences between samples within the same range of protein molecular weight ($p < 0.05$).

consistent amino acid composition, with only minor differences in amounts. Additionally, all samples contained a higher percentage of hydrophilic amino acids, which may enhance peptide solubility (Trevino et al., 2007). This increased solubility is attributed to the hydrophilic nature of these amino acids, which interact effectively with water molecules. This property is extremely important in various biological processes and applications, including pharmaceutical formulations, cosmetic ingredients, and functional food, where solubility affects the absorption and function of proteins and peptides. Structural characteristics like molecular weight, hydrophobicity, amino acid composition, and peptide sequence are generally thought to be indicators of a peptide's bioactive potential (Zou et al., 2016).

3.2. Protein molecular weight distribution

The molecular weight distribution of sandfish protein hydrolysate was analyzed by HPSEC, and the results are presented in Fig. 1. Sandfish protein hydrolysate has a low molecular weight, comprising mostly peptides with Mw below 500 Da, followed by peptides with Mw between 500 and 1000 Da, and peptides with Mw greater than 1000 Da. Peptides vary in structure and function based on their molecular weight (Mw). Peptides with Mw below 500 Da, such as dipeptides and tripeptides, have simple linear structures, making them highly bioavailable and effective in basic functions like antioxidant and antimicrobial activities. Their small size allows for rapid absorption and quick biological effects, which is particularly beneficial in functional foods. In contrast, peptides in the 500–1000 Da range exhibit more complex structures, enabling interactions with specific biological targets such as enzymes and receptors, and demonstrating bioactivities like ACE inhibition. Larger peptides, with Mw above 1000 Da, have intricate tertiary or quaternary structures, contributing to food texture, gelation, and serving as carriers for bioactive compounds. Thus, peptide size directly influences their structural complexity, bioavailability, and functional potential.

The study results demonstrate a similar pattern. Mw below 500 Da showed the highest percentages: N-NA (89.60 %), N-PN (49.40 %), N-PA (78.85 %), C-NA (89.70 %), C-PN (48.50 %), and C-PA (77.60 %). For Mw between 500 and 1000 Da, the percentages were: N-NA (8.50 %), N-PN (28.40 %), N-PA (18.31 %), C-NA (9.00 %), C-PN (31.70 %), and C-PA (18.80 %). The peptides with Mw greater than 1000 Da showed the lowest percentage compared to those with Mw below 500 Da and between 500 and 1000 Da. It is noteworthy that the use of the NA enzyme resulted in a higher percentage of peptides with Mw below 500 Da compared to the use of PA and PN enzymes for both sources of sandfish. The main factor influencing the molecular weight distribution might relate to the type and mechanism of the enzymes used for the hydrolysis process. The mechanism of action of neutral protease enzymes closely resembles that of alcalase. Both enzymes are serine proteases that break down proteins by forming an acyl-enzyme intermediate through a catalytic triad consisting of serine, histidine, and aspartate or glutamate residues to catalyze the hydrolysis of peptide bonds within protein substrates (Tacias-Pascacio et al., 2020). This process releases smaller peptides and amino acids, which are often more biologically active. The results indicate that the use of alcalase predominantly produces smaller peptides compared to when alcalase is not used. e Silva et al. (2017) confirmed that alcalase digested mostly small peptides, with 70 % of the peptides in the range of 500–1500 Da, and total sizes less than 4000 Da.

Sea cucumber peptides tend to be found as small molecules, as in our study, the highest percentage peptides molecular weights were determined to be below 500 Da. Wang et al. (2022) reported sea cucumber (*Cucumaria frondosa*) gonad hydrolysates are mainly composed of peptides with Mw below 3 kDa (98.84 %), and peptides below 500 Da accounted for 32.53 %. The molecular weight of the peptide can affect its biological activity. In a study by Mildenberger et al. (2021), the molecular weights of the Norwegian sea cucumber (*P. tremulus*) fractions ranged from 103 kDa to below 1 kDa. The fractions exhibit antioxidant activity and are suitable for hydrogel production.

There have been reports that peptides with a molecular weight below 1 kDa from sea cucumber exhibit many bioactivities, such as ACE inhibitory effect, antioxidant activity, immunomodulatory effect, and inhibition of the growth of MCF-7 tumor cells in zebrafish (He et al., 2016; Li et al., 2019; Wei et al., 2021; Zhong et al., 2018). Sun et al. (2017) reported that as the molecular weight decreased, the ability of sea cucumber (*Stichopus japonicus*) ovum hydrolysates to bind iron increased significantly from 55.7 % to 92.1 %. Furthermore, the percentage of the small fraction between 200 and 1000 Da showed a positive association with iron-binding activities, using correlation analysis. According to their structural properties, amino acid composition, charge, and sequence, these low molecular weight molecules exhibit a range of biological properties. The molecular weight of these molecules plays a crucial role in determining their specific biological activities and interactions (Korhonen & Pihlanto, 2006).

3.3. FTIR spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was employed to provide more details regarding the functional groups and examine molecular vibrations of the peptides. The FTIR spectra of the six samples are shown in Fig. 2. The functional groups of peptides from both sources were mainly observed at wavenumbers 1658 cm^{-1} (amide I), 1541 cm^{-1} (amide II), and 1246 cm^{-1} (amide III). The ranges of amide I, II, and III are important for determining the structure of the peptide (Ji et al., 2020). The most crucial aspect of analyzing the structure of proteins is the amide I wavenumber, which ranges from 1600 to 1660 cm^{-1}. This wavenumber is associated with the carbonyl (C=O) stretching vibration of the peptide chain. Amide II (1550–1480 cm^{-1}) is associated with C—N stretching and N—H bending, whereas amide III (1320–1220 cm^{-1}) plays an important role in the triple helix structure, this information can be used to predict that the material may originate from a starting substance with a triple helix or collagen structure. It is related to C—N stretching and N—H bending (Berisio et al., 2002; Nalinanon et al., 2011). The functional groups identified in our samples coincide with those found in sea cucumbers. Po-Hsien Li et al. (2020) reported that collagen from the body wall of the sea cucumber *H. cinerascens* exhibited similar results: 1656 cm^{-1} (amide I), 1534 cm^{-1} (amide II), 1237 cm^{-1} (amide III), 3400 cm^{-1} (amide A), and 3084 cm^{-1} (amide B). Fitri et al. (2024) investigated the FTIR analysis for functional groups between fresh and dried sandfish (*H.scabra*). FTIR spectra of sandfish extracts reveal strong absorptions at 3000–3500 cm^{-1} for carbonyl (C=O) and hydroxyl (OH), with a distinct peak at 1700 cm^{-1} for carbonyl. The 2800–2900 cm^{-1} range shows methylene (CH_2) stretching vibrations, while 1600 cm^{-1} indicates olefinic groups (alkene). Fresh sandfish extracts exhibit additional peaks at 1400 cm^{-1} for aromatic groups, 1100 cm^{-1} for C—N stretching amine, and 600 cm^{-1} potentially for hydrogen bending in hydroxyl groups (Fitri et al., 2024).

However, the side chains of amino acids are also crucial for analyzing FTIR results, as they can help predict amino acid composition. While the exact wavenumbers may not match perfectly, they can still provide comparable information when considered within a range. Specifically, the amide II region is effective in determining the profiles of glutamic acid and asparagine at wavenumbers 1570 cm^{-1} and 1559 cm^{-1}, respectively. These are the main amino acids found in sandfish protein hydrolysates. In addition, the contribution of the arginine side chain is more accurately identified in the amide I region, with bands at 1673 and 1646 cm^{-1} (Meutter & Goormaghtigh, 2021). The results indicated that the functional groups identified in sea cucumber peptides signify the presence of amino acids, carboxyl groups, and peptide bonds.

3.4. Proteomic analysis of the sandfish protein hydrolysate by LC-MSMS

Proteomic techniques that utilize data from LC-MS/MS to analyze protein identification have been developed and applied to identify potential biomarker candidates, without requiring prior knowledge of the

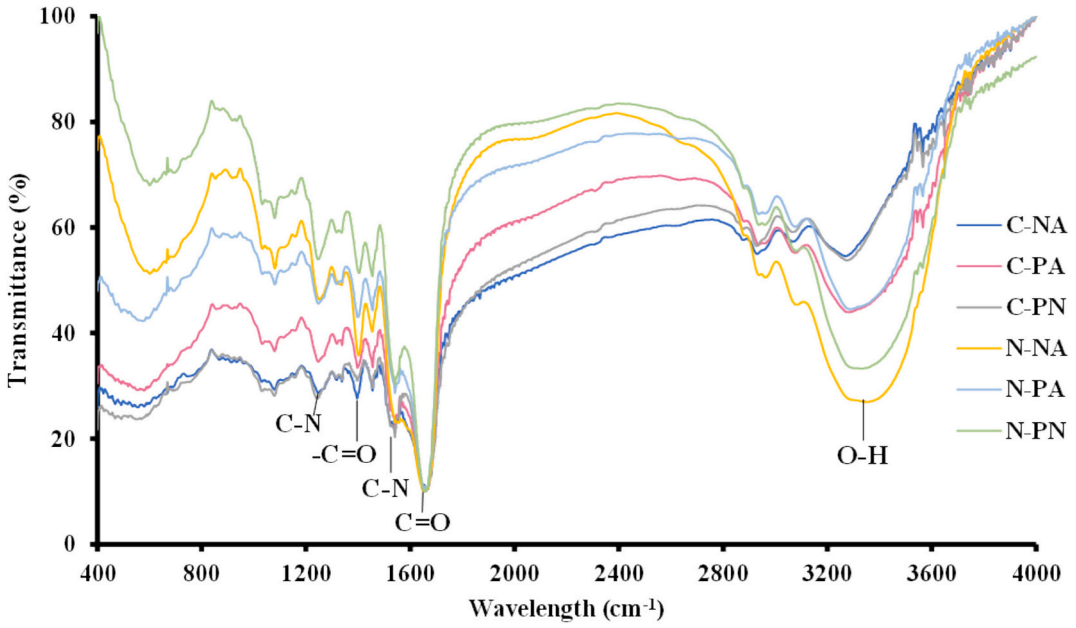

Fig. 2. FTIR spectra of protein hydrolysates from natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using two-step enzymatic hydrolysis; NA: neutral protease-alcalase, PA: papain-alcalase, and PN: papain- neutral protease.

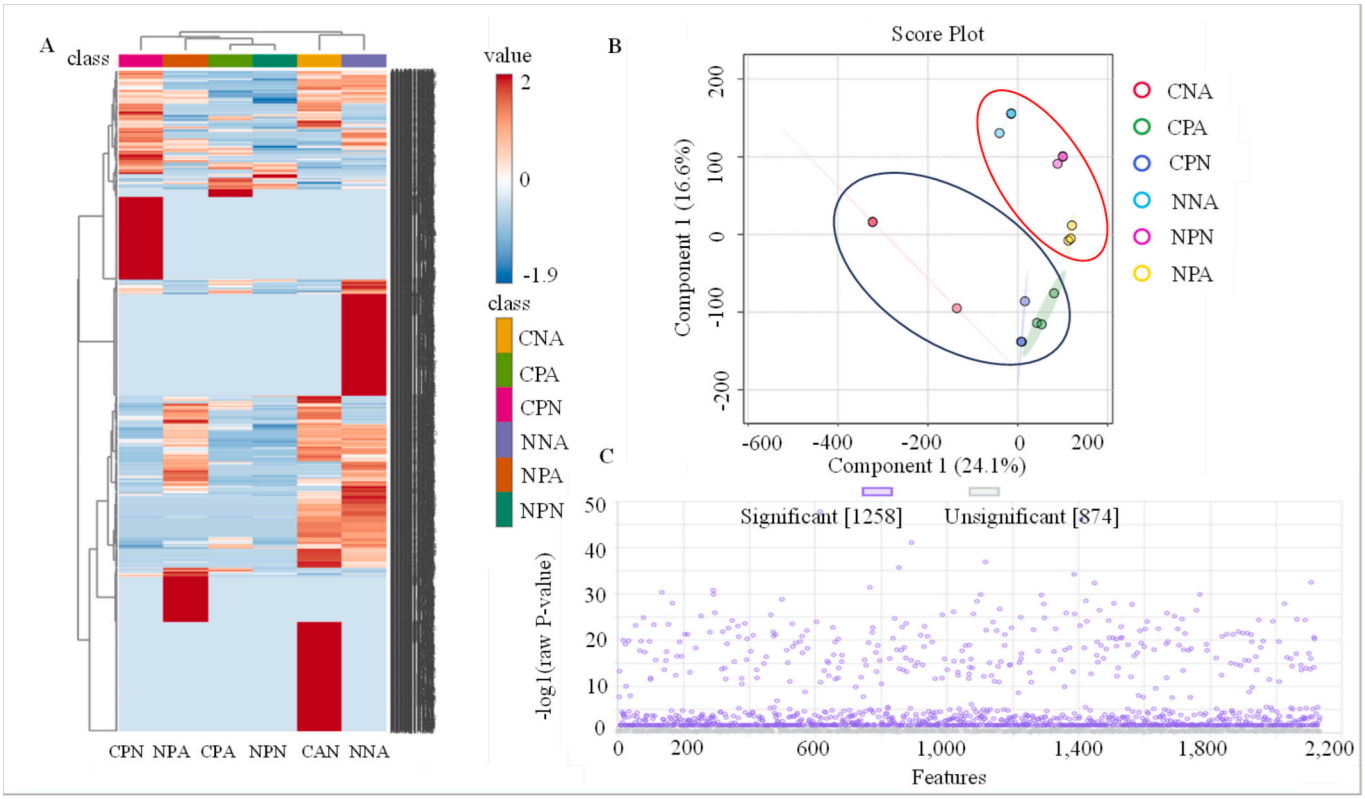

Fig. 3. A. Heat map of all identified proteins of protein hydrolysates from the natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using two-step enzymatic hydrolysis; NA: neutral protease-alcalase, PA: papain-alcalase, and PN: papain- neutral protease. The identified proteins are shown in rows, whereas the groups are organized in columns. Protein intensity is represented by colour, ranging from very low (deep blue) to extremely high (red), with the colour scale on the right indicating the range of expression value; **B.** Partial least squares-discriminant analysis (PLS-DA) was conducted on all identified proteins, with samples clustered according to groups: N-NA, N-PA, N-PN, C-NA, C-PA, and C-PN; **C.** The Analysis of Variance (ANOVA) plot illustrates the comparison among the six sample groups of sandfish for significantly identified proteins. The purple dots indicate significantly expressed proteins with $p < 0.05$, while the grey dots represent proteins without statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

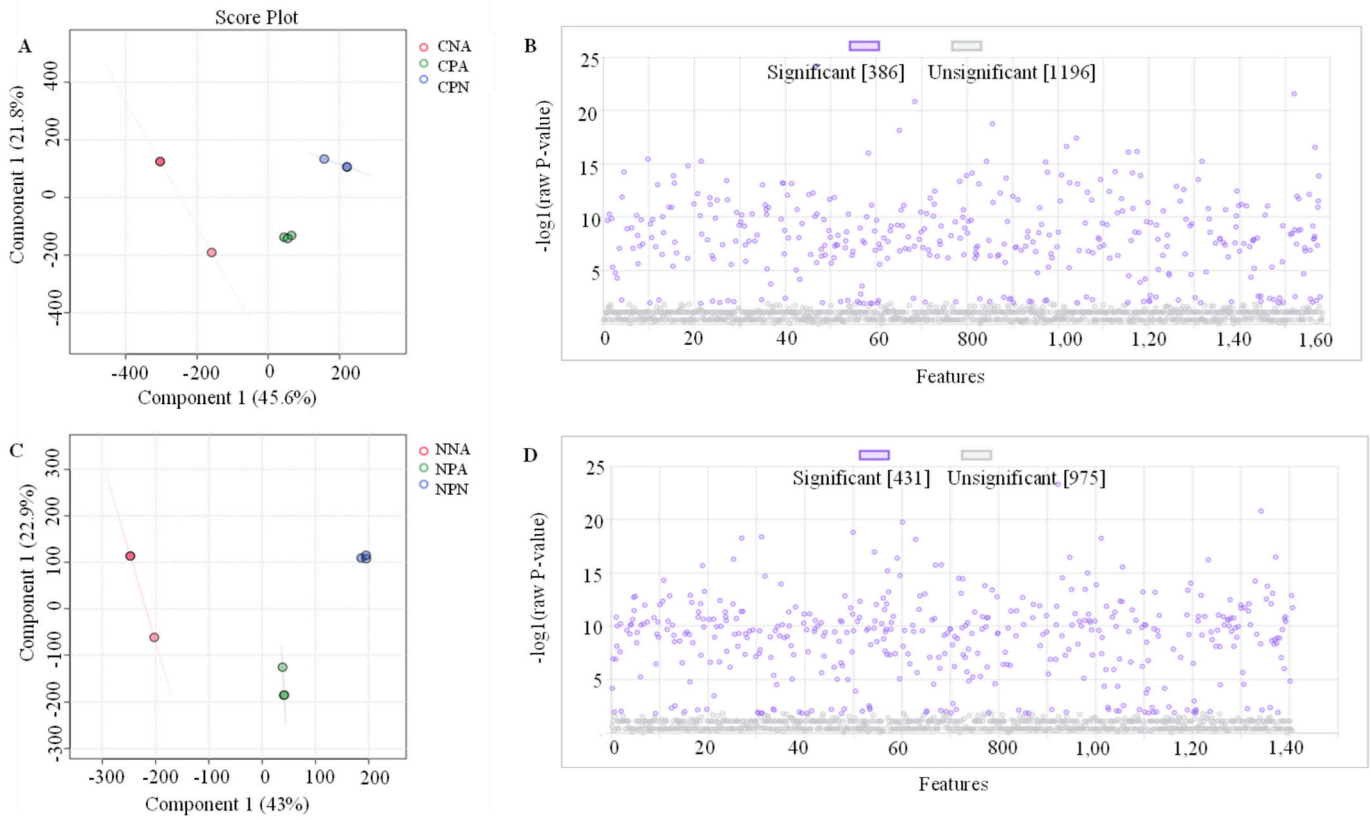

Fig. 4. **T**he PLS-DA score plot of the proteomics dataset of protein hydrolysates compares sea cucumbers sourced from the same origins but subjected to different enzymes in natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using a two-step enzymatic hydrolysis; NA: neutral protease-alcalase, PA: papain-alcalase, and PN: papain- neutral protease. The samples clustered according to groups: **A.** The cultured sandfish and **C.** natural sandfish. The Analysis of Variance (ANOVA) plot illustrates the comparison among the sample groups of **B.** cultured sandfish and **D.** natural sandfish for significantly identified proteins. An ANOVA test exhibited significant differences among the groups. The purple dots indicate significantly expressed proteins with $p < 0.05$, while the grey dots represent proteins without statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

target proteins. These methods allow for the identification of a wide range of proteins from samples (Wheelock et al., 2013) and can also provide valuable information. Fig. 3–5 demonstrated the comparisons of identified protein based on the source of the samples and the enzymes used in the hydrolysis process. A total of 2132 proteins were identified, as shown in the heat map (Fig. 3A). The partial least squares-discriminant analysis (PLS-DA) was conducted on all identified proteins, with samples clustered according to groups: N-NA, N-PA, N-PN, C-NA, C-PA, and C-PN (Fig. 3B). The PLS-DA results demonstrated a good separation of identified proteins between groupings. There was no overlap between cultured sandfish and natural sandfish. Similarly, there was no overlap between natural and cultured samples digested with different enzymes. Based on the database search, 1832 peptides were detected as part of identified proteins, meanwhile the remaining 300 peptides exhibited uncharacterized proteins. The ANOVA test with Tukey's post hoc analysis revealed that 1258 out of 2132 proteins showed significant difference ($p < 0.05$, FDR < 0.05) among the natural and cultured sandfish groups (Fig. 3C).

To underline the point that different enzymes produce various proteins patterns, Fig. 4. shows significant support. The overall count of recognized proteins was 1582 (Fig. 4B) and 1406 (Fig. 4D) for the cultured and natural sandfish, respectively. Sandfish from the same source but treated to different enzymes, were compared using a PLS-DA score plot on a proteomics dataset. The samples divided into two groups, cultured sandfish (Fig. 4A) and natural sandfish (Fig. 4C). The Analysis of Variance (ANOVA) plot illustrates the comparison among the sample groups of cultured sandfish (Fig. 4B) revealed that 386 out of 1582 identified proteins and natural sandfish (Fig. 4D) revealed that 431 out of 1406 identified proteins showed significant difference ($p < 0.05$, FDR < 0.05) among the groups. The results indicated that enzymatic modification of proteins depends significantly on the specific enzyme employed during hydrolysis, as mentioned by Senadheera et al. (2021). However, each enzyme has limitations in achieving optimal peptide fragmentation through the cleavage of peptide bonds. Papain, cleaves peptide bonds within polypeptide chains, works poorly in alkaline conditions and may cause over-hydrolysis at higher concentrations, reducing peptide functionality (Yuan et al., 2018). Alcalase, while selective for hydrophobic residues, produces a narrow peptide profile and its activity decreases at higher concentrations, limiting its ability to effectively cleave bonds and thus reducing bioactivity (Bun et al., 2020). Neutral protease, although efficient at neutral pH, often leads to incomplete hydrolysis, resulting in less control over peptide size, especially at higher concentrations (Sarmadi et al., 2015). To address these limitations, combining these enzymes in double enzymatic hydrolysis helps mitigate these drawbacks by enhancing the efficiency and diversity of peptide fragmentation, thus improving bioactivity through complementary actions (Schlegel et al., 2019).

To emphasize that different sandfish sources result in different proteins pattern, Fig. 5. demonstrates the PLS-DA results for identified protein of sandfish hydrolysates hydrolyzed with the same enzymes: NA (Fig. 5A), PN (Fig. 5C), and PA (Fig. 5E). The identified proteins were well-separated among the various groups, with no overlap between samples in each group. The Volcano plot based on proteomics data sets identified the protein exhibited significant differences among the groups of NA (Fig. 5B), C-NA illustrated that 95 identified proteins showed significant difference ($p < 0.05$, FDR < 0.05), while N-NA indicated that 122 identified proteins exhibited significant difference ($p < 0.05$, FDR < 0.05). For the PN group (Fig. 5D), C-PN and N-PN revealed 210 and 8

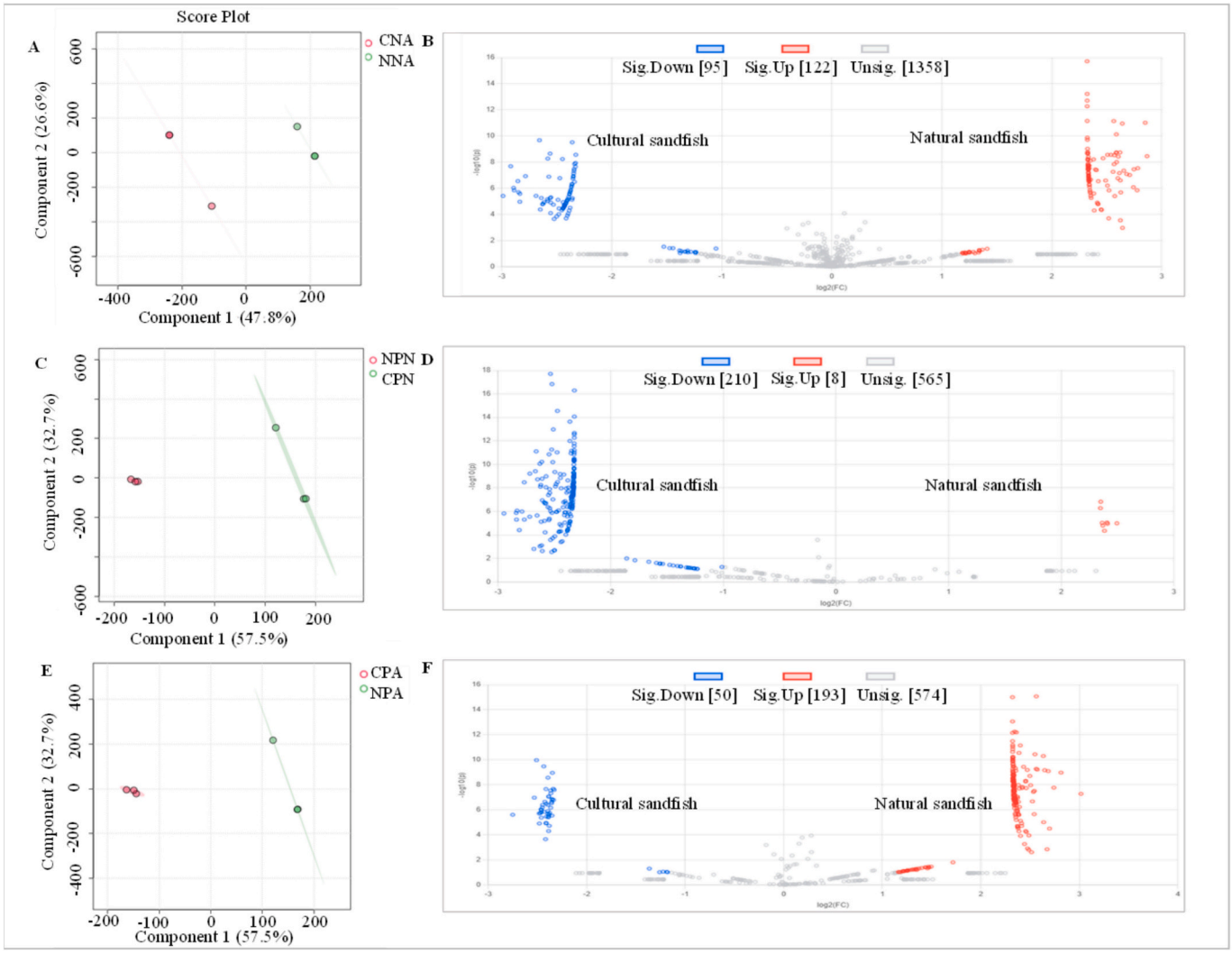

Fig. 5. The PLS-DA score plot, based on the proteomics dataset of protein hydrolysates, compares sandfish sourced from different origins but subjected to the same enzyme in natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using a two-step enzymatic hydrolysis; NA: neutral protease-alcalase, PA: papain-alcalase, and PN: papain- neutral protease for significantly identified proteins. The samples clustered according to groups: **A.** The NA; **C.** PN; and **E.** PA groups. The Volcano plot identified the protein exhibited significant differences among the groups of **B.** NA; **D.** PN; and **F.** PA. The blue dots demonstrate significantly expressed proteins with $p < 0.05$ of cultured sandfish, while the red dots demonstrate significantly expressed proteins with $p < 0.05$ of natural sandfish, and the grey dots represent proteins without statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identified proteins, respectively, with significant difference ($p < 0.05$, FDR < 0.05). For the PA group (Fig. 5F), C-PA and N-PA revealed 50 and 193 identified proteins, respectively, showing significant difference ($p < 0.05$, FDR < 0.05).

The protein identification results indicate that peptide patterns in sandfish is influenced by various factors, including habitat, feed, water quality, farming conditions and other environmental conditions. Additionally, genetic factors, such as different genotypes or species variants, also play a role. Ninwichian and Klinbunga (2020) studied the population genetics of sandfish from four locations in the Andaman Sea, Thailand. Their findings reveal that the gene pool of *H. scabra* in this region is genetically divided into two groups: Group A, consisting of samples from Phang Nga, and Group B, comprising samples from Krabi, Satun, and Trang. This suggests that sandfish from different sources can express distinct genes. Genetic differences affect the sequence of peptides. Different genes can code for proteins with varying amino acid sequences, resulting in peptides produced by hydrolysis having different sequences. Therefore, variations in gene sequences can lead to changes in both the sequence and nature of the resulting peptides (Harris & Wang, 2015). For the sandfish obtained from aquaculture can control the farming conditions. Currently, many countries have successfully cultivated sandfish. Purcell and Wu (2017) report the Large-scale sandfish aquaculture in multitrophic polyculture ponds in southern China. Serang et al. (2016) demonstrated that density stocking significantly effected on sandfish growth and survival. Broom et al. (2021) investigated alternative sources other than the use of macroalgal powder for formulating sea cucumber feed, fed with three diets containing different amounts of nutrients. The results of the experiment indicated that feeding different foods affects the growth of sandfish differently (Broom et al., 2021). Previous studies have identified various factors related to farming conditions that affect the growth of sandfish. However, there is currently limited proteomic research on sandfish in relation to these conditions. Therefore, it can only be speculated that farming conditions impact protein pattern. Meanwhile, the experimental results in our study clearly demonstrate that sandfish from different sources exhibit variations in factors affecting protein pattern and peptide sequences, as well as differences in the enzymes used for hydrolysis.

The results indicate a relationship between the analyzed factors of the experiment, showing that the amino acid composition had high

levels of glycine, alanine, glutamic acid, and aspartic acid, which are commonly associated with bioactive peptides. The molecular weight distribution suggested that enzymatic treatments reduced peptide size, thereby enhancing bioactivity. FTIR spectra confirmed structural changes in the hydrolysates, while LC-MS/MS analysis identified peptides and proteins, revealing differences between natural and cultured sandfish. Furthermore, the mineral profile may support these findings, as essential minerals could play a role in enhancing peptide bioactivity, which is why the mineral profile was analyzed, as discussed further.

3.5. ICP-MS

Eleven elements were demonstrated in the sandfish raw materials and sandfish protein hydrolysate by ICP-MS as shown in Table 2. Comparing sandfish raw materials and sandfish protein hydrolysate, hydrolyzed sandfish showed significantly different results with raw materials. All sandfish samples showed the following main minerals: sodium (Na), calcium (Ca), and magnesium (Mg). It found that sodium was the major component, which is consistent with previous research that studied the mineral composition of various types of sea cucumbers, Song et al. (2016) examined the sea cucumber (*Apostichopus japonicus*), and Haider et al. (2015) examined the sea cucumber (*Actinopyta mauritiana*). Rasyid et al. (2020) reported that in *H. scabra* without hydrolysis, calcium was the predominant element, with the order of abundance being Ca > Na > Mg > P > Fe. Haider et al. (2015) reported on minerals from *H.arenicola*, *Actinopyta mauritiana,* both species are rich sources of calcium, magnesium, sodium, and potassium. Gacia et al. (2019) examined the nutritional composition and peptide profile of two-step protein hydrolysate from whole *H. forskali* organisms and reported high value of the minerals calcium, potassium, and magnesium (examined 3 minerals).

In our study, we observed that the sodium elemental composition doubled after the hydrolysis process. This increase in sodium content may be attributed to the addition of NaCl used to adjust the pH during enzymatic hydrolysis. Potassium content showed a slight increase, magnesium and calcium, along with all other remaining elements, showed a decrease. The reduction in calcium content can be attributed to the presence of spicules, primarily composed of calcium carbonate, in the sandfish body wall. After scrubbing, some spicules may remain and contribute to the high calcium levels. However, the enzymatic hydrolysis process removes these spicules, leaving only the calcium naturally present in the body, which leads to the observed reduction in calcium content. Information on the mineral profile of sea cucumbers, particularly in sea cucumber protein hydrolysate, is limited. Gocer et al. (2018) investigated the mineral content in sea cucumbers, identifying key elements such as sodium, calcium, and magnesium.

Table 2
Elemental composition of peptides from natural (N-) and cultured (C-) sandfish (*Holothuria scabra*) using two-step enzymatic hydrolysis.

Element	Correlation	N-RAW	C-RAW	N-NA	C-NA
Na (g/kg)	Y = 1185.5720× + 202.0533 (R = 0.9997)	25.72 ± 0.97^{d}	31.72 ± 0.47^{c}	50.46 ± 0.35^{b}	60.57 ± 0.18^{a}
Mg (g/kg)	Y = 542.8598× - 40.0067 (R = 0.9997)	17.27 ± 0.44^{b}	21.13 ± 0.28^{a}	12.16 ± 0.74^{d}	13.79 ± 0.27^{c}
Al (g/kg)	Y = 179.2798× + 4.6633 (R = 0.9997)	0.30 ± 0.71^{a}	0.23 ± 0.72^{b}	0.18 ± 0.27^{d}	0.23 ± 0.27^{c}
K (g/kg)	Y = 275.3318× + 2456.3700 (R = 0.9997)	2.34 ± 0.67^{d}	3.43 ± 0.45^{b}	2.68 ± 0.64^{c}	4.14 ± 0.37^{a}
Ca (g/kg)	Y = 4.8685× + 1.6700 (R = 0.9996)	36.87 ± 0.18^{b}	50.31 ± 0.37^{a}	13.78 ± 0.74^{c}	12.2 ± 0.18^{d}
Cr (mg/kg)	Y = 5102.6194× + 101.6833 (R = 0.9992)	10.49 ± 0.27^{a}	9.81 ± 0.20^{b}	3.49 ± 0.18^{d}	5.94 ± 0.18^{c}
Mn (mg/kg)	Y = 2671.6035× + 45.0100 (R = 0.9993)	40.20 ± 0.74^{c}	76.05 ± 0.27^{a}	22.28 ± 0.20^{d}	62.70 ± 0.18^{b}
Fe (mg/kg)	Y = 4657.8761× - 20.7067 (R = 0.9994)	465.49 ± 0.35^{a}	326.81 ± 0.38^{c}	191.25 ± 0.45^{c}	337.05 ± 0.21^{b}
Zn (mg/kg)	Y = 1218.2328× - 199.0333 (R = 0.004)	127.33 ± 0.62^{c}	175.89 ± 0.18^{b}	88.60 ± 0.45^{d}	187.50 ± 0.18^{a}
Se (mg/kg)	Y = 36.9354× - 2.9967 (R = 0.9996)	27.65 ± 0.67^{a}	16.65 ± 0.45^{b}	9.11 ± 0.27^{d}	14.08 ± 0.27^{c}
Ba (mg/kg)	Y = 1311.0954× + 175.6933 (R = 0.9999)	25.94 ± 0.37^{a}	11.68 ± 0.47^{b}	2.42 ± 0.18^{d}	8.78 ± 0.18^{c}

NA: neutral protease-alcalase, PA: papain-alcalase, and PN: papain-neutral protease. Values are presented as mean ± SD (n = 3). Different lowercase letters indicate significant differences between samples within the same row ($p < 0.05$).

4. Conclusions

Using double enzymatic hydrolysis to produce peptides from both natural and cultured sandfish resulted in similar amino acid profiles, characterized by high levels of glycine, alanine, and glutamic acid. Approximately 70 % of the composition consisted of hydrophilic amino acids, indicating excellent water solubility. Peptides with molecular weights below 500 Da predominated in every sample, highlighting their small molecular size. LC-MSMS analysis, coupled with bioinformatics database comparisons, confirmed that enzymatic hydrolysis with different enzymes and sources of sandfish significantly affects protein and peptide pattern. The analysis also revealed the presence of elements such as sodium (Na), magnesium (Mg), potassium (K), and calcium (Ca). These findings emphasize the importance of considering variations in source and hydrolysis conditions when evaluating the functional properties of sandfish peptides. Given their amino acid composition and small molecular size, these peptides show potential as functional ingredients in food products, with possible in enhancing nutritional value, improving texture, and promoting health benefits.

CRediT authorship contribution statement

Pawitporn Daopa: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Chakkapat Aenglong:** Writing – review & editing, Investigation. **Sittiruk Roytrakul:** Methodology, Formal analysis. **Teerasak E-kobon:** Formal analysis. **Xue Zhao:** Supervision, Funding acquisition, Conceptualization. **Wanwimol Klaypradit:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The MS proteomics data from this study have been submitted to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via jPOSTrepo partner (https://repository.jpostdb.org/entry/JPST003277) and can be found under the dataset identifier PXD054884.

References

Agudo, N. S. (2006). *Sandfish hatchery techniques*. Secretariat of the Pacific Community and WorldFish Center, Nouméa, New Caledonia: Australian Centre for International Agricultural Research.

Althunibat, O. Y., Ridzwan, B. H., Taher, M., Jamaludin, M. D., Ikeda, M. A., & Zali, B. I. (2009). In vitro antioxidant and antiproliferative activities of three Malaysian sea cucumber species. *European Journal of Scientific Research, 37*, 376–387.

Ardiansyah, A., Rasyid, A., Siahaan, E. A., Pangestuti, R., & Murniasih, T. (2020). Nutritional value and heavy metals content of sea cucumber Holothuria scabra commercially harvested in Indonesia. *Nutritional Food Science Journal, 8*(3), 765–773. https://doi.org/10.12944/CRNFSJ.8.3.09

Berisio, R., Vitagliano, L., Mazzarella, L., & Zagari, A. (2002). *Crystal structure of the collagen triple helix model, 1*(2), 262–270. https://doi.org/10.1110/ps.32602

Berthomieu, C., & Hienerwadel, R. (2009). Fourier transform infrared (FTIR) spectroscopy. *Photosynthesis Research, 101*, 157–170. https://doi.org/10.1007/s11120-009-9439-x

Berti, C., Roncoroni, L., Falini, M. L., Caramanico, R., Dolfini, E., Bardella, M. T., & Forlani, F. (2007). Celiac-related properties of chemically and enzymatically modified gluten proteins. *Journal of Agricultural and Food Chemistry, 55*(6). https://doi.org/10.1021/jf062623n, 548 2482-2488.

Bordbar, S., Anwar, F., & Saari, N. (2011). High-value components and bioactives from sea cucumbers and functional foods – A review. *Marine Drugs, 9*, 1761–1805. https://doi.org/10.3390/md9101761

Broom, M., Gabr, M. H., Al-Harbi, M., & Satheesh, S. (2021). Effect of different diets on growth of the sub-adult sea cucumber Holothuria scabra. *Aquaculture International, 29*, 2403–2414. https://doi.org/10.1007/s10499-021-00756-9

Bun, S., Kannan, P., & Lee, J. (2020). Characterization of alcalase hydrolysis on marine proteins for bioactive peptides. *Food Chemistry, 320*, Article 126564. https://doi.org/10.1016/j.foodchem.2020.126564

Doungapai, C., Siriwoharn, T., Malila, Y., Autsavapromporn, N., Makkhun, S., Yarnpakdee, S., Jantanasakulwong, K., Regenstein, J. M., & Wangtueai, S. (2022). UV-B protective and antioxidant activities of protein hydrolysate from sea cucumber (Holothuria scabra) using enzymatic hydrolysis. *Frontiers in Marine Science, 9*, Article 892255. https://doi.org/10.3389/fmars.2022.892255

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics, 11*(1), 1–42. https://doi.org/10.2307/3001478

e Silva, F. G. D., Hernandez-Ledesma, B., Amigo, L., Netto, F. M., & Miralles, B. (2017). Identification of peptides released from flaxseed (Linum usitatissimum) protein by Alcalase® hydrolysis: Antioxidant activity. *Lwt – Food Science and Technology, 76*, 140–146. https://doi.org/10.1016/j.lwt.2016.10.049

Fitri, D. A., Purwanti, N., Nursid, M., & Patantis, G. (2024). Effects of sun and oven drying on the physicochemical composition of Indonesian sandfish (Holothuria scabra). *Squalen Bulletin of Marine Fishery and Postharvest Biotechnology, 19*(1), 13–21. https://doi.org/10.15578/squalen.886

García, J., Méndez, D., Álvarez, M., Sanmartin, B., Vázquez, R., Regueiro, L., & Atanassova, M. (2019). Design of novel functional food products enriched with bioactive extracts from holothurians for meeting the nutritional needs of the elderly. *LWT - Food Science and Technology, 109*(2019), 55–62. https://doi.org/10.1016/j.lwt.2019.03.097

Gocer, M., Olgunoglu, I. A., & Olgunoglu, M. P. (2018). A study on fatty acid profile and some major mineral contents of sea cucumber (Holothuria platyperona sanctori) from Medirerranean Sea (Turkey). *Food Science and Quality Management, 72*, 1–5.

González-Ortiz, M., Medina-Santillán, R., Martínez-Abundis, E., & von Drateln, C. R. (2001). Effect of glycine on insulin secretion and action in healthy first-degree relatives of type 2 diabetes mellitus patients. *Hormone and Metabolic Research, 33*(6), 358–360. https://doi.org/10.1055/s-2001-15421

Gowda, N. M., Goswami, U., & Khan, M. I. (2008). T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (Holothuria scabra): Possible involvement in differential recognition of bacteria. *Journal of Invertebrate Pathology, 99*(2), 141–145. https://doi.org/10.1016/j.jip.2008.04.003

Guo, L., Li, X., & Tang, Q. Q. (2015). Transcriptional regulation of adipocyte differentiation: A central role for CCAAT/enhancer-binding protein (C/EBP) β. *Journal of Biological Chemistry, 290*(2), 755–761. https://doi.org/10.1074/jbc.R114.619957

Haider, M. S., Sultana, R., Jamil, K., E-Zehra, L., Tarar, O. M., Shirin, K., & Afzal, W. (2015). *A study on proximate composition, amino acid profile, fatty acid profile and some mineral contents in two species of sea cucumber. The Journal of Animal & Plant Sciences, 25(1): 2015, Page: 168–175, (ISSN: 1018-7081)*.

Harris, R. A., & Wang, L. (2015). Genomics and proteomics in cancer research: Challenges and opportunities. *Journal of Proteome Research, 14*(5), 2313–2327. https://doi.org/10.1021/pr501235a

He, L. X., Zhang, Z. F., Sun, B., Chen, Q. H., Liu, R., Ren, J. W., & Li, Y. (2016). Sea cucumber (Codonopsis pilosula) oligopeptides: Immunomodulatoryeffects based on stimulating Th cells, cytokine secretion and antibody production. *Food & Function, 7*(2), 1208–1216. https://doi.org/10.1039/c5fa01480h

Janakiram, N. B., Mohammed, A., & Rao, C. V. (2015). Sea cucumber metabolites as potent anti- cancer agents. *Marine Drugs, 13*, 2909–2923. https://doi.org/10.3390/md13052909

Ji, Y., Yang, X. L., Ji, Z., Zhu, L. H., Ma, N., Chen, D. J., & Cao, Y. (2020). DFT-calculated IR Spectrum amide I, II, and III band contributions of N-methylacetamide fine components. *ACS Omega, 5*(15), 8572–8578. https://doi.org/10.1021/acsomega.9b04421

Junus, S., Kwong, P. J., & Khoo, G. (2018). A review on the recent advances in the biology and aquaculture technology of Holothuria scabra. *Journal of Survey in Fisheries Sciences.*. https://doi.org/10.18331/SFS2018.4.2.2

Kareh, M., Nahas, R. E., Al-Aaraj, L., Al-Ghadban, S., Al Deen, N. N., Saliba, N., El-Sabban, M., & Talhouk, R. (2018). Anti-proliferative and anti-inflammatory activities of the sea cucumber Holothuria polii aqueous extract. *SAGE Open Medicine, 6*. https://doi.org/10.1177/2050312118809541, 2050312118809541.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*(9), 945–960. https://doi.org/10.1016/j.idairyj.2005.10.012

Kumar, R., Chaturvedi, A. K., Shukla, P. K., & Lakshmi, V. (2007). Antifungal activity in triterpene glycosides from the sea cucumber Actinopyga lecanora. *Bioorganic & Medicinal Chemistry Letters, 17*(15), 4387–4391. https://doi.org/10.1016/j.bmcl.2006.12.052

Li, M., Gao, Y., Qi, Y. X., Song, Z. Y., Li, Z. B., Lin, Y. T., & Zhang, Y. (2021). Assessment of the nutritional value of Cultured Sea cucumber Apostichopus Japonicus. *Journal of Aquatic Food Product Technology, 30*(7), 868–879. https://doi.org/10.1080/10498850.2021.1949769

Li, P. H., Lu, W. C., Chan, Y. J., Ko, W. C., Jung, C. C., Le Huynh, D. T., & Ji, Y. X. (2020). Extraction and characterization of collagen from sea cucumber (Holothuria cinerascens) and its potential application in moisturizing cosmetics. *Aquaculture, 515*, Article 734590. https://doi.org/10.1016/j.aquaculture.2019.734590

Li, Y., Li, J., Lin, S. J., Yang, Z. S., & Jin, H. X. (2019). Preparation of antioxidant peptide by microwave-assisted hydrolysis of collagen and its protective effect against H_2O_2 - induced damage of RAW264.7 cells. *Marine Drugs, 17*(11), 642. https://doi.org/10.3390/md17110642

Meutter, J. D., & Goormaghtigh, E. (2021). Evaluation of protein secondary structure from FTIR spectra improved after partial deuteration. *European Biophysics Journal, 50*(3–4). https://doi.org/10.1007/s00249-021-01502-y

Mildenberger, J., Remm, M., & Atanassova, M. (2021). Self-assembly potential of bioactive peptides from Norwegian sea cucumber Parastichopus tremulus for development of functional hydrogels. *LWT - Food Science and Technology, 148*(2021), Article 111678. https://doi.org/10.1016/j.lwt.2021.111678

Munprasit, R. (2009). Sea cucumbers fisheries, utilization, and trade in Thailand. In *Report of the regional study on sea cucumber fisheries, utilization, and trade in Southeast Asia, 2007–2008* (pp. 95–112). Secretariat, Southeast Asian Fisheries Development Center: Bangkok, Thailand.

Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi.. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry, 124*(4), 1354–1362. https://doi.org/10.1016/j.foodchem.2010.07.089

Ninwichian, P., & Klinbunga, S. (2020). Population genetics of sandfish (Holothuria scabra) in the Andaman Sea, Thailand inferred from 12S rDNA and microsatellite polymorphism. *Regional Studies in Marine Science, 35*, Article 101189. https://doi.org/10.1016/j.rsma.2020.101189

Phetchthumrongchai, T., Tachapuripunya, V., Chintong, S., Roytrakul, S., E-kobon, T., & Klaypradit, W. (2022). Properties of protein hydrolysates and bioinformatics prediction of peptides derived from thermal and enzymatic process of skipjack tuna (Katsuwonus pelamis) roe. *Fishes, 7*, 255. https://doi.org/10.3390/fishes7050255

Popov, A., Popov, A., Artyukov, A., Krivoshapko, O., & Kozlovskaya, E. (2013). Biological activities of collagen peptides obtained by enzymic hydrolysis from far-eastern holothurians. *American Journal of Biomedical and Life Sciences, 1*(1), 17–26. https://doi.org/10.11648/j.ajbls.20130101.14

Prescott, J. (2001). Taste hedonics and the role of umami. *Food Australia ·, 53*, 550–554.

Purcell, S. W., & Wu, M. (2017). *Large-scale sandfish (Holothuria scabra) aquaculture in multitrophic polyculture ponds in southern China. SPC Beche-de-mer Information Bulletin, 37 – March 2017*.

Rahael, K. P., Rahantoknam, S. P. T., & Hamid, S. K. (2019). The amino acid of sandfish sea cucumber (Holothuria scabra): Dry method with various feeding enzyme. *Journal of Physics: Conference Series, 1424*(2019), Article 012005. doi:1088/1742- 6596/1424/1/012005.

Rasyid, A., Murniasih, T., Putra, M. Y., Pangestuti, R., Harahap, I. A., Untari, F., & Sembiring, S. B. M. (2020). Evaluation of nutritional value of sea cucumber Holothuria scabra cultured in Bali. *Indonesia. AACL Bioflux, 13*(4).

Rod-In, W., Monmai, C., Shin, I. S., You, S., & Park, W. J. (2020). Neutral lipids, glycolipids, and phospholipids, isolated from sandfish (Arctoscopus japonicus) eggs, exhibit anti-inflammatory activity in LPS-stimulated RAW264.7 cells through NF-κB and MAPKs pathways. *Marine Drugs, 18(9):480*. https://doi.org/10.3390/md18090480

Roggatz, C. C., Gonzalez-Wanguemert, M., Pereira, H., Vizetto-Duarte, C., Rodriguez, M. J., & Barreira, L. (2018). A first glance into the nutritional properties of sea cucumber Parastichopus regalis from the Mediterranean Sea (SE Spain). *Natural Product Research, 32*(2), 116–120. https://doi.org/10.1080/14786419.2017.1331224

Sarmadi, B. H., Ismail, A., & Goh, K. L. (2015). Neutral protease: Effect of concentration and pH on the production of bioactive peptides. *Food Chemistry, 176*, 137–144. https://doi.org/10.1016/j.foodchem.2015.01.027

Schlegel, K., Sontheimer, K., Hickisch, A., Wani, A. A., Eisner, P., & Schweiggert-Weisz, U. (2019). Enzymatic hydrolysis of lupin protein isolates—Changes in the molecular weight distribution, technofunctional characteristics, and sensory attributes. *Food Science & Nutrition, 7*, 2747–2759. https://doi.org/10.1002/fsn3.1139

Senadheera, T. R. L., Dave, D., & Shahidi, F. (2021). Antioxidant potential and physicochemical properties of protein hydrolysates from body parts of North Atlantic Sea cucumber (Cucumaria frondosa). *Food Production, Processing and Nutrition, 3*(3). https://doi.org/10.1186/s43014-020-00049-3

Serang, A. M., Rahantoknam, S. P. T., & Tomatala, P. (2016). Effect of different stocking densities on growth and survival rates of sea cucumber Holothuria scabra seedlings. *Aquaculture Indonesian., 17*(1), 30–34. https://doi.org/10.21534/ai.v17il.44

Song, Z., Li, P., Wang, J., Huang, B., Li, B., Wang, S., & Tan, Q. (2016). Effects of seaweed replacement by hydrolyzed soybean meal on growth, metabolism, oxidation resistance and body composition of sea cucumber Apostichopus japonicus. *Aquaculture, 463*, 135–144. https://doi.org/10.1016/j.aquaculture.2016.05.020

Sroyraya, M., Hanna, P. J., Siangcham, T., Tinikul, R., Jattujan, P., Poomtong, T., & Sobhon, P. (2017). Nutritional components of the sea cucumber Holothuria scabra. *Functional Foods in Health and Disease, 7*(3), 168–181. doi:31989/ffhd.v7i3.303.

Sun, N., Cui, P., Jin, Z., Wu, H., Wang, Y., & Lin, S. (2017). Contributions of molecular size, charge distribution, and specific amino acids to the iron-binding capacity of sea cucumber (Stichopus japonicus) ovum hydrolysates. *Food Chemistry, 230*, 627–636. https://doi.org/10.1016/j.foodchem.2017.03.077

Tacias-Pascacio, V. G., Morellon-Sterling, R., Siar, E.-H., Tavano, O., Berenguer-Murcia, Á., & Fernandez-Lafuente, R. (2020). Use of Alcalase in the production of bioactive peptides: A review. International Journal of Biological Macromolecules. *BIOMAC, 16950*. https://doi.org/10.1016/j.ijbiomac.2020.10.060

Trevino, S. R., Scholtz, J. M., & Pace, C. N. (2007). Amino acid contribution to protein solubility: Asp, Glu, and Ser contribute more favorably than the other hydrophilic amino acids in RNase Sa. *Journal of Molecular Biology, 366*, 449–460. https://doi.org/10.1016/j.jmb.2006.10.026

Wang, T., Zheng, L., Wang, S., Zhaoa, M., & Liua, X. (2022). Anti-diabetic and anti-hyperlipidemic effects of sea cucumber (Cucumaria frondosa) gonad hydrolysates in type II diabetic rats. *Food Science and Human Wellness, 11*, 1614–1622. https://doi.org/10.1016/j.fshw.2022.06.020

Wang, T., Zheng, L., Zhao, T., Zhang, Q., Liu, Z., Liu, X., & Zhao, M. (2020). Anti-diabetic effects of sea cucumber (Holothuria nobilis) hydrolysates in streptozotocin and high-fat-diet induced diabetic rats via activating the PI3K/Akt pathway. *Journal of Functional Foods, 75*(2020), Article 104224. https://doi.org/10.1016/j.jff.2020.104224

Wei, W., Fan, X. M., Jia, S. H., Zhang, X. P., Zhang, Z., Zhang, X. J., & Zhang, Y. W. (2021). Sea cucumber intestinal peptide induces the apoptosis of MCF-7cells by inhibiting PI3K/AKT pathway. *Frontiers in Nutrition, 8*, Article 763692. https://doi.org/10.103389/fnut.2021.763692

Wen, J., Hu, C., & Fan, S. (2010). Chemical composition and nutritional quality of sea cucumbers. *Journal of the Science of Food and Agriculture, 90*, 2469–2474. https://doi.org/10.1002/jsfa.4108

Wheelock, C. E., Goss, V. M., Balgoma, D., Nicholas, B., Brandsma, J., Skipp, P. J., & Djukanovic, R. (2013). Application of 'omics technologies to biomarker discovery in inflammatory lung diseases. *European Respiratory Journal, 42*, 802–825. https://doi.org/10.1183/09031936.00078812

Yuan, Y., Cheng, L., Liu, Q., & Zhang, J. (2018). Effect of papain concentration and pH on the hydrolysis of fish proteins. *Food Chemistry, 240*, 1084–1091. https://doi.org/10.1016/j.foodchem.2017.08.045

Zhong, C., Sun, L. C., Yan, L. J., Lin, Y. C., Liu, G. M., & Cao, M. J. (2018). Production, optimisation and characterisation of angiotensin converting enzyme inhibitory peptides from sea cucumber (Stichopus japonicus) gonad. *Food & Function, 9*(1), 594–603. https://doi.org/10.1039/c7fo01388d

Zou, T. B., He, T. P., Li, H. B., Tang, H. W., & Xia, E. Q. (2016). The structure-activity relationship of the antioxidant peptides from natural proteins. *Molecules, 21*, 72. https://doi.org/10.3390/molecules21010072