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Food Functionality Evaluation of Enzymatic Hydrolysates Prepared from Edible Sea Cucumber Body Walls

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***Abstract*— Bioactive peptides from marine organisms, particularly sea cucumbers, represent a promising source of functional compounds with potential health benefits. This study investigated the bioactivities of hydrolysates prepared from several sea cucumber species using five proteolytic enzymes: Alcalase, bromelain, trypsin, papain, and pepsin. The hydrolysates were analyzed for angiotensin-converting enzyme (ACE) inhibitory activity, dipeptidyl peptidase-IV (DPP-IV) inhibitory activity, antioxidant capacity, and molecular weight distribution. Among the samples, 24-h Alcalase hydrolysates of red giant and Kinko sea cucumbers exhibited the highest combined ACE and DPP-IV inhibitory activities. Antioxidant analysis revealed that low DPPH radical scavenging activity showed relatively high oxygen radical absorbance capacity (ORAC) values, indicating different mechanisms of antioxidant action. Ethanol fractionation effectively enhanced ACE and DPP-IV inhibition as well as antioxidant activity. Amino acid composition analysis indicated proteins consistent with type I collagen as the primary source. Enzyme kinetics showed competitive inhibition for ACE and mixed-type inhibition for DPP-IV. Simulated gastrointestinal digestion caused only minor reductions in activity, suggesting that sea cucumber-derived peptides retain their bioactivity under physiological conditions. These findings highlight the potential of sea cucumber hydrolysates as nutraceutical ingredients.**

***Keywords*— Sea cucumber; bioactive peptides; ACE inhibition; DPP-IV inhibition; antioxidant activity**

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I. INTRODUCTION

Diabetic patients have a higher incidence of hypertension than non-diabetic individuals, and diabetes can contribute to hypertension as a complication [1]. Moreover, epidemiological studies have shown that hypertension approximately doubles the risk of coronary heart disease events, including angina and myocardial infarction [2]. In Indonesia, the prevalence of hypertension was reported to be 26.9 % and 34.7% for men and women, respectively, in 2023 [3], while nearly 12% of adults were reported to have diabetes in 2024 [4]. The frequent coexistence of hypertension and diabetes highlights the need for effective strategies to support disease management and prevention. Although pharmacological treatments remain the primary approach for controlling these conditions, adverse effects and drug-drug interactions may occur. For example, the combined use of captopril and sitagliptin has been associated with an increased risk of angioedema [5,6], while both drugs may also cause headache and dizziness [7,8]. Therefore, increasing attention has been given to functional foods and nutraceuticals containing bioactive compounds that may complement conventional therapies.

Among these compounds, food-derived bioactive peptides have attracted considerable interest due to their potential health-promoting properties. Multifunctional peptides are promising because they can target multiple pathological mechanisms associated with chronic diseases. Angiotensin-converting enzyme (ACE) inhibitory peptides may help regulate blood

pressure by reducing the formation of angiotensin II, whereas dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides may improve glycemic control by prolonging the activity of incretin hormones [9,10]. Some peptides also exhibit antioxidant activity, which may help reduce oxidative stress associated with hypertension and diabetes [11,12].

Sea cucumbers are a promising source of such bioactive peptides due to their high protein content and favorable amino acid composition. Previous studies have shown that enzymatic hydrolysis of sea cucumber proteins can generate peptides with ACE inhibitory and DPP-IV inhibitory activities, as well as other bioactivities [13-18]. Compared with other marine protein sources, sea cucumber-derived proteins are particularly rich in collagen-like structures, which may influence peptide profiles and bioactivity generation during hydrolysis [19]. Despite increasing interest, the relationship between enzyme specificity and the production of multifunctional peptides from different sea cucumber species remains insufficiently understood. Therefore, this study aimed to develop sea cucumber body wall hydrolysates possessing ACE inhibitory, DPP-IV inhibitory, and antioxidant activities. Hydrolysates were prepared using various enzymes, and their bioactivities were evaluated. To enrich low-molecular-weight peptide fractions, ethanol fractionation was performed, and the resulting fractions were subsequently evaluated to identify active peptide fractions and characterize their functional properties.

I. MATERIAL AND METHODS

A. Material

Four species of sea cucumbers, *Apostichopus armata* (blue sea cucumber), *Apostichopus japonicus* (red sea cucumber), *Stichopus naso Semper* (red giant sea cucumber), and *Cucumaria frondosa* (Kinko sea cucumber) in **Figure 1** were purchased from markets across Japan.

Five enzymes were used for enzymatic hydrolysis: bromelain, pepsin, papain, trypsin and chymotrypsin purchased from Fujifilm Wako Pure Chemical Co., Ltd., and Alcalase purchased from Sigma-Aldrich. Apparent molecular weights were estimated by comparison with a prestained protein molecular weight marker (ExcelBand™ All Blue Regular Range Protein Marker, PM150021, COSMO BIO Co., Ltd., Tokyo, Japan), covering a molecular weight range of 10-180 kDa. ACE was purchased from Sigma Aldrich Co. (Tokyo, Japan). N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FA-Phe-Gly-Gly) and captopril were purchased from FUJIFILM WAKO PURE CHEMICAL Co. (Osaka, Japan). DPP-IV was purchased from Sigma Aldrich Co. (Tokyo, Japan). Glycyl-L-proline 7-amino-4-methyl-coumarin (Gly-Pro-AMC) and Diprotin A (Ile-Pro-Ile) were purchased from PEPTIDE INSTITUTE, Inc. (Osaka, Japan). All other chemical reagents used were of analytical grade.

B. Methods

Preparation of peptide extraction

For each sea cucumber species, three independent body wall samples (n = 3) were prepared. The body walls were separated from the viscera, cut into small pieces, freeze-dried for 48-72 h, and stored until use. Freeze-dried body wall powder (0.5 g) was mixed with 5 mL of water and 10 mM HCl. The suspension was pretreated at 105°C for 10 min using an autoclave to inactivate endogenous enzymes and facilitate protein extraction, followed by cooling to room temperature. The initial pH of the reaction mixture was adjusted to the optimal pH of each enzyme before enzyme addition and was maintained throughout the hydrolysis period (pH 7.0 for bromelain and papain, pH 8.0 for Alcalase and trypsin, and pH 2.0 for pepsin). Enzymes were added at an enzyme-to-substrate ratio of 1:100 (w/w), and the mixtures were incubated for 24 h with agitation at 120 rpm under the respective optimal conditions: bromelain (37°C, pH 7.0), Alcalase (37°C, pH 8.0), trypsin (37°C, pH 8.0), papain (50°C, pH 7.0), and pepsin (37°C, pH 2.0). Enzymatic reactions were terminated by heating the mixtures in boiling water (100°C) for 10 min, followed by centrifugation (13,000 × g, 20 min). The resulting supernatants were mixed with ethanol to a final concentration of 70% (v/v) to precipitate high-molecular-weight peptides. After centrifugation, the ethanol-soluble fraction was collected and dried by freeze-drying to obtain the final hydrolysate powder for subsequent analyses.

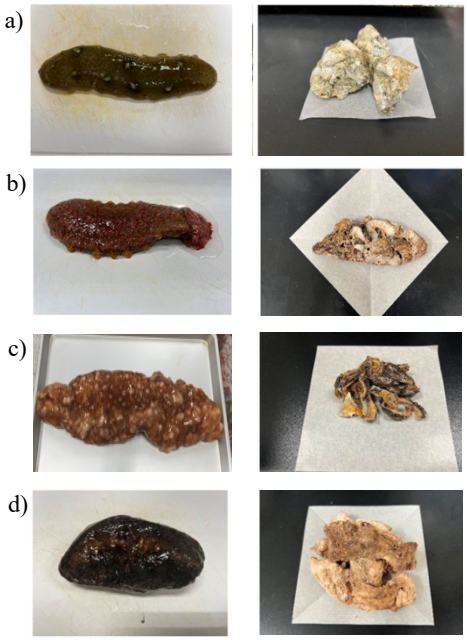

Fig 1. Fresh and dried forms of sea cucumbers used in this research. a) *Apostichopus armata* (blue sea cucumber), b) *Apostichopus japonicus* (red sea cucumber), c) *Stichopus naso Semper* (red giant sea cucumber), d) *Cucumaria*

ACE inhibitory activity measurement

ACE inhibitory activity was measured using a previously published method with minor modifications [20]. Briefly, 5 µL of ACE (final concentration 1.4 mg/mL) was added to a 96-well

microplate, followed by 20 µL of sample, 3.4 mM captopril (positive control), or water (control). The plate was stirred for 15 sec using a microplate shaker and preincubated at 37°C for 5 min. Then, 110 µL of 0.96 mM FA-Phe-Gly-Gly was added, followed by agitation for 15 seconds and incubation at 37°C for 60 min. 125 µL of 1M NaOH was added to terminate the reaction. Absorbance was then measured at 340 nm using microplate reader. ACE inhibitory activity was calculated as:

$$\text{ACE inhibitory activity (\%)} = \{1 - \frac{\Delta \text{ Sample}}{\Delta \text{ Control}}\} \times 100 \quad \text{eq (1)}$$

where ΔSample and ΔControl are the absorbance changes measured in the presence and absence of sample, respectively. ACE specific activity was expressed as ACE inhibitory activity (%) per milligram of sample protein.

DPP-IV inhibitory activity measurement

DPP-IV inhibitory activity was measured using purified porcine DPP-IV and a slight modification of a previously reported method [21]. DPP-IV inhibitory activity was assessed by measuring 7-amino-4-methyl-coumarin (AMC) released from the fluorescent substrate Gly-Pro-AMC. Samples (10 µl) were mixed with 30 µl of 0.05 M Tris-HCl buffer at pH 7.5 and 50 µl of substrate (100 µM), then 10 µl of purified porcine DPP-IV (0.8 mU/ml) was added to initiate the reaction, followed by incubation at 37°C for 30 min. Fluorescence was then measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm. Diprotin A was used as a positive control. DPP-IV inhibitory activity was calculated as:

$$\text{DPP} - \text{IV inhibitory activity (\%)} = \{1 - \frac{(A-B)-(C-B)}{(A-B)}\} \times 100 \quad \text{eq (2)}$$

where A, B, and C represent the fluorescence intensities of the initial activity, background, and sample, respectively. The half-life maximum inhibitory concentration (IC_{50}) was evaluated by plotting a nonlinear regression curve corresponding to the reaction process.

The mode of DPP-IV inhibition was determined according to Berger et al. [22] with minor modifications. Briefly, DPP-IV was incubated with peptide samples at three concentrations (0, 41, and 82 µM). Following pre-incubation, Gly-Pro-AMC was added as the substrate at final concentrations of 12.5, 25, 50, 100, and 200 µM. The reaction was conducted under the same conditions used for the DPP-IV inhibitory activity assay. Fluorescence generated by the release of AMC was measured using a microplate reader (Ex 360 nm, Em 460 nm), and the initial reaction rates were calculated. For kinetic analysis, Lineweaver-Burk plots were constructed by plotting the reciprocal of the reaction rate (1/V) against the reciprocal of substrate concentration (1/[S]) for each inhibitor concentration. The apparent Vmax and Km values were determined from the slope and intercept of the regression lines. The mode of inhibition (competitive, non-competitive, uncompetitive, or mixed) was assigned based on changes in Vmax and Km in the presence of the inhibitor.

DPPH radical scavenging activity measurement

DPPH radical scavenging activity was analyzed as trolox equivalent antioxidant capacity (TEAC) by the modified method of Shimamura et al. [23]. Samples (300 µl, in ultrapure water) were mixed with 900 µl of 133 µM DPPH-66.7 mM MES-40% ethanol, incubated 10 min at room temperature, and absorbance at 517 nm was measured (NanoDrop). Trolox (0-160 µM in 80% ethanol) was used as standard; 80% ethanol served as blank. The result was calculated as:

$$\text{TEAC (µmol TE/g)} = \frac{\text{IC50 of Trolox (µmol/ml)}}{\text{IC50 of Sample (µmol/ml)}} \times 1000 \quad \text{eq (3)}$$

ORAC value measurement

ORAC values were determined using a modified method of Prior et al. [24], Dávalos et al. [25], and Barreca et al. [26] Samples (20 µl, in ultrapure water) were mixed with 120 µl of 0.117 µM fluorescein in a 96-well black plate, pre-incubated at 37 °C for 15 min, then 60 µl of 40 mM AAPH was added. Fluorescence (Ex: 485 nm, Em: 520 nm) was recorded every minute for 90 min at 37 °C using a microplate reader (SH9000, Hitachi).

Trolox (6.25–100 µM, prepared from a 500 µM stock in 75 mM sodium phosphate buffer, pH 7.4) was used as standard, and buffer alone served as control. ORAC values were calculated from the Trolox calibration curve:

$$\text{ORAC (µmol TE/g)} = \frac{[A \times (B-C)]-D}{E} \times 1000 \quad \text{eq (4)}$$

A is defined as the slope of the Trolox calibration curve, B represents the total fluorescence intensity of the sample, C corresponds to the total fluorescence intensity of the control, D denotes the intercept of the Trolox calibration curve, and E indicates the protein concentration of the sample (mg/mL)

Size exclusion chromatography

The weight average molecular weight of sea cucumber hydrolysate was analyzed by size exclusion column chromatography (SEC) as previously reported method by Miyamoto et al. [27]. The sample dissolved in HPLC eluent, 0.1% [v/v] trifluoroacetic acid-30% acetonitrile, were used for SEC. The HPLC analysis conditions were Shimadzu LC-20 system, Superdex™ peptide 10/300GL (Cytiva), column temperature of 40°C, flow rate of 0.5 ml/min, and absorption wavelength of 230 nm. The Labsolution GPC software (Shimadzu Co.) was used as SEC analysis software, and a calibration curve of standard substances (Lysozyme (Mw 14300), insulin (Mw 5800), substance P (Mw 1348), L-Anserin (Mw 240), and Glycylglycine (Mw 132)) was created to calculate the weight average molecular weight.

Amino acid composition analysis

Amino acid composition was determined according to the method of Saito et al. [28]. Briefly, the sample was hydrolyzed in 6 M HCl at 110 °C for 24 h. The resulting hydrolysate was subjected to HPLC analysis with post-column derivatization using o-phthalaldehyde (OPA) at a flow rate of 0.6 mL/min.

Amino acids were detected by fluorescence, with excitation and emission wavelengths set at 350 nm and 450 nm, respectively.

Artificial digestion test

Simulated gastrointestinal digestion was conducted to evaluate the stability of the hydrolysate under digestive conditions. The pH of the hydrolysate was first adjusted to 2.0 using 6 M HCl and preincubated at 37 °C. Pepsin was then added as the gastric enzyme at an enzyme-to-substrate ratio of 1:100, and the mixture was incubated for 2 h. Enzymatic activity was terminated by heating the solution at 100 °C for 10 min. Subsequently, the pH was adjusted to 8.0 to mimic intestinal conditions, then trypsin and chymotrypsin (E:S 1:100) were added, followed by incubation at 37 °C for an additional 2 h. After digestion, samples were collected for the determination of protein content, ACE, and DPP-IV inhibitory activity.

Statistical analysis

All results are presented as the mean ± standard error (SE) from three independent experiments (n = 3). Statistical analyses were performed using SPSS Statistics version 28 (IBM, Armonk, NY, USA). Student's *t*-test was used to compare two groups when appropriate, whereas differences among multiple groups were evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Statistical significance was accepted at $p < 0.05$.

II. Result and Discussion

C. SDS-PAGE Analysis of Sea Cucumber Protein Hydrolysates

Extensive hydrolysis generally increases the formation of low-molecular-weight peptides, which are often associated with ACE inhibitory, DPP-IV inhibitory, and antioxidant activities. Therefore, the hydrolysis patterns of proteins from *A. armata, A. japonicus, S. naso*, and *C. frondosa* after 24 h of enzymatic treatment were evaluated by SDS-PAGE (10% gel) (**Figure 2**). Apparent molecular weights were estimated using a protein molecular weight marker. Compared with the untreated controls, all enzymes reduced the intensity of high-molecular-weight protein bands and generated lower-molecular-weight fragments, indicating successful hydrolysis. The most extensive protein degradation was observed with bromelain, Alcalase and trypsin, which markedly reduced protein bands in the higher molecular-weight region, whereas pepsin-treated samples retained more visible bands.

Differences among sea cucumber species likely reflect variations in protein composition and enzyme accessibility. Furthermore, the observed hydrolysis patterns can be explained by enzyme specificity. Alcalase, a broad-spectrum serine endopeptidase, produced the most extensive degradation, while bromelain, a cysteine protease, also effectively hydrolyzed sea cucumber proteins. Trypsin showed substantial hydrolysis through cleavage at lysine and arginine residues, whereas pepsin exhibited lower activity due to its narrower substrate preference and acidic pH requirement [29,30]. Similar findings have been reported for marine protein hydrolysates, where Alcalase generally produces greater protein degradation and lower-molecular-weight peptides than more substrate-specific enzymes [30,31]. These results demonstrate that enzyme selection strongly influences peptide generation and should be optimized to produce bioactive sea cucumber hydrolysates.

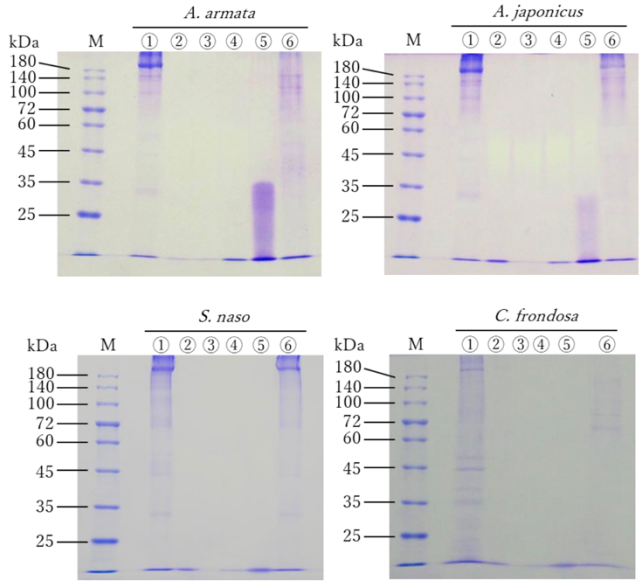

Fig. 2. SDS-10%PAGE analysis of sea cucumber hydrolysates treated with various enzymes. Lane M: molecular weight marker, lane 1: control (no enzyme treatment), lane 2: bromelain, lane 3: Alcalase, lane 4: trypsin, lane 5: papain, lane 6: pepsin. Each lane was loaded with 20 µg of protein.

TABLE 1
ACE INHIBITORY ACTIVITY OF SEA CUCUMBER HYDROLYSATE AFTER ENZYMATIC HYDROLYSIS

Enzyme	Time (h)	Specific activity (% inhibition/mg protein)			
		A. armata	*A. japonicus*	*S. naso*	*C. frondosa*
Bromelain	2	$83.2\pm3.9^{Aa\alpha}$	$62.5\pm0.7^{Aa\beta}$	$37.1\pm0.4^{Aa\gamma}$	ND
Bromelain	4	$96.8\pm3.2^{Ab\alpha}$	$73.1\pm1.5^{Ab\beta}$	$37.1\pm1.5^{Aa\gamma}$	ND
Bromelain	6	$121.4\pm2.0^{Ac\alpha}$	$88.0\pm4.0^{Ac\beta}$	$35.0\pm1.5^{Aa\gamma}$	ND
Bromelain	24	$95.8\pm3.5^{Ab\alpha}$	$89.4\pm3.9^{Ac\alpha\beta}$	$82.4\pm3.3^{Ab\beta}$	ND
Alcalase	2	$66.4\pm0.6^{Ba\alpha}$	$71.9\pm3.2^{Ba\alpha}$	$89.8\pm4.2^{Ba\beta}$	$23.5\pm0.9^{a\gamma}$
Alcalase	4	$69.0\pm1.4^{Bab\alpha}$	$81.1\pm2.5^{Ba\beta}$	$109.9\pm2.4^{Bb\gamma}$	$51.6\pm0.9^{b\delta}$
Alcalase	6	$72.8\pm1.3^{Bc\alpha}$	$112.0\pm3.5^{Bb\beta}$	$109.0\pm2.8^{Bb\beta}$	$58.2\pm2.9^{b\gamma}$
Alcalase	24	$72.8\pm1.2^{Bbc\alpha}$	$116.3\pm5.1^{Bb\beta}$	$103.6\pm2.4^{Bb\gamma}$	$75.0\pm3.2^{c\alpha}$
Trypsin	2	$60.9\pm2.9^{BCa\alpha}$	$59.1\pm1.8^{Aa\alpha}$	$77.8\pm2.5^{Ca\beta}$	$34.8\pm1.3^{a\gamma*}$
Trypsin	4	$82.8\pm3.1^{Cb\alpha}$	$65.1\pm2.8^{Ca\beta}$	$77.8\pm3.2^{Ca\alpha}$	$35.1\pm1.3^{a\gamma*}$
Trypsin	6	$81.0\pm3.9^{Cb\alpha}$	$65.6\pm2.1^{Ca\beta}$	$82.1\pm3.7^{Ca\alpha}$	$42.9\pm1.7^{b\gamma*}$
Trypsin	24	$78.4\pm3.5^{Bb\alpha}$	$65.9\pm2.5^{Ca\beta}$	$86.4\pm3.1^{Aa\alpha}$	$43.7\pm1.1^{b\gamma*}$
Papain	2	$56.2\pm2.8^{Ca\alpha}$	$16.6\pm0.4^{Ca\beta}$	$12.9\pm0.4^{Da\beta}$	ND
Papain	4	$56.8\pm1.7^{Da\alpha}$	$18.6\pm0.7^{Db\beta}$	$24.4\pm1.2^{Db\gamma}$	ND
Papain	6	$45.3\pm2.1^{Db\alpha}$	$29.0\pm0.7^{Dc\beta}$	$25.4\pm1.2^{Db\beta}$	ND
Papain	24	$43.4\pm1.0^{Cb\alpha}$	$29.2\pm0.6^{Dc\beta}$	$24.8\pm0.9^{Cb\gamma}$	ND
Pepsin	2	$28.0\pm0.0^{Da\alpha}$	$33.7\pm0.0^{Da\beta}$	$12.5\pm0.4^{Da\gamma}$	ND
Pepsin	4	$53.0\pm2.1^{Db\alpha}$	$57.4\pm1.8^{Eb\alpha}$	$13.2\pm0.4^{Ea\beta}$	ND
Pepsin	6	$44.2\pm1.8^{Dc\alpha}$	$62.4\pm1.8^{Cb\beta}$	$13.5\pm0.4^{Ea\gamma}$	ND
Pepsin	24	$41.2\pm0.0^{Cc\alpha}$	$71.2\pm3.1^{Cc\beta}$	$12.5\pm0.4^{Da\gamma}$	ND

All data are shown as the means±SE, n=3. Specific activity: ACE inhibitory activity per mg of peptide or protein. ND: Not determined. Different uppercase letters indicate statistically significant differences among the control, bromelain, Alcalase, trypsin, papain, and pepsin treatments, as determined by the Tukey–Kramer test ($p < 0.05$). The significant difference alcalase and trypsin of *C. frondosa* is shown by asterisks by Student's t -test ($p<0.05$). Different small letters indicate significant differences among the 2, 4, 6 and 24 h by Tukey -Kramer($p<0.05$). Different greek characters indicate significant differences between *A. armata, A. japonicus, S. naso and C. frondosa* by Tukey-Kramer($p<0.05$).

TABLE 2
DPP-IV INHIBITORY ACTIVITY OF SEA CUCUMBER HYDROLYSATE AFTER ENZYMATIC HYDROLYSIS

Enzyme	Time (h)	Specific activity (x10, %/mg)			
		A. armata	*A. japonicus*	*S. naso*	*C. frondosa*
Bromelain	2	$23.3\pm1.0^{ABa\alpha}$	$17.2\pm0.5^{Ba\beta}$	$48.2\pm2.3^{Ba\gamma}$	$48.3\pm2.2B^{Ca\gamma}$
Bromelain	4	$26.7\pm0.8^{Ab\alpha}$	$18.6\pm0.7^{Ba\beta}$	$50.9\pm2.0^{Ba\gamma}$	$47.1\pm1.1^{Ba\gamma}$
Bromelain	6	$27.5\pm1.3^{Ab\alpha}$	$22.6\pm0.3^{Bb\alpha}$	$54.5\pm2.4^{Aa\beta}$	$44.0\pm1.6^{Ba\gamma}$
Bromelain	24	$27.9\pm0.5^{Bb\alpha}$	$21.2\pm0.4^{Ab\beta}$	$67.5\pm0.8^{Bb\gamma}$	$67.5\pm1.8^{BCb\gamma}$
Alcalase	2	$48.6\pm1.0^{Ca\alpha}$	$41.7\pm0.9^{Ca\beta}$	$62.9\pm1.3^{Aa\gamma}$	$61.0\pm2.1^{Ba\gamma}$
Alcalase	4	$49.9\pm0.5^{Bab\alpha}$	$42.5\pm1.9^{Ca\beta}$	$66.9\pm0.7^{Ab\gamma}$	$61.2\pm0.5^{Ca\delta}$
Alcalase	6	$53.0\pm1.5^{Cb\alpha}$	$49.4\pm1.4^{Cb\alpha}$	$66.6\pm1.0^{Bb\beta}$	$62.9\pm0.4^{Ca\beta}$
Alcalase	24	$52.6\pm1.8^{Cab\alpha}$	$49.1\pm0.3^{Bb\alpha}$	$75.8\pm1.1^{Cc\beta}$	$69.5\pm1.3^{Bb\gamma}$
Trypsin	2	$44.7\pm2.2^{Da\alpha}$	$37.9\pm1.1^{Da\beta}$	$50.2\pm2.0^{Ba\gamma}$	$39.2\pm0.5^{Ca\beta}$
Trypsin	4	$43.7\pm1.0^{Ca\alpha}$	$38.2\pm0.6^{Da\beta}$	$50.5\pm1.4^{Ba\gamma}$	$44.1\pm0.7^{Bb\alpha}$
Trypsin	6	$43.6\pm1.2^{Da\alpha}$	$42.8\pm1.7^{Db\alpha}$	$48.0\pm2.0^{Ca\alpha}$	$48.3\pm2.2^{Bc\alpha}$
Trypsin	24	$45.3\pm1.3^{Da\alpha}$	$43.6\pm1.6^{Cb\alpha}$	$52.0\pm0.9^{Aa\beta}$	$47.1\pm1.1B^{Cbc\alpha}$
Papain	2	$20.3\pm0.2^{Ba\alpha}$	$7.9\pm0.2^{Ea\beta}$	$20.3\pm0.8^{Ca\alpha}$	$44.0\pm1.6^{Ca\gamma}$
Papain	4	$23.5\pm0.2^{Db\alpha}$	$12.7\pm0.2^{Eb\beta}$	$19.0\pm0.5^{Ca\gamma}$	$67.5\pm1.8^{Cb\delta}$
Papain	6	$22.1\pm0.2^{Bc\alpha}$	$12.7\pm0.3^{Eb\beta}$	$14.6\pm0.1^{Db\gamma}$	$39.2\pm0.5^{Bc\delta}$
Papain	24	$24.8\pm0.1^{Bd\alpha}$	$13.9\pm0.2^{Dc\beta}$	$15.6\pm0.8^{Db\beta}$	$44.1\pm0.7^{Ca\gamma}$
Pepsin	2	$23.3\pm0.6^{Aba\alpha}$	$18.2\pm0.6^{Ba\alpha}$	$30.8\pm1.5^{Da\alpha}$	$210.6\pm9.1^{Aa\beta}$
Pepsin	4	$26.6\pm0.3^{Ab\alpha}$	$24.4\pm0.8^{Fb\alpha}$	$21.6\pm0.8^{Cb\alpha}$	$178.9\pm6.6^{Db\beta}$
Pepsin	6	$26.5\pm0.8^{Ab\alpha}$	$25.9\pm0.4^{Ab\alpha}$	$35.1\pm1.1^{Ec\alpha}$	$187.5\pm6.3^{Aab\beta}$
Pepsin	24	$26.9\pm0.4^{Bbc\beta}$	$10.9\pm0.2^{Ec\alpha}$	$31.1\pm0.7^{Ea\beta}$	$259.1\pm12.2^{Dc\gamma}$

All data are shown as the means±SE, n=3. Specific activity: DPP- IV inhibitory activity per mg of peptide or protein. Different uppercase letters indicate statistically significant differences among the control, bromelain, Alcalase, trypsin, papain, and pepsin treatments, as determined by the Tukey–Kramer test ($p < 0.05$). Different lowercase letters indicate statistically significant differences among the 2, 4, 6 and 24 h treatments, as determined by the Tukey–Kramer ($p < 0.05$). Different greek characters indicate significant differences between *A. armata, A. japonicus, S. naso* and *C. frondosa* by Tukey-Kramer($p<0.05$).

D. Bioactivity and molecular weight profile of sea cucumber hydrolysates

ACE, DPP-IV inhibitory, and antioxidant activities

The ACE inhibitory activities of sea cucumber hydrolysates were influenced by enzyme type, hydrolysis time, and species (**Table 1**). Bromelain produced the highest ACE inhibitory activity in *A. armata*, whereas Alcalase was most effective for *A. japonicus* and *S. naso*, particularly after 6-24 h of hydrolysis. Trypsin also generated relatively high ACE inhibitory activity in *S. naso*, while papain and pepsin generally showed lower activities. Similarly, DPP-IV inhibitory activity (**Table 2**) was highest in Alcalase- and trypsin-treated hydrolysates from *A. armata*, *A. japonicus*, and *S. naso*. In contrast, pepsin-treated *C. frondosa* exhibited exceptionally high DPP-IV inhibitory activity, suggesting the generation of species-specific bioactive peptides.

The observed differences are likely attributable to enzyme specificity and the resulting peptide profiles. Control samples without enzymatic treatment exhibited low bioactivities (data not shown), highlighting the importance of proteolysis for generating bioactive peptides. Alcalase, a broad-spectrum serine endopeptidase, has been widely used to produce ACE inhibitory, DPP-IV inhibitory, and antioxidant peptides from fish, walnut, and pecan proteins [32,33]. Its broad substrate specificity enables extensive protein degradation and the release of diverse peptide sequences [29,30]. Consistent with this property, Alcalase generally produced hydrolysates with higher ACE and DPP-IV inhibitory activities than the other enzymes. Bromelain also generated strong ACE inhibitory activity, particularly in *A. armata*, which agrees with previous reports demonstrating potent ACE inhibitory and antioxidant activities in bromelain-derived protein hydrolysates [34]. The species-dependent responses further suggest differences in protein composition and amino acid sequence among sea cucumber species.

Antioxidant activities were evaluated using DPPH and ORAC assays (**Tables 3** and **4**). DPPH values ranged from 8.7 to 114.2 µmol TE/g, whereas ORAC values ranged from 70.1 to 538.8 µmol TE/g. The highest DPPH activity was observed in Alcalase-treated *A. japonicus* (114.2 µmol TE/g), which also exhibited relatively high ORAC activity (475.4 µmol TE/g), suggesting that Alcalase generated peptides capable of acting through multiple antioxidant mechanisms. In contrast, pepsin-treated *C. frondosa* exhibited relatively low DPPH activity (27.9 µmol TE/g) but the second-highest ORAC value (509.5 µmol TE/g). This discrepancy indicates that the peptides generated by pepsin were more effective at scavenging peroxyl radicals than donating electrons to DPPH radicals. These findings suggest that antioxidant activity depends not only on the extent of hydrolysis but also on the structural characteristics and amino acid composition of the peptides produced. Hydrolysis time did not consistently enhance antioxidant activity, as prolonged proteolysis may either release bioactive peptides or further degrade active sequences depending on the enzyme-substrate combination. The contrasting antioxidant profiles observed for Alcalase-treated *A. japonicus* and pepsin-treated *C. frondosa* highlight the importance of evaluating antioxidant capacity using multiple assays based on different reaction mechanisms [35,36].

TABLE 3
DPPH RADICAL SCAVENGING ACTIVITY OF SEA CUCUMBER HYDROLYSATE AFTER ENZYMATIC HYDROLYSIS

Enzyme	Time (h)	DPPH (µmol TE/g)			
		A. armata	*A. japonicus*	*S. naso*	*C. frondosa*
Bromelain	6	109.9 ± 2.65^{Ba}	89.6 ± 1.94^{Bb}	12.8 ± 0.35^{Bc}	17.9 ± 0.24^{Bd}
Bromelain	24	110.6 ± 0.28^{Ba}	$72.6 \pm 2.96^{Bb*}$	13.2 ± 0.29^{Bc}	$24.7 \pm 0.27^{Bd*}$
Alcalase	6	104.3 ± 0.45^{Ca}	114.2 ± 1.17^{Cb}	14.5 ± 0.24^{Cc}	19.9 ± 0.91^{Cd}
Alcalase	24	104.5 ± 0.11^{Ca}	$77.9 \pm 2.33^{Cb*}$	$21.4 \pm 0.17^{Cc*}$	20.4 ± 0.58^{Cc}

Enzyme	Time (h)	DPPH (µmol TE/g)			
		A. armata	*A. japonicus*	*S. naso*	*C. frondosa*
Trypsin	6	21.7 ± 0.47^{Da}	41.6 ± 0.83^{Db}	13.4 ± 0.40^{BCc}	16.6 ± 0.16^{Bd}
Trypsin	24	18.1 ± 0.66^{Da*}	47.0 ± 0.59^{Db*}	12.2 ± 0.20^{Dc}	31.0 ± 0.42^{Dd*}
Papain	6	19.1 ± 0.83^{Ea}	33.5 ± 0.62^{Eb}	11.3 ± 0.06^{Ec}	10.3 ± 0.42^{Dc}
Papain	24	14.2 ± 0.38^{Ea*}	29.5 ± 0.45^{Eb*}	8.7 ± 0.21^{Ec*}	10.1 ± 0.40^{Ed}
Pepsin	6	15.3 ± 0.72^{Fab}	10.4 ± 0.32^{Fc}	13.9 ± 0.51^{BCa}	16.8 ± 0.77^{Bb}
Pepsin	24	14.0 ± 0.66^{Ea*}	10.3 ± 0.36^{Fb}	9.8 ± 0.08^{Fb*}	27.9 ± 0.30^{Fc*}

All data are shown as the means ± SE, n=3. DPPH (µmol TE/g): DPPH radical-scavenging activities are expressed as trolox equivalent antioxidant capacity (TEAC), µmol trolox equivalent (TE) per protein of 1g. Different uppercase letters indicate statistically significant differences among the control, bromelain, Alcalase, trypsin, papain, and pepsin treatments, as determined by the Tukey–Kramer test ($p < 0.05$). Different small letters indicate significant differences between *A. armata, A. japonicus, S. naso* and *C. frondosa* by Tukey-Kramer($p<0.05$). The significant difference at 6 and 24 h is shown by asterisks by Student's t -test($p<0.05$).

Molecular weight of hydrolysates

The molecular-weight distributions of selected hydrolysates were analyzed by SEC (**Table 5**). Pepsin-treated hydrolysates of A. armata, A. japonicus, and S. naso were excluded from SEC analysis because SDS-PAGE showed little or no protein degradation, indicating limited hydrolysis. Therefore, only the pepsin-treated *C. frondosa* hydrolysate was included. Weight-average molecular weight generally decreased with hydrolysis time, particularly in bromelain and Alcalase-treated samples. Bromelain reduced the apparent molecular weight from 1540-1820 Da at 2 h to 570-1430 Da at 24 h, while Alcalase generated the lowest-molecular-weight peptides across all species (320-400 Da after 24 h). Trypsin also reduced molecular weight, although the decrease was generally less pronounced than that observed for bromelain and Alcalase.

These molecular-weight profiles support the bioactivity results. Previous studies have shown that peptides with molecular weights below 1000 Da are effective ACE and DPP-IV inhibitors because they can readily interact with enzyme active sites [37,38], while antioxidant peptides are commonly found below 3000 Da [30]. Accordingly, the extensive hydrolysis achieved by Alcalase and bromelain likely contributed to the generation of low-molecular-weight peptides responsible for the enhanced ACE inhibitory, DPP-IV inhibitory, and antioxidant activities observed in the present study.

TABLE 4
ORAC VALUE OF SEA CUCUMBER HYDROLYSATE AFTER ENZYMATIC HYDROLYSIS

Enzyme	Time (h)	ORAC (µmol TE/g)			
		A. armata	*A. japonicus*	*S. naso*	*C. frondosa*
Bromelain	6	300.2 ± 4.3^{Ba}	184.8 ± 2.2^{Bb}	160.8 ± 2.2^{Bb}	122.0 ± 5.3^{Bc}
Bromelain	24	292.0 ± 13.6^{Ba}	300.1 ± 4.8^{Ba*}	196.4 ± 6.7^{Bb}	229.4 ± 4.8^{Bab}
Alcalase	6	538.8 ± 15.9^{Ca}	239.8 ± 8.8^{Cb}	248.7 ± 2.8^{Cb}	504.0 ± 21.8^{Ca}
Alcalase	24	396.5 ± 16.4^{Ca}	475.4 ± 13.4^{Ca*}	456.4 ± 18.7^{Ca*}	375.2 ± 6.2^{Ca}
Trypsin	6	472.9 ± 21.4^{Da}	340.5 ± 8.1^{Db}	283.1 ± 5.4^{Cc}	459.8 ± 3.9^{Da}
Trypsin	24	168.0 ± 4.3^{Da*}	278.3 ± 12.1^{Bb}	304.3 ± 6.2^{Db}	206.4 ± 4.3^{Bab*}
Papain	6	221.8 ± 6.2^{Ea}	229.0 ± 3.7^{Ca}	165.6 ± 1.2^{Bb}	162.8 ± 5.6^{Bb}
Papain	24	281.8 ± 11.2^{Ba*}	238.9 ± 7.7^{Db}	292.0 ± 4.9^{Da*}	290.7 ± 4.1^{Bca*}
Pepsin	6	93.4 ± 3.5^{Fa}	97.5 ± 0.9^{Ea}	70.1 ± 2.2^{Da}	426.3 ± 20.6^{Db}
Pepsin	24	103.5 ± 3.6A^{Da*}	97.1 ± 2.9^{Ea}	91.7 ± 4.2^{Ea*}	509.5 ± 10.6^{Db*}

All data are shown as the means±SE, n=3. ORAC value（µmol TE/g）: µmol Trolox equivalent (TE) per 1g of protein. Different uppercase letters indicate statistically significant differences among the control, bromelain, Alcalase, trypsin, papain, and pepsin treatments, as determined by the Tukey–Kramer ($p < 0.05$). Different small letters indicate significant differences between *A. armata, A. japonicus, S. naso* and *C. frondosa* by Tukey-Kramer($p<0.05$). The significant difference at 6 and 24 h is shown by asterisks by Student's t -test($p<0.05$).

TABLE 5
WEIGHT-AVERAGE MOLECULAR WEIGHT OF SEA CUCUMBER HYDROLYSATE

Enzyme	Time (h)	Weight-average molecular weight			
		A. armata	*A. japonicus*	*S. naso*	*C. frondosa*
Bromelain	2	1540±30^{a}	1530±10^{a}	1820±30^{a}	1320±10^{a}
Bromelain	4	1530±100^{ab}	1310±40^{b}	1730±10^{b}	1280±0^{b}
Bromelain	6	1470±100^{ab}	1190±20^{c}	1640±10^{c}	1270±0^{b}
Bromelain	24	1160±30^{b}	1120±0^{d}	1430±10^{d}	570±10^{c}
Alcalase	2	670±20^{a}	520±10^{a}	620±0^{a}	620±10^{a}
Alcalase	4	540±40^{ab}	410±10^{b}	550±0^{b}	510±10^{b}
Alcalase	6	500±30^{ab}	500±30^{a}	470±0^{c}	440±0^{c}
Alcalase	24	400±70^{b}	350±0^{b}	360±0^{d}	320±0^{d}
Trypsin	2	1580±50^{a}	1580±20^{a}	1790±20^{a}	1600±20^{a}
Trypsin	4	1520±30^{ab}	1510±10^{a}	1600±20^{b}	1520±10^{a}
Trypsin	6	1370±20^{bc}	1420±10^{b}	1520±10^{c}	1600±20^{a}
Trypsin	24	1300±10^{c}	1200±10^{c}	1270±10^{d}	780±10^{b}
Pepsin	24	-	-	-	1160 ± 0

All data are shown as the means ± SE, n=3. Different letters indicate significant differences among the 2, 4, 6 and 24 h by Tukey-Kramer($p<0.05$)

TABLE 6
ACE INHIBITORY ACTIVITY, DPP-IV INHIBITORY ACTIVITY AND WEIGHT-AVERAGE MOLECULAR WEIGHT OF SEA CUCUMBER HYDROLYSATE BEFORE AND AFTER ETHANOL FRACTIONATION

Sample	ACE inhibitory activity (%/mg)	DPP-IV inhibitory activity (%/mg)	Molecular weight
Before ethanol fractionation			
A. armata			
Alcalase, 24h	72.8±1.2*	525.6±18.4*	400±70
Trypsin, 6h	81.0±3.9*	435.7±11.7	1370±20*
A. japonicus Alcalase, 6h	112.0±3.5*	493.6±14.4*	500±30
S. naso Alcalase, 24h	103.6±2.4*	757.6±10.5	360±0
C. frondosa			
Alcalase, 24h	75.0±1.2*	694.6±10.3	320±0
Pepsin, 24h	-	2590.9±121.9*	1160±0*
After ethanol fractionation			
A. armata			

Sample	ACE inhibitory activity (%/mg)	DPP-IV inhibitory activity (%/mg)	Molecular weight
Alcalase, 24h	92.7±2.9*	688.5±12.4*	370±0
Trypsin, 6h	118.0±2.9*	448.5±22.2	1570±10*
A. japonicus Alcalase, 6h	66.0±2.2*	635.1±19.9*	440±0
S. naso Alcalase, 24h	186.5±1.0*	769.0±5.7	360±0
C. frondosa			
Alcalase, 24h	9.8±0.3*	642.0±10.0	320±10
Pepsin, 24h	1.5±0.4	729.6±10.3*	940±0*

All data are shown as the means±SE, n=3. The significant difference before and after ethanol fractionation is shown by asterisks by Student's t -test($p<0.05$). ACE and DPP-IV inhibitory activity(%/mg): specific activity per mg of peptide or protein. Mw: weight-average molecular weight.

E. ACE and DPP-IV inhibitory activity and weight average molecular weight of ethanol fractions

Ethanol fractionation was performed to enrich peptide fractions and further characterize the inhibitory components of selected sea cucumber hydrolysates with high ACE and DPP-IV inhibitory activities. Following ethanol treatment and removal of precipitates, ACE inhibitory activity, DPP-IV inhibitory activity, and apparent molecular weight were determined (**Table 6**). ACE inhibitory activity increased significantly in *A. armata* (Alcalase, 24 h) and *S. naso* (trypsin, 6 h), while DPP-IV inhibitory activity increased in *A. armata* (Alcalase, 24 h), *A. japonicus* (trypsin, 6 h), and *S. naso* (Alcalase, 6 h). In contrast, apparent molecular weight decreased significantly in *C. frondosa* after fractionation.

Ethanol fractionation separates peptides according to multiple physicochemical properties, including molecular weight, polarity, amino acid composition, and conformation [39,40]. Consequently, the supernatant is enriched in peptides with higher ethanol solubility, while less soluble peptides and larger proteinaceous components are preferentially precipitated. Most supernatant fractions exhibited apparent molecular weights below 2000 Da, suggesting that low-molecular-weight peptides contributed substantially to the observed ACE and DPP-IV inhibitory activities.

TABLE 7
AMINO ACID COMPOSITION PERCENTAGE OF ETHANOL FRACTION OF SEA CUCUMBER HYDROLYSATE AND ITS BODY WALL

Amino acid	Amino acid composition (%)			
	A. armata **(Alcalase, 24h)**	***A. japonicus*** **(Alcalase, 6h)**	***S. naso*** **(Alcalase, 24h)**	***C. frondose*** **(Pepsin, 24h)**
Asp	6.8 ± 0.0^{AB} (8.2 ± 0.5^{a})	6.8 ± 0.0^{A} (8.3 ± 0.4^{a})*	6.6 ± 0.1^{B} (8.1 ± 0.2^{a})*	5.3 ± 0.1^{C} (7.7 ± 0.2^{a})*
Hyp	3.9 ± 0.1^{A} (3.7 ± 1.6^{a})	3.7 ± 0.3^{A} (4.6 ± 0.4^{a})*	4.2 ± 0.1^{A} (4.9 ± 0.1^{a})*	4.8 ± 0.1^{B} (4.0 ± 0.1^{a})*
Thr	4.5 ± 0.1^{A} (4.7 ± 0.4^{a})	4.5 ± 0.1^{A} (4.9 ± 0.2^{a})	3.9 ± 0.1^{B} (4.5 ± 0.1^{ab})*	2.7 ± 0.1^{C} (3.8 ± 0.1^{b})*
Ser	4.2 ± 0.1^{A} (5.1 ± 0.2^{a})	4.1 ± 0.3^{A} (5.0 ± 0.2^{a})	2.8 ± 0.1^{B} (4.1 ± 0.0^{b})*	4.5 ± 0.2^{A} (5.6 ± 0.1^{c})*
Glu	10.8 ± 0.0^{AB} (12.2 ± 0.5^{a})	11.0 ± 0.4^{A} (12.4 ± 0.5^{a})*	11.7 ± 0.2^{C} (12.4 ± 0.3^{a})	10.3 ± 0.1^{B} (12.2 ± 0.3^{a})*
Pro	9.2 ± 0.1^{AB} (8.0 ± 2.3^{a})	9.2 ± 0.1^{A} (8.1 ± 1.2^{a})	9.4 ± 0.1^{B} (8.3 ± 0.3^{a})*	10.4 ± 0.1^{C} (7.7 ± 0.2^{a})*
Gly	23.7 ± 0.4^{A} (26.2 ± 2.1^{a})	23.9 ± 0.7^{A} (26.3 ± 0.4^{a})	24.9 ± 0.3^{A} (25.5 ± 0.6^{a})	27.1 ± 0.2^{B} (26.6 ± 0.6^{a})
Ala	9.0 ± 0.1^{A} (8.8 ± 0.4^{a})	9.1 ± 0.3^{A} (9.0 ± 0.2^{ab})	10.3 ± 0.1^{B} (9.8 ± 0.2^{c})	8.8 ± 0.0^{A} (9.8 ± 0.2^{bc})*
Val	3.8 ± 0.1^{A} (3.9 ± 0.3^{a})	3.8 ± 0.1^{A} (3.5 ± 0.1^{ab})	3.5 ± 0.0^{B} (3.2 ± 0.1^{b})	2.7 ± 0.0^{C} (3.4 ± 0.1^{ab})*
Cys	0.0 ± 0.0^{A} (0.0 ± 0.0^{a})	0.0 ± 0.0^{A} (0.0 ± 0.0^{a})	0.0 ± 0.0^{A} (0.1 ± 0.1^{a})	0.0 ± 0.0^{A} (0.1 ± 0.1^{a})
Met	0.9 ± 0.0^{A} (1.1 ± 0.1^{a})	0.9 ± 0.1^{A} (1.0 ± 0.1^{a})	0.5 ± 0.0^{B} (0.8 ± 0.0^{a})*	0.4 ± 0.0^{B} (0.8 ± 0.2^{a})
Ile	2.9 ± 0.0^{A} (2.7 ± 0.1^{a})	2.9 ± 0.0^{A} (2.8 ± 0.1^{a})	2.5 ± 0.0^{B} (2.3 ± 0.0^{b})	1.7 ± 0.0^{C} (2.4 ± 0.1^{b})*
Leu	3.8 ± 0.0^{A} (3.8 ± 0.2^{a})	3.8 ± 0.1^{A} (3.6 ± 0.1^{a})	3.7 ± 0.0^{A} (3.5 ± 0.1^{a})	2.8 ± 0.0^{B} (3.6 ± 0.1^{a})*
Tyr	1.6 ± 0.0^{A} (1.8 ± 0.3^{a})	1.5 ± 0.2^{AB} (1.6 ± 0.1^{ab})	1.3 ± 0.0^{B} (1.3 ± 0.1^{b})	0.9 ± 0.0^{C} (1.4 ± 0.0^{ab})*
Phe	1.8 ± 0.0^{A} (1.9 ± 0.1^{a})	1.8 ± 0.1^{A} (1.8 ± 0.1^{ab})	1.6 ± 0.0^{B} (1.5 ± 0.1^{bc})	1.0 ± 0.0^{C} (1.5 ± 0.1^{c})*
His	1.2 ± 0.1^{A} (0.7 ± 0.1^{a})*	1.2 ± 0.1^{A} (0.6 ± 0.1^{a})*	1.2 ± 0.0^{A} (0.8 ± 0.1^{a})*	0.8 ± 0.0^{B} (0.7 ± 0.0^{a})
Hyl	1.3 ± 0.1^{AB} (0.7 ± 0.2^{a})	1.3 ± 0.0^{A} (0.7 ± 0.2^{a})	1.0 ± 0.1^{B} (0.9 ± 0.0^{a})	1.8 ± 0.1^{C} (0.8 ± 0.1^{a})*
Lys	3.6 ± 0.2^{A} (2.3 ± 0.9^{a})	3.2 ± 0.2^{AB} (2.1 ± 1.0^{a})	2.8 ± 0.1^{B} (2.7 ± 0.7^{a})	2.2 ± 0.1^{C} (2.4 ± 0.5^{a})
Arg	7.3 ± 0.4^{A} (4.1 ± 0.9^{a})	7.2 ± 1.0^{A} (3.9 ± 1.0^{a})*	8.0 ± 1.0^{A} (5.2 ± 0.2^{a})	12.8 ± 1.3^{B} (5.5 ± 0.2^{a})*
Total	100	100	100	100
Amino acid amounts#	21.6 ± 4.4	22.4 ± 5.0	34.3 ± 5.6	45.1 ± 5.0

Ethanol fractions were obtained from hydrolysates of *A. armata* (Alcalase, 24 h), *A. japonicus* (Alcalase, 6 h), *S. naso* (Alcalase, 24 h), and *C. frondosa* (Pepsin, 24 h). Values in parentheses represent the corresponding measurements for the body wall. All data are expressed as means ± SE (n = 3). Significant differences between body wall and ethanol fractions are indicated by asterisks (*), based on Student's t-test ($p < 0.05$). Different uppercase and lowercase letters indicate statistically significant differences among ethanol fractions and body wall samples as determined by the Tukey–Kramer ($p < 0.05$). #: Calculated protein content (g/100 g dry weight) for the body wall is based on amino acid composition.

F. Amino acid profiles and bioactivity-linked in sea cucumber hydrolysates

The amino acid compositions of ethanol fractions from *A. armata* (Alcalase, 24 h), *A. japonicus* (Alcalase, 6 h), *S. naso*(Alcalase, 24 h), and *C. frondosa* (Pepsin, 24 h), which exhibited high ACE and DPP-IV inhibitory activities, were compared with those of the corresponding body wall tissues (Table 7). Gly, Glu, Pro, and Ala were the predominant amino acids in all species, consistent with the composition of type I collagen, the major structural protein in sea cucumber body walls [41]. However, significant differences in the abundance of several amino acids were observed among species. C. *frondosa* contained higher proportions of Gly (27.1%), Pro (10.4%), Hyp (4.8%), Hyl (1.8%), and Arg (12.8%) than the other species.

Comparison of the ethanol fractions with the corresponding body wall tissues revealed compositional changes following hydrolysis and fractionation. Notably, Lys content increased approximately two-fold in the ethanol fractions of *A. armata*and *A. japonicus*, while Arg content increased in all species, particularly in *C. frondosa*. These changes suggest selective enrichment of peptide populations containing basic amino acid residues.

The distinctive composition of *C. frondosa* may explain its unique bioactivity profile. This species exhibited the highest protein content (45.1 g/100 g dry weight), the strongest DPP-IV inhibitory activity, and was the only species for which pepsin-generated hydrolysates showed high bioactivity. The elevated Arg content may have contributed to these activities, as ACE inhibitory peptides containing C-terminal Lys or Arg are reported to bind effectively to the active site of ACE and exhibit strong inhibitory activity [42]. In addition, DPP-IV inhibitory peptides frequently contain Pro or Ala at the second position from the N-terminus, while Pro residues at the C-terminus can enhance peptide stability against enzymatic degradation [38]. The ethanol fractions contained relatively high levels of Pro (9.2-10.4%) and Ala (8.8-10.3%), suggesting that enzymatic hydrolysis followed by ethanol fractionation enriched peptides possessing structural features associated with both ACE and DPP-IV inhibition. Together, these findings indicate that species-specific amino acid composition and peptide sequences contribute to the observed bioactivities of sea cucumber hydrolysates.

To evaluate whether hydrolyzed ethanol fractions of sea cucumbers retain or enhance ACE inhibitory and DPP-IV inhibitory activities after digestion in the human gastrointestinal tract, simulated digestion experiments were conducted using digestive enzymes. **Table 8** presents the results of enzymatic digestion of hydrolyzed ethanol fractions from various sea cucumber species using pepsin, pepsin-trypsin, pepsin-chymotrypsin, or pepsin-trypsin-chymotrypsin combinations.

TABLE 8
BIOACTIVITY AND PROTEIN CHARACTERISTICS OF SEA CUCUMBER ETHANOL FRACTIONS AFTER SIMULATED DIGESTION

Sample	Protein yield (mg)	ACE inhibitory activity (%/mg)	DPP-IV inhibitory activity (%/mg)	Weight-average Molecular weight
A. armata, Alcalase 24h				
Ethanol fraction	150^{a}	92.7±2.9^{a}	688.5±12.4^{a}	370±0^{a}
Pepsin	113.3±2.1^{b}	126.3±4.1^{b}	881.9±5.7^{b}	400±0^{ab}
Pepsin-Trypsin	105.8±1.4^{c}	139.6±4.5^{c}	874.8±1.4^{b}	490±10^{c}
Pepsin-Chymotrypsin	109.0±1.6b^{c}	149.9±1.4^{c}	875.4±0.4^{b}	420±10^{b}
Pepsin-Trypsin-Chymotrypsin	114.1±4.0^{b}	169.9±3.8^{d}	865.6±1.0^{b}	520±0^{d}
A. japonicus, Alcalase 6h				
Ethanol fraction	150^{a}	66.0±2.2^{a}	635.1±19.9^{a}	440±0^{a}
Pepsin	114.8±2.4^{b}	151.0±3.2^{b}	896.1±1.1^{b}	490±0^{b}
Pepsin-Trypsin	115.2±1.6^{b}	181.3±1.6^{c}	888.7±3.5^{b}	520±0^{c}
Pepsin-Chymotrypsin	113.0±3.0^{b}	190.5±1.9^{d}	882.0±1.0^{b}	510±0^{bc}
Pepsin-Trypsin-Chymotrypsin	144.3±2.1^{b}	212.7±1.9^{e}	876.8±1.6^{b}	540±10^{d}
S. naso, Alcalase 24h				
Ethanol fraction	150^{a}	186.5±1.0^{a}	769.0±5.7^{a}	360±0^{a}
Pepsin	146.0±1.9^{a}	169.3±3.2^{b}	923.5±3.0^{b}	380±0^{b}
Pepsin-Trypsin	149.4±1.6^{a}	167.2±1.8^{b}	920.3±3.1^{b}	410±10^{c}
Pepsin-Chymotrypsin	145.6±5.6^{a}	134.4±4.1^{c}	921.4±1.4^{b}	410±0^{c}
Pepsin-Trypsin-Chymotrypsin	140.3±3.9^{a}	130.1±0.9^{c}	927.9±1.4^{b}	450±0^{d}
C. frondosa, pepsin 24h				
Ethanol fraction	150^{a}	1.5±0.4^{a}	729.6±10.3^{a}	940±0^{a}
Pepsin	132.9±1.4^{ab}	32.3±1.4^{b}	736.2±5.0^{ab}	1150±0^{b}
Pepsin-Trypsin	126.0±1.6^{b}	37.1±0.5b^{bc}	748.8±4.1^{bc}	830±0^{c}
Pepsin-Chymotrypsin	132.9±2.1^{ab}	40.0±1.9^{c}	739.6±2.6^{abc}	710±0^{d}
Pepsin-Trypsin-Chymotrypsin	129.3±2.6^{b}	51.6±2.4^{d}	757.9±1.6^{c}	800±0^{e}

Data are presented as means ± SE (n = 3). Different letters indicate statistically significant differences among the ethanol fraction, pepsin, pepsin–trypsin, pepsin–chymotrypsin, and pepsin–trypsin–chymotrypsin treatments, as determined by the Tukey–Kramer ($p < 0.05$). Mw: weight-average molecular weight.

All samples, except for *S. naso*, showed a significant decrease in protein recovery following enzymatic digestion. Specific ACE inhibitory activity significantly increased in all samples post-digestion, except for *S. naso*. In contrast, specific DPP-IV inhibitory activity significantly increased in all samples after digestion.

All sea cucumbers, except for *C. frondosa*, exhibited significantly higher weight-average molecular weights after digestion compared to their pre-digestion values. This suggests that the hydrolyzed ethanol fractions may contain small amounts of macromolecular components resistant to enzymatic hydrolysis. Since both ACE and DPP-IV inhibitory activities were enhanced in the ethanol fractions of *A. armata, A. japonicus* and *C. frondosa* after simulated digestion, these bioactivities are likely to be preserved or even amplified as peptides undergo further enzymatic degradation in the gastrointestinal tract, consistent with the findings of Hossain et al. [43].

To clarify the inhibition mechanisms of the bioactive components, IC_{50} values and Lineweaver-Burk plots were determined for ethanol fractions prepared from *A. armata* (Alcalase, 24 h), *A. japonicus* (Alcalase, 6 h), *S. naso* (Alcalase, 24 h), and *C. frondosa* (Pepsin, 24 h). The ACE inhibitory activity of the *C. frondosa* fraction was not proportional to concentration; therefore, its IC_{50} value and inhibition mode could not be reliably determined and were excluded from further ACE kinetic analysis.

TABLE 9
IC_{50} OF ACE AND DPP-IV INHIBITORY ACTIVITY

Sample	IC_{50}	
	ACE inhibitory activity	DPP-IV inhibitory activity
Positive control	3.76±0.01 nM	2.01±0.09 μM
A. armata	3.19±0.01 mg/ml	0.58±0.02 mg/ml
A. japonicus	4.23±0.04 mg/ml	0.60±0.01 mg/ml
S. naso	3.34±0.02 mg/ml	0.54±0.02 mg/ml
C. frondosa	-	0.32±0.01 mg/ml

For ACE inhibition, captopril exhibited competitive inhibition with an IC_{50} of 3.76 nM (**Table 9**). The ethanol fractions

from *A. armata*, *A. japonicus*, and *S. naso* showed IC_{50} values of 3.19, 4.23, and 3.34 mg/mL, respectively. In all cases, changes in Km with relatively constant Vmax (**Table 10**) were consistent with a competitive inhibition mechanism, indicating that the peptides compete with the substrate for binding to the ACE active site.

For DPP-IV inhibition, Diprotin A exhibited competitive inhibition with an IC_{50} of 2.01 µM. The ethanol fractions from *A. armata*, *A. japonicus*, *S. naso*, and *C. frondosa* showed IC_{50} values of 0.58, 0.60, 0.54, and 0.32 mg/mL, respectively. Both Vmax and Km changed in the presence of the fractions, indicating a mixed inhibition mechanism involving both competitive and non-competitive interactions with DPP-IV.

TABLE 10
VMAX, KM AND INHIBITON PATTERN OF ACE AND DPP-IV INHIBITORY ACTIVITY

	Sample		Vmax	Km	Inhibition pattern
ACE	Captopril	0 nM	0.004486	0.282	Competitive
		3.76(IC_{50})	0.003869	1.1	
		7.52	0.0044	2.494	
	A. armata (Alcalase, 24h)	0 mg/ml	0.004847	0.231	Competitive
		3.19(IC_{50})	0.003322	0.72	
		6.38	0.002737	1.21	
	A. japonicus (Alcalase, 6h)	0 mg/ml	0.004847	0.231	Competitive
		4.23(IC_{50})	0.0031	0.626	
		8.46	0.002441	0.922	
	S. naso (Alcalase, 24h)	0 mg/ml	0.004847	0.231	Competitive
		3.34(IC_{50})	0.003183	0.64	
		6.68	0.002418	0.845	
DPP-IV	DiprotinA	0 µM	123.5	55.11	Competitive
		2.01(IC_{50})	122	131.7	
		4.02	123.5	210.9	
	A. armata (Alcalase, 24h)	0 mg/ml	156.3	90.52	Mix
		0.58(IC_{50})	108.7	138	
		1.16	104.2	191.7	
	A. japonicus (Alcalase, 6h)	0 mg/ml	156.3	90.66	Mix
		0.60(IC_{50})	238.1	311.4	
		1.2	204.1	447.3	
	S. naso (Alcalase, 24h)	0 mg/ml	151.5	88.09	Mix
		0.54(IC_{50})	153.8	184.7	
		1.08	111.1	204.2	
	C. frondose (Pepsin, 24h)	0 mg/ml	151.5	88.47	Mix
		0.32(IC_{50})	185.2	232.1	
		0.64	147.1	308.9	

Previously reported IC_{50} values for protein hydrolysates from mussels and sesame seeds were 0.79 and 0.15 mg/mL for ACE inhibition and 0.66 and 0.78 mg/mL for DPP-IV inhibition, respectively [44,45]. The DPP-IV IC_{50} values of the sea cucumber ethanol fractions (0.32-0.60 mg/mL) were lower than those reported for mussels and sesame seeds, indicating greater inhibitory potency under the assay conditions used. Collectively, these results suggest that the ethanol fractions contain multiple bioactive peptides that inhibit ACE competitively and DPP-IV through mixed inhibition mechanisms.

III. CONCLUSION

This study demonstrated that the bioactivities of sea cucumber hydrolysates were strongly influenced by enzyme type, hydrolysis conditions, and species. Alcalase generally produced the lowest molecular-weight peptide fractions, while bromelain-generated hydrolysates from *A. armata* exhibited the strongest ACE inhibitory activity. The highest DPP-IV inhibitory activity was observed in pepsin hydrolysates from *C. frondosa*, indicating species- and enzyme-specific differences in bioactive peptide generation. Ethanol fractionation further enhanced ACE and DPP-IV inhibitory activities in several hydrolysates, suggesting enrichment of active peptide fractions. Importantly, these bioactivities were largely retained following simulated gastrointestinal digestion, indicating their potential stability during digestion. Enzyme kinetic analyses showed that ACE inhibition occurred through a competitive mechanism, whereas DPP-IV inhibition followed a mixed inhibition mechanism. Overall, the results demonstrate that sea cucumber body wall proteins are promising sources of multifunctional peptides with antihypertensive, antidiabetic, and antioxidant properties, supporting their potential application as functional food ingredients and nutraceuticals. Further studies are warranted to identify the active peptide sequences and evaluate their efficacy in vivo.

USE OF ARTIFICIAL INTELLIGENCE (AI) TOOLS STATEMENT

To improve grammatical accuracy and readability, we utilized Grammarly (Grammarly Inc., 2025). The authors carefully reviewed and approved all modifications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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