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Research Article

Amino acid profile, antioxidant activity, and hybridization levels of gelatine hydrolysates prepared with single or combined enzymes from fish skin and scales

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Abstract

In the present study, gelatin was extracted from the skin and scales obtained from the processing of pangasius fish and sea bass, respectively. Collagen peptides (gelatin hydrolysates) were produced from gelatin using different single enzymes or combinations of enzymes. Samples obtained from fish skin contained 22.81 to 32.80 mg.100 g⁻¹ essential amino acids, whereas those obtained from fish scales had 22.24 to 29.52 mg.100 g⁻¹ essential amino acids. In terms of antioxidant activity, the collagen peptide samples obtained from the skin have DPPH values of 141.00 to 240.21 mg Trolox.100 g⁻¹ and ABTS values of 14.62 to 16.97 mM Trolox.100 g⁻¹. For the fish scale samples, the DPPH values ranged from 154.32 to 223.24 mg Trolox.100 g⁻¹ and ABTS values from 13.70 to 16.34 mM Trolox.100 g⁻¹. The best results for degree of hydrolysis, molecular weight and antioxidant activities were achieved with the Alcalase + Neutrase + Flavourzyme (ANF) enzyme combination. Samples obtained from fish skin and scales hydrolyzed with the ANF enzyme combination were found to contain the highest amounts of essential amino acids, also. Collagen hydrolysates obtained from sea bass fish scales and pangasius fish skin have high potential applications in food and pharmaceutical industry.

Keywords

collagen peptides, enzyme, degree of hydrolysis, antioxidant activity, amino acids

Abbreviations

DPPH – 2,2-diphenyl-1-picrylhydrazyl; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DH – degree of hydrolysis; ANF – Alcalase + Neutrase + Flavourzyme; A – alcalase; C – collagenase; P – papain; N – neutrase; A.F – alcalase + flavourzyme; A.C – alcalase + collagenase; A.P – alcalase + papain; A.N.F – alcalase + neutrase + flavourzyme; A.N.F.C – alcalase + neutrase + flavourzyme + collagenase; FTIR – fourier transform infrared spectroscopy; HPLC – high-performance liquid chromatography; GME – gelatine manufacturers of Europe; MW – molecular weight; MN – molecular weight distribution.

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Introduction

The increasing awareness of the relationship between diet and health has led to a growing interest in functional foods. Advances in nutrition and medical sciences, along with improved living standards, have contributed to this trend. Functional foods, particularly those enriched with bioactive compounds, are gaining prominence due to their potential health benefits, leading to a rapid expansion of the global functional food market (Chobdar Rahim et al. 2021; Christiansen et al. 2024). Collagen, a structural protein abundant in connective tissues such as skin, bone, cartilage, and tendons, plays a crucial role in maintaining the integrity of these tissues. It constitutes approximately 30% of the total protein content in mammals (Zamorano-Apodaca et al. 2020). Collagen-derived peptides have gained significant attention due to their potential health benefits, including antioxidant, anti-inflammatory, antihypertensive, and antiosteoporosis properties (Leon-Lopez et al. 2019; Moghadam et al. 2025).

Collagen hydrolysate, recognized as a functional ingredient, has gained popularity as a food supplement aimed at enhancing bone and skin health through its capacity to stimulate collagen synthesis within the body (Khan et al. 2009; Shigemura et al. 2023; de Souza Pereira et al. 2023). The synthesis of collagen in the body begins to decrease from the mid-20s onward, primarily due to factors such as aging, smoking, alcohol consumption, inadequate oxygen levels, nutritional deficiencies, sun exposure, and other external influences (Bolke et al. 2019). As collagen synthesis diminishes - a vital source of protein and amino acids - symptoms such as delayed wound healing, fatigue, and reduced performance may also manifest. Collagen hydrolysate exhibits the potential to mitigate this prevalent condition by alleviating joint pain and thwarting the onset of arthritis (Guo et al. 2025). For this reason, it is important to consume it as a food supplement in certain amounts (Barnett et al. 1998; Bonjour 2005; Guillerminet et al. 2010; Karaguzel and Holick 2010; Bruyère et al. 2012).

The primary sources of collagen traditionally utilized are porcine and bovine. However, due to escalating concerns regarding the transition of infectious diseases such as Transmissible

Spongiform Encephalopathy (TSE), and religious beliefs, the utilization of this source has declined. Consequently, the exploration for alternative collagen sources has commenced, encompassing options such as algae and marine organisms, including fish (Lim et al. 2019; Farooq et al. 2024). According to the Food and Agriculture Organization (2023), approximately 30-50% of the total fish biomass is generated as processing by-products such as skin, scales, bones, and viscera. Globally, this accounts for more than 60 million tons of waste annually, representing a valuable yet underutilized source for collagen recovery (FAO 2023).

Marine-derived collagen offers several practical advantages over mammalian collagen, making it an increasingly preferred alternative in functional food and nutraceutical applications. Fish collagen typically has a lower molecular weight and a less compact triple-helix structure, which results in faster gastrointestinal absorption and higher bioavailability compared to bovine or porcine collagen (Gutierrez-Canul et al. 2024; Kim and Park 2024). Additionally, it is sourced from fish-processing by-products such as skin and scales, providing a sustainable and environmentally responsible option while avoiding religious restrictions and the risks associated with transmissible diseases like BSE/TSE commonly linked to mammalian tissues (Rajabimashhadi et al. 2023). These characteristics, combined with its excellent solubility and compatibility in cold beverages and nutraceutical formulations, have positioned marine collagen as a promising candidate for next-generation collagen-based products.

In recent years, research has increasingly focused on the enzymatic hydrolysis of marine by-products such as fish skin, scales, and bones to obtain collagen peptides with enhanced bioactive and functional properties. These studies demonstrate that the enzyme type, degree of hydrolysis, and molecular weight distribution critically influence the resulting biological activities. To provide a concise and quantitative perspective on these advances, the most recent investigations conducted between 2023 and 2025 are summarized in Table 1 (Pires et al. 2024; Xiang et al. 2024; Panjaitan et al. 2024; Chang et al. 2024; Tekle et al. 2024; Ng et al. 2024).

Table 1. Recent studies on enzymatic hydrolysis of marine and fish-derived collagen peptides and their bioactivities

Year	Source material	Enzyme(s) used	DH, % / MW range, Da	Key findings	Reference
2024	Salmon heads & Cape hake trimmings (FPH)	Alcalase	DH = 10.7–36.4 %; small-peptide profile (<3 kDa prominent)	Strong DPPH/ABTS, Fe ²⁺ /Cu ²⁺ chelation; ACE-inhibitory activity; heavy metals below legal limits. Identified ACE-	(Gutierrez-Canul et al. 2024)
2024	Eel (<i>Anguilla japonica</i>) bone collagen hydrolysates	Alcalase → UF fractions	Fractions: >3 kDa, 1–3 kDa, <1 kDa; 1–3 kDa most active	inhibitory peptide (e.g., GPPGPPGL); IC ₅₀ ≈ 0.54 mM; protective effects on HUVECs. Highest ACE-I	(Kim and Park 2024)
2024	Commercial bovine/porcine/tilapia gelatins → enzymatic hydrolysates	Bromelain, Papain, Collagenase (comparative)	DH up to ~54 % (collagenase: porcine 53.89 %, bovine 48.95 %, tilapia 45.44 %); active <1 kDa fraction (PP1)	inhibition observed in papain-tilapia (<1 kDa) fraction; in vivo anti-amnestic & antioxidant effects.	(Rajabima shhadi et al. 2023)
2024	Fish skin collagen solution (5 % w/v)	Alcalase (3000 U/g, pH 10, 55 °C, 2 h); Papain (3000 U/g, pH 6, 55 °C, 2 h); Natural protease (3000 U/g, pH 7, 55 °C, 2 h)	(Process conditions explicit; low-MW peptides targeted)	Multi-enzyme/condition optimization to enhance hydrolysis efficiency; sets up AA/PP/NN hydrolysate panels for bioactivity testing.	(Pires et al. 2024)
2024	Sea bream fish-skin gelatin → hydrolysates (FGH)	Alcalase & Savinase	(Trend) ↑DH → lower MW peptides; emulsifying/foaming properties reported	Alcalase-treated FGH showed higher zeta potential & emulsifying stability (ESI); Savinase improved foaming & fat-binding. DPPH 87.4 %, ABTS 83.2 %, ACE-I IC ₅₀ = 0.52 mg.mL ⁻¹ ; in vivo antioxidant effects confirmed.	(Xiang et al. 2024)
2024	Tilapia skin & scales	Papain + Flavourzyme	DH ≈ 24 %; MW < 1 kDa		(Panjaitan et al. 2024)

Marine collagen is mainly extracted from marine sources such as fish skin, bone, cartilage, and scales, encompassing both marine vertebrates and invertebrates. It demonstrates similarity to traditional bovine and porcine collagen in terms of amino acid composition and biocompatibility but also possesses enhanced bioavailability in comparison to collagen sourced from bovine or porcine origins. This heightened bioavailability stems from their lower molecular weight and smaller particle size, facilitating up to 1.5 times more efficient absorption into the body and accelerated bloodstream circulation. While

extracting fish collagen, various fish by-products like fishbone, scales, and skins are used as raw materials so that substantial waste ranging from 30% to 50% of the raw materials processed from industrial fish facilities can be re-evaluated sustainably (Khan et al. 2009; Aleman and Martinez-Alvarez 2013; Blanco et al. 2017; Carvalho et al. 2018; Jafari et al. 2020).

Collagen hydrolysis can be achieved through two distinct methods: chemical and enzymatic processes. In the chemical method, collagen hydrolysis involves subjecting the collagen to acid

or alkaline conditions at elevated temperatures (around 120°C) and pressures (approximately 100 kPa). While this method offers cost-effective extraction, its popularity has waned in recent years due to drawbacks such as the loss of desired functional properties of the hydrolysate and equipment corrosion. Conversely, the enzymatic method circumvents many of these disadvantages by employing lower temperatures, pressures, and a pH range typically between 5 and 8. This approach has become increasingly attractive for hydrolysate extraction due to its ability to mitigate the drawbacks associated with the chemical method (Sathivel et al. 2005; Šližyte et al. 2004; Korkmaz et al. 2021; Nagai and Suzuki 2000). In this study, collagen bioactive peptides were extracted from fish skin and scales, which are by-products of fish processing, using enzymatic methods. The effects of different enzyme types and their combinations were evaluated in terms of molecular weight, antioxidant properties, and amino acid composition. This study contributes to sustainability and the circular economy by promoting waste valorization and the use of green technology in extraction.

Materials and Methods

Materials. Sea bass fish scales were provided by the local fish farm in Trabzon, Turkey. The dried pangasius fish skin was purchased from Viet Delta Industrial Co. in Vietnam. The dried skins were brought to the laboratory in airtight bags and stored at -40°C. The study utilized four different enzyme

types provided by Novozymes: Alcalase® at 2.4-4.0 AU-A.g⁻¹, Flavourzyme® at 500-1000 LAPU.g⁻¹, Collagenase® at > 100,000 U.g⁻¹, and Neutrase® at 0.5 AU.g⁻¹. All the other analytical grade chemicals used in experiments were purchased from Merck, USA.

Fish scale demineralization. The demineralization method described by Nagai and Suzuki (2000) was used with slight modifications. Firstly, fish scales were washed with water to remove any contaminants and dried in an oven at 45°C for 24 h. Subsequently, the dried fish scales underwent treatment with 0.1M NaOH at a concentration of 10% w/v of raw material for 1 h. They were then rinsed with distilled water until reaching a pH of 7. Following this, the scales were subjected to treatment with 1M HCl for 1.5 h to complete the demineralization process. Post-demineralization, the scales were washed with distilled water to achieve pH neutralization. Finally, the demineralized flakes were dried at 45°C for 24 h.

Gelatin extraction from fish scale. Gelatin extraction from the scales of sea bass was adapted from Izzati et al. (2017) with slight modifications. The gelatin was extracted from demineralized fish scales by using hot water extraction at pH 5.5 and 55°C for 2 h, followed by coarse filtration, activated carbon treatment, and linter filtration to purify the solution (Fig. 1). After filtration samples were dried via lyophilized and stored at room temperature until characterization.

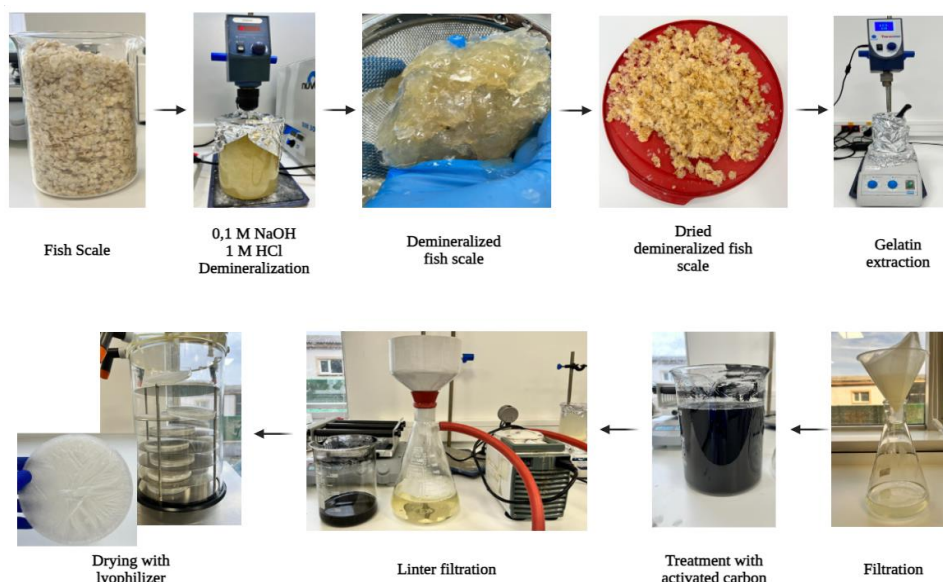


Figure 1. Fish scale gelatin extraction

Gelatin extraction from fish skin. During the gelatin extraction from the Pangasius fish skin, firstly degreasing was performed to remove fats from fish skin. The degreasing was conducted using 10% (v/v) isopropyl alcohol for 5 h. Then, for the ripening process, the fish skins were soaked in 0.1 M NaOH for 1.5 h, followed by treatment with 0.05 M acetic acid for 2 h. After the pretreatment, ripened fish skin was mixed with deionized water and hydrothermally extracted at pH 6.0 and 70°C for 2 h. After the extraction process, the same filtration steps were applied to the solution like fish scale extraction (Mendis et al. 2005).

Enzymatic hydrolysis of gelatin obtained from fish scales and skin. In order to obtain gelatin hydrolysates, after a 2 h extraction, the pH of the gelatin solution was measured and adjusted properly for further hydrolysis under the existence of various enzymes. The hydrolysis process was carried out at 55°C, the optimal temperature for all enzymes involved and was monitored for 6 h with continuous stirring (Azizi Khesal et al. 2019). At the end of the hydrolysis, the solution was heated at 90°C for 15 min to deactivate the enzymes. Subsequently, the resulting hydrolyzed collagen solution was subjected to anion exchange treatment using Purolite A420S anion resin (1:1 w/v) under continuous stirring at room temperature (25°C) for 45 min to reduce conductivity. Following this, the solution was mixed with activated charcoal (Merck 102184.1000, extra pure food-grade, 2 g.L⁻¹) at 50°C for 45 min to remove impurities and unwanted color compounds. The mixture was then filtered through a cellulose-based linter. Finally, hydrolyzed samples obtained with the different enzymes were frozen at -80°C and lyophilized for 72 h. The detailed hydrolysis conditions are given in Table 2.

The processing workflow in this study followed a clear sequence of raw material → gelatin extraction → enzymatic hydrolysis → collagen hydrolysates, ensuring that all enzymatic treatments were performed on a standardized gelatin substrate obtained from fish skin and scales.

Proximate composition. The moisture, ash, crude protein, fat, and hydroxyproline analysis were performed on fish skin and scales before and after the treatments to evaluate the effects of pretreatment such as demineralization, degreasing, and ripening process. Moisture content was assessed by placing

approximately 2 g of the sample into a pre-weighed aluminum dish (AOAC 2003).

Table 2. Enzyme combination and hydrolyzation conditions.

Enzyme	Raw material concentration, %	Enzyme concentration, %	Time, h
A	10	1.5	5
C	10	1.5	5
P	10	1.5	5
N	10	1.5	5
A.F	10	0.75 + 0.75	4 + 4
A.C	10	0.75 + 0.75	4 + 4
A.P	10	0.75 + 0.75	4 + 4
A.N.F	10	0.5 + 0.5 + 0.5	4 + 4 + 4
A.N.F.C	10	0.35 + 0.35 + 0.35 + 0.35	4 + 4 + 4 + 4

The samples were then dried in an oven at 105°C until a constant weight was achieved. Ash content was estimated by incinerating the pre-dried sample in a crucible at 600°C until white ash was formed (AOAC 2005). Total crude protein ($N \times 6.25$) in the raw material was determined using the Kjeldahl method (Latimer 2023). Hydroxyproline content was determined according to AOAC method 990.26-1993 (AOAC 1993).

Degree of hydrolysis. The degree of hydrolysis (DH) for the samples treated with different enzymes was assessed using the trichloroacetic acid (TCA) solubility method described by Liu et al. (2015). In this approach, the collagen hydrolysates produced after enzymatic treatment were mixed with 20% (w/v) TCA to separate the precipitable proteins from the soluble peptide fraction. The mixtures were then centrifuged at 7800 g for 15 min., and the nitrogen content of the resulting supernatant was determined by the Kjeldahl procedure (Latimer 2023). The DH value was expressed as the percentage of TCA-soluble nitrogen relative to the total nitrogen in the sample, as shown in Equation 1:

$$DH = \frac{10\% \text{ TCA soluble nitrogen in the sample}}{\text{Total nitrogen in the sample}} \times 100, \% \quad (1)$$

Fourier transform infrared (FTIR) analysis. FTIR analysis was performed using an Agilent Cary

630 spectrometer to examine the chemical characteristics of collagen obtained from fish scales and skin. The samples were analyzed directly in the 500-4000 cm^{-1} spectral range with the aid of a diamond-based Horizontal Attenuated Total Reflectance (HATR) unit. This approach enabled the evaluation of how different enzymatic treatments influenced the major collagen-specific vibrational bands, particularly those corresponding to amide A, amide I, amide II, and amide III, which reflect the structural and conformational properties of the hydrolyzed collagen.

Molecular weight analysis (HP-SEC). Molecular weight distribution of the collagen hydrolysates was assessed by High-Performance Size Exclusion Chromatography (HP-SEC). The analyses were carried out on an Agilent 1200 HPLC system (Germany) equipped with a TSK-gel 2000 SW XL column (Tosoh, Japan). For each run, a 10 μL sample was injected and eluted with a mobile phase composed of acetonitrile, water, and trifluoroacetic acid (40: 60: 0.05, v/ v/ v) at a constant flow rate of 0.5 $\text{mL}\cdot\text{min}^{-1}$. Detection was performed at 220 nm using a UV detector. Calibration of the system was achieved using a set of GME porcine collagen peptide standards with molecular weights of 1920 Da (893614), 2850 Da (899598), 3310 Da (899154), 4560 Da (891727), and 7765 Da (890482). The number-average molecular weight (M_n) of each sample was calculated based on a narrow-range calibration curve derived from these standards.

Amino acid composition. To determine the amino acid composition of the collagen peptides obtained from fish scale and fish skin, samples were digested for 16 h in 20 mL of 6N HCl at 110°C. The hydrolysate was then dissolved in deionized water and filtered. The amino acid profile was determined using high-performance liquid chromatography (HPLC), equipped with a Waters 410 Scanning Fluorescence detector and an AccQ Tag column (3.9 $\times$ 150 mm). AccQ Tag Eluent A and AccQ Tag Eluent B, or 60% acetonitrile with acid, were used as the mobile phases (flow rate = 1 $\text{mL}\cdot\text{min}^{-1}$) (See et al. 2011).

Antioxidant activity, DPPH assays. The antioxidant activity of the peptide fractions was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay (Chobdar Rahim et al. 2021). A DPPH stock solution

($6 \times 10^{-5}\text{M}$) was freshly prepared in methanol, and 4.9 mL of this solution was combined with 0.1 mL of appropriately diluted collagen hydrolysate. The mixtures were kept in the dark for 30 min to allow the reaction to proceed, after which their absorbance at 517 nm was measured using a Shimadzu 1240 spectrophotometer. A Trolox calibration curve (1-5 mM) was used for quantification, and the antioxidant capacity of the samples was expressed as mg Trolox equivalents ($\text{mM Trolox}\cdot 100\text{ g}^{-1}$). The percentage of DPPH radical inhibition was calculated using the equation provided in Equation 2:

$$\text{DPPH inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100, \% \quad (2)$$

Antioxidant activity, ABTS⁺ assays. The ABTS⁺ radical scavenging capacity was determined according to the procedure outlined by Chobdar Rahim et al. (2021). To generate the ABTS⁺ radical cation, a 7 mM ABTS stock solution was reacted with 2.45 mM potassium persulfate and kept at room temperature in the dark for 12-16 h. Before use, this mixture was diluted with distilled water until an absorbance value of 0.700 at 734 nm was obtained. For the assay, 2 mL of the adjusted ABTS⁺ solution was combined with 0.1 mL of the collagen hydrolysate and allowed to stand in the dark for 6 min. The absorbance was then recorded at 734 nm using a Shimadzu 1240 spectrophotometer. A Trolox calibration curve was employed for quantification, and the antioxidant activity was expressed as millimoles of Trolox equivalents per 100 g (Trolox $\text{mM}\cdot 100\text{ g}^{-1}$). The percentage inhibition of the ABTS⁺ radical was calculated as shown in Equation 3:

$$\text{ABTS} = \frac{A_{\text{ABTS}} - A_{\text{Sample}}}{A_{\text{ABTS}}} \times 100, \% \quad (3)$$

Statistical analysis. The experimental design consisted of three independent randomized groups, each analyzed in duplicate, resulting in a total of six observations ($n = 6$). Statistical evaluation of the data was carried out using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, implemented in SPSS software (version 25.0; SPSS Inc., Chicago, USA). Differences among samples were considered statistically significant when the confidence level exceeded 95% ($p < 0.05$).

Results and Discussion

Chemical characterization of fish skin and fish scale. The results of the characterization tests conducted on fish skin and fish scales before and after pretreatment are summarized in Table 3. Fish skin and scales are valuable raw materials due to their naturally high protein content. [Muyonga et al. \(2004\)](#) reported that the elevated ash levels found in fish scales mainly originate from calcium phosphate and other inorganic minerals, highlighting the importance of an effective demineralization step to improve collagen yield. In the present study, the substantial reduction of ash from 32.51% to 4.54% confirms that minerals were efficiently removed. Similar observations were made by [Syandri et al. \(2023\)](#), who noted that demineralization concentrates the organic fraction and consequently increases the relative protein content. Therefore, the rise in protein levels from 42.70% to 73.40% in fish scales is an expected outcome of both mineral loss and collagen enrichment.

In fish skin, degreasing leads to a different but equally important transformation. [Scheja et al. \(2024\)](#) demonstrated that removing the lipid phase exposes collagen fibrils and enhances the tissue's water-binding capacity. Consistent with this, the

decrease in fat content from 35.90% to 1.73% in the present study-accompanied by an increase in moisture-is likely due to collagen absorbing more water once the lipids are eliminated. The marked increase in hydroxyproline content further supports this structural concentration. [Wang et al. \(2025\)](#) similarly documented that demineralization exposes collagen chains, resulting in higher Hyp levels. Overall, the pretreatments applied to both skin and scales successfully enriched collagen and aligned well with the values reported in recent literature.

FTIR analysis. The FTIR spectral profiles of collagen peptides derived from fish skin and scales are presented in Table 4. Distinct vibrational bands characteristic of collagen were identified in both materials. In peptides obtained from fish skin, Amide A appeared between 3421-3465 cm^{-1} , Amide B between 2881-2931 cm^{-1} , Amide I between 1618-1650 cm^{-1} , Amide II between 1502-1587 cm^{-1} , and Amide III between 1212-1244 cm^{-1} . Similarly, peptides produced from fish scales displayed Amide A at 3292-3311 cm^{-1} , Amide B at 2926-2954 cm^{-1} , Amide I at 1622-1652 cm^{-1} , Amide II at 1528-1566 cm^{-1} , and Amide III at 1216-1239 cm^{-1} . These characteristic amide regions confirm the preservation of the collagen backbone following enzymatic hydrolysis.

Table 3. Proximate composition and hydroxyproline content of fish skin and scales

	Fish skin (dried)	Skin (after degreasing)	Fish scale (dried)	Fish scale (after demineralization)
Moisture, %	11.20±0.46 ^b	52.80±2.21 ^{*d**}	19.30±0.77 ^c	9.30±0.73 ^a
Ash, %	0.63±0.08 ^b	0.46±0.25 ^a	32.51±2.10 ^c	4.54±0.21 ^b
Crude Protein, %	44.85±2.05 ^a	41.30±4.04 ^b	42.70±3.06 ^b	73.40±2.37 ^c
Fat, %	35.90±2.73 ^b	1.73±0.12 ^a	-	-
Hydroxyproline, %	6.69±0.03 ^a	3.45±0.13 ^b	4.43±0.11 ^b	11.19±0.33 ^c

* The values are the mean and the standard deviation;

**Different letters in each line indicate statistical difference ($p < 0.05$)

Table 4. FTIR results of gelatin hydrolysates

	Skin										Scale									
	A	C	P	N	AF	AC	AP	ANF	ANFC	A	C	P	N	AF	AC	AP	ANF	ANFC		
Amide I	1650	1632	1647	1620	1638	1645	1618	1634	1635	1623	1643	1637	1641	1652	1644	1628	1622	1631		
Amide II	1540	1555	1537	1517	1558	1546	1502	1587	1523	1555	1537	1546	1566	1528	1542	1537	1532	1537		
Amide III	1240	1218	1234	1226	1234	1228	1212	1244	1229	1231	1228	1225	1235	1239	1222	1216	1228	1220		
Amide A	3365	3351	3360	3346	3360	3358	3321	3354	3344	3301	3298	3292	3308	3311	3293	3300	3302	3293		
Amide B	2910	2885	2930	2892	2934	2928	2881	2843	2921	2926	2928	2932	2954	2943	2928	2933	2946	2934		

More noticeable and broadened peaks were observed in the Amide A and B regions, particularly in hydrolyzed samples. The broadening of Amide A is mainly attributed to O–H stretching vibrations involved in hydrogen bonding, which typically intensifies after peptide chains are fragmented. Peaks around $2843\text{--}2954\text{ cm}^{-1}$ originate from N–H and CH_2 stretching, while those near $2934\text{--}3078\text{ cm}^{-1}$ also reflect similar stretching modes. The Amide I band ($1618\text{--}1652\text{ cm}^{-1}$), primarily associated with C=O stretching, is widely recognized as a key indicator of protein secondary structure; the positions detected in this study suggest partial unfolding of the triple helix during

hydrolysis. The Amide II region ($1502\text{--}1587\text{ cm}^{-1}$), corresponding to N–H bending and C–N stretching, and the Amide III region ($1212\text{--}1244\text{ cm}^{-1}$), reflecting O=C–N and N–H bending, further support that the peptide products retained the fundamental features of collagen while undergoing controlled structural modification (Fig. 2a–b). Overall, the spectral patterns obtained here are highly consistent with previously published FTIR profiles of collagen-derived peptides reported by several authors (Izzati et al. 2017; Kirdat and Dandge 2021; Zhang et al. 2019), confirming both the successful extraction of collagen and the structural integrity of the resulting hydrolysates.

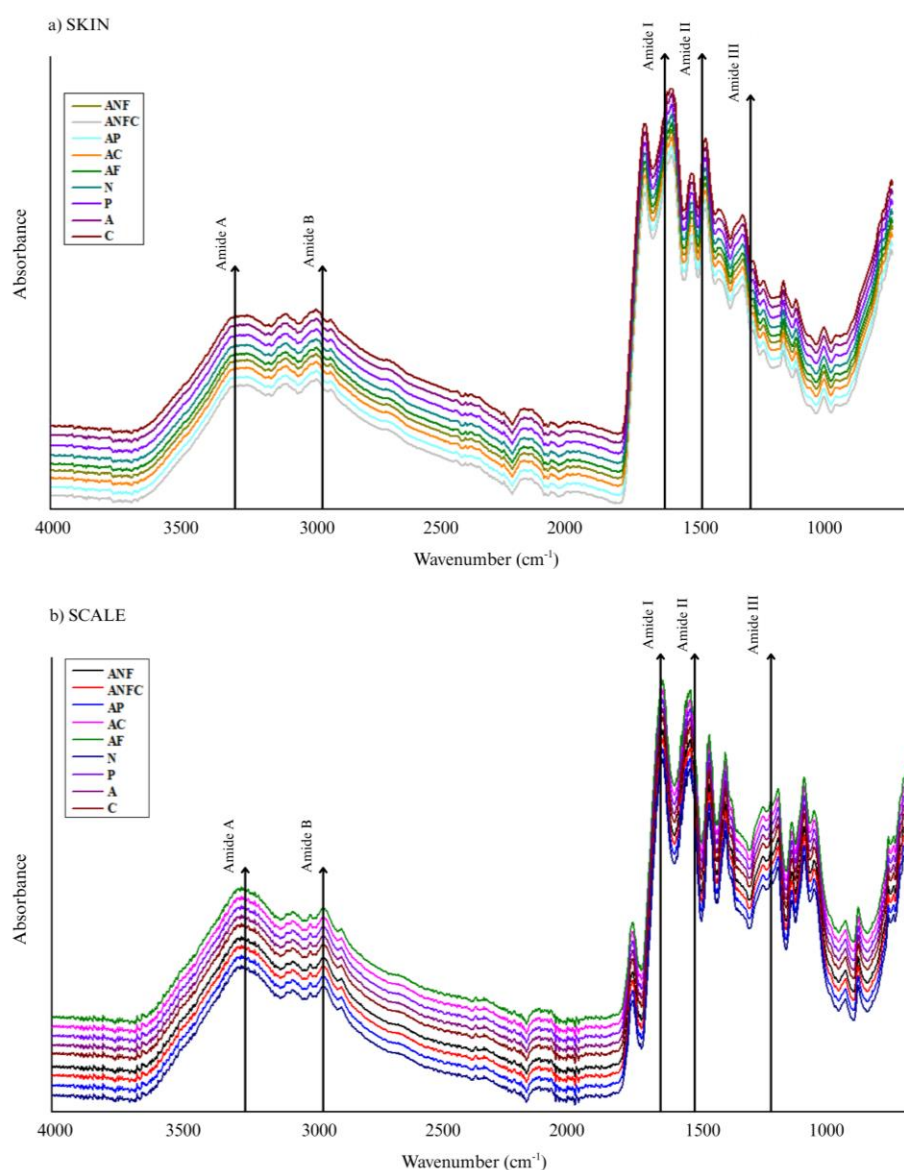


Figure 2. FTIR spectra of gelatin hydrolysates from a) skin and b) scales

Degree of hydrolysis. The degree of hydrolysis (DH) obtained from different enzyme combinations is summarized in Table 5. Significant differences were observed among treatments ($p < 0.05$). For collagen hydrolysates produced from fish skin, the lowest DH was recorded in samples treated solely with collagenase (17.00 ± 4.04), whereas the combination of Alcalase, Neutrase, and Flavourzyme (ANF) resulted in the highest hydrolysis level (47.00 ± 5.13). A similar trend was

evident in collagen hydrolysates derived from demineralized fish scales. Collagenase again yielded the lowest DH value (9.00 ± 3.03), while the ANF combination showed the highest hydrolytic activity, reaching 41.00 ± 3.28 ($p < 0.05$). These results indicate that the multi-enzyme blend (ANF) was consistently the most effective system for breaking down collagen structures in both raw materials.

Table 5. Hydrolysis degrees of collagen peptide samples

	A	C	P	N	DH, %				
					AF	AC	AP	ANF	ANFC
Skin	$39.00 \pm 5.01^{***}$	17.00 ± 4.04^a	28.00 ± 3.63^b	31.00 ± 4.47^b	34.00 ± 5.69^{bc}	30.00 ± 4.19^b	33.00 ± 4.56^b	47.00 ± 5.13^d	40.00 ± 6.18^c
Scale	32.00 ± 3.57^c	9.00 ± 3.03^a	21.00 ± 4.56^b	24.00 ± 5.40^b	33.00 ± 6.87^c	30.00 ± 4.73^c	32.00 ± 5.85^{cd}	41.00 ± 3.28^d	39.00 ± 3.84^d

*Standard Deviation; ** Different letters in each column indicate that the difference is statistically significant ($p < 0.05$).

Comparable outcomes have been reported in previous studies. Umi Hartina et al. (2019) applied Alcalase and Bromelain to milkfish (*Chanos chanos*) skin and reported DH values between 7.24% and 9.23%, depending on hydrolysis time. In another investigation, Nilsuwan et al. (2021) used an Alcalase–Papain combination on Sockeye salmon skin, obtaining DH levels ranging from 20% to 40%. These findings, together with the present results, demonstrate that Alcalase especially when used in combination with other proteases-is a highly efficient enzyme system for producing collagen-

derived bioactive peptides from both fish skin and scales.

Molecular weight analysis (HP-SEC). The molecular weight of collagen peptides is a critical factor influencing their absorption and biological activity in the human body (Hong et al. 2019). In this study, the molecular weight profiles of gelatin hydrolysates produced with different enzyme combinations were determined using HP-SEC, and the results for both fish skin and scale hydrolysates are shown in Table 6.

Table 6. Molecular weights of skin and scale gelatin hydrolysates

	Weight average molecular weight (MW)								
	A	C	P	N	AF	AC	AP	ANF	ANFC
Skin	1083	1459	1324	1233	1114	1265	1129	950	1034
Scale	1186	1706	1334	1286	1112	1306	1142	969	1092

Among all treatments, the Alcalase–Neutrase–Flavourzyme (ANF) combination generated the smallest peptide fractions, yielding molecular weights of 969 Da for fish skin and 950 Da for fish scales (Fig. 3a and 3b).

These values indicate a high degree of peptide fragmentation, which is typically associated with improved bioavailability and enhanced functional properties.

A strong negative correlation was observed between DH and molecular weight for both raw materials. As the degree of hydrolysis increased, peptide size decreased proportionally, with correlation coefficients of -0.9656 for fish skin and -0.9710 for scales (Fig. 4a and 4b). This relationship confirms that the multi-enzyme ANF system was the most effective at breaking collagen chains into low-molecular-weight peptides.

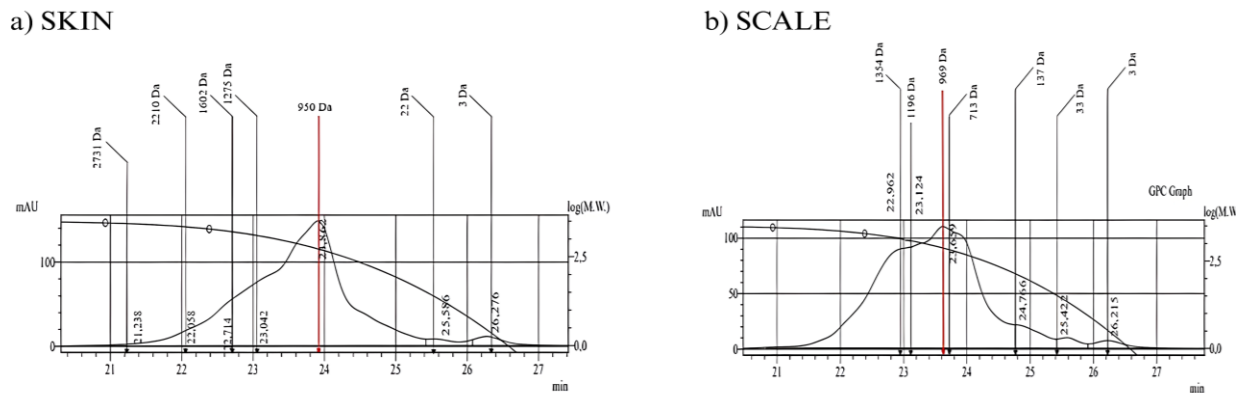


Figure 3. Average molecular weight of collagen peptide samples obtained from a) fish skin and b) scales

This relationship confirms that the multi-enzyme ANF system was the most effective at breaking collagen chains into low-molecular-weight peptides. Previous research supports these findings. Nilsuwan et al. (2021) reported molecular weights between 1460 and 1975 Da when using Alcalase and Papain on salmon skin, which are higher than the values obtained in the present study. Chotphruethipong et al. (2021) demonstrated that collagen hydrolysates with molecular weights below 3000 Da can stimulate osteoblast

proliferation and alkaline phosphatase activity, indicating their suitability for functional food or nutraceutical applications. This is further supported by evidence showing that low-molecular-weight peptides exhibit superior digestibility and physiological activity (Sibilla et al. 2015). Overall, the present findings confirm that the ANF combination is highly effective at producing small, bioactive collagen peptides from both fish skin and scales.

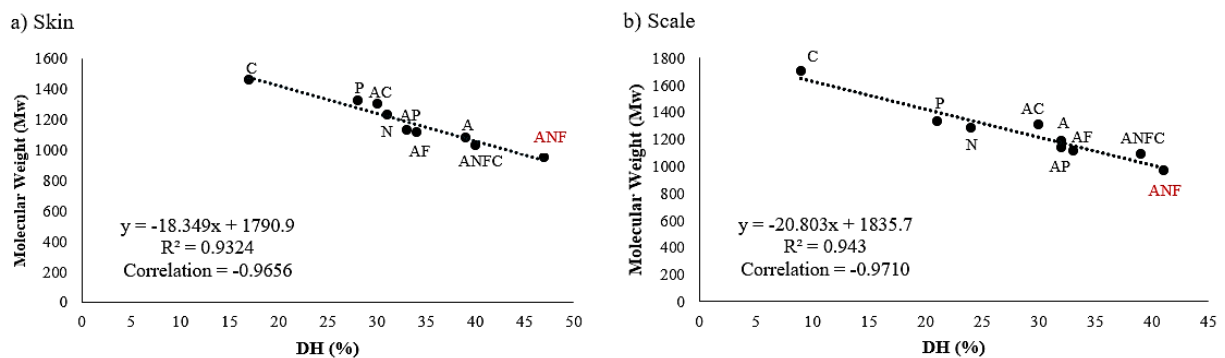


Figure 4. Correlation chart of the degree of hydrolysis and molecular weight for a) fish skin and a) fish scale collagen hydrolysates

Amino acid composition. Amino acids such as glycine (Gly), proline (Pro), hydroxyproline (Hyp), and glutamic acid (Glu) are central to the structural and nutritional qualities of collagen-derived peptides. Their distribution can vary considerably depending on the raw material and the enzymes used during hydrolysis. In this study, the essential amino acid profiles of collagen peptides obtained from fish skin and scales were compared across different enzyme combinations (Table 7). Among all treatments, the ANF combination produced

hydrolysates with the highest essential amino acid content, reaching 32.80 mg.100 g⁻¹ in fish skin and 26.91 mg.100 g⁻¹ in fish scales. This indicates that the multi-enzyme system was particularly effective in releasing indispensable amino acids from the collagen matrix.

Amino acid analysis further showed that hydrolysis performed with Alcalase generated peptide mixtures enriched in hydrophobic residues. This is consistent with the enzyme's specificity, as

Alcalase preferentially cleaves peptide bonds adjacent to hydrophobic and aromatic amino acids such as leucine (Leu), tryptophan (Trp), and proline (Pro). Papain also targets similar residues, although its catalytic efficiency is generally lower than that of Alcalase. The presence of aromatic amino acids, including phenylalanine (Phe) and tyrosine (Tyr), was more pronounced in papain-treated samples, which may contribute to enhanced bioactivity due to their radical-scavenging potential. Overall, the amino acid composition trends observed in this study align well with previously reported findings on enzymatically produced collagen hydrolysates (Izzati et al. 2017; Mohammad et al. 2014; Abuine et al. 2019; Najafian and Babji 2012).

Antioxidant activity. The antioxidant capacities of collagen peptides produced from fish skin and scales using different enzyme combinations are summarized in Table 8, and the differences among treatments were statistically significant ($p < 0.05$).

For fish skin hydrolysates, the Alcalase–Neutrase–Flavourzyme (ANF) combination yielded the

strongest antioxidant effect, reaching 240.21 mg Trolox.100 g⁻¹ in the DPPH assay and 16.97 mM Trolox.100 g⁻¹ in the ABTS+ assay. The antioxidant ranking for skin-derived peptides followed the order: ANF > ANFC > AP > AF > A > P > N > AC > C.

A similar trend was observed for fish scale hydrolysates. The ANF enzyme system again produced the most potent antioxidant response, with DPPH and ABTS+ values of 223.24 mg Trolox.100 g⁻¹ and 16.34 mM Trolox.100 g⁻¹, respectively. The activity order for scale-derived peptides was as follows: ANF > AP > ANFC > A > P > N > AF > AC > C. These findings agree with earlier studies on fish-derived collagen peptides (Mendis et al. 2005; Abuine et al. 2019; Yaghoobzadeh and Peyravii Ghadikolaii 2020; Azizah et al. 2020).

A strong negative association was detected between antioxidant activity (ABTS and DPPH) and the molecular weight of the hydrolysates. As peptide size decreased, radical-scavenging activity increased (Fig. 5).

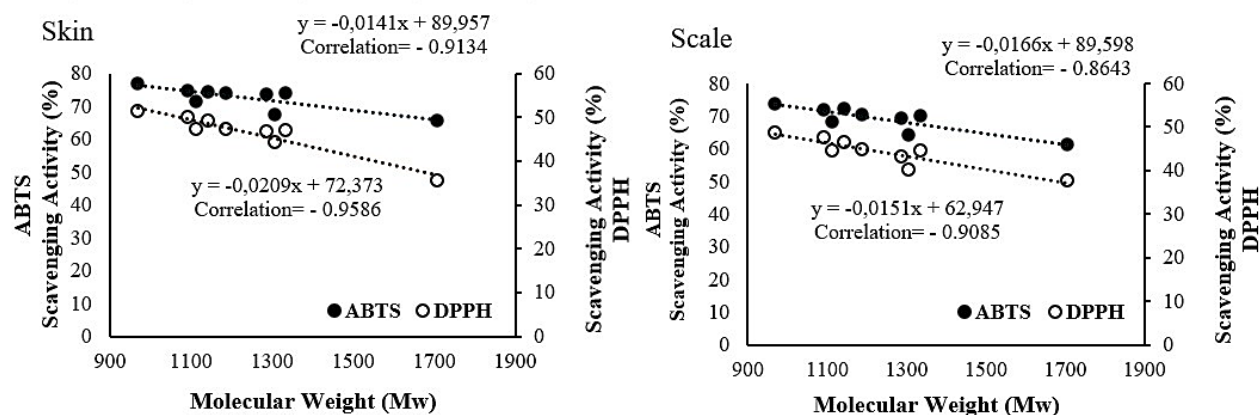


Figure 5. Molecular weight correlation chart with antioxidant activity (DPPH) (ABTS⁺)

Correlation coefficients were -0.9134 (ABTS) and -0.9586 (DPPH) for skin samples, and -0.8643 (ABTS) and -0.9085 (DPPH) for scale samples. This pattern underscores that smaller peptides generally exhibit greater antioxidant potential. The enhanced activity of low-molecular-weight peptides is likely related to their structural features, including

amino acid composition and hydrophobicity. Peptides rich in hydrophobic or aromatic residues can more effectively interact with free radicals. Moreover, the protease specificity directly influences peptide size and sequence, ultimately shaping their antioxidant characteristics (Nurilmala et al. 2020).

Table 7. Amino acid composition of fish skin and scale gelatin hydrolysates

	Amino acid composition of fish skin and scale collagen hydrolysate, mg.100 g ⁻¹																	
	A		C		P		N		AF		AC		AP		ANF		ANFC	
	Skin	Scale	Skin	Scale	Skin	Scale	Skin	Scale	Skin	Scale	Skin	Scale	Skin	Scale	Skin	Scale	Skin	Scale
Asp	5.87	6.73	4.13	5.12	5.34	4.38	5.72	6.00	5.88	5.42	5.80	6.00	5.90	6.95	6.04	7.00	6.00	7.00
Ser	5.52	4.87	3.72	3.65	6.12	4.43	5.13	4.17	6.07	4.95	5.48	4.27	6.64	6.50	7.21	7.00	6.86	6.75
Glu	8.76	9.72	6.64	5.43	7.52	6.00	7.68	7.80	8.79	9.12	7.13	8.00	7.90	8.89	8.94	9.00	8.83	9.00
Gly	11.14	11.38	9.48	10.00	11.53	13.81	11.72	12.00	11.68	11.52	10.49	11.12	11.92	12.25	12.61	13.00	12.04	12.70
His*	4.32	5.52	3.91	1.91	4.00	4.49	4.12	4.68	4.16	4.88	3.98	4.25	4.41	5.00	4.52	5.10	4.47	5.00
Arg	5.12	4.74	4.22	3.85	5.00	3.82	5.03	4.48	5.27	4.82	5.00	4.37	5.36	5.06	5.84	5.23	5.67	5.11
Thr*	6.41	5.83	5.16	3.46	5.68	5.06	5.88	5.00	6.86	5.00	6.00	5.69	6.24	6.00	7.07	6.71	6.94	6.12
Ala	6.12	5.49	5.53	3.35	6.63	6.24	6.00	5.78	6.90	6.21	6.34	5.00	6.71	6.24	7.00	6.74	6.88	6.68
Pro	13.64	12.43	9.98	10.93	14.03	13.76	13.88	14.85	13.91	13.64	13.47	12.00	14.58	14.67	15.27	15.74	14.65	15.00
Hyp	7.13	10.53	6.66	9.23	7.21	11.00	7.04	9.18	7.15	9.00	7.13	10.00	7.32	11.00	7.51	11.48	7.46	11.28
Tyr	2.54	2.13	2.00	1.86	2.52	1.84	2.31	2.07	2.61	2.22	2.55	2.08	2.78	2.34	3.00	3.17	2.98	3.10
Val*	3.42	2.58	2.86	2.26	3.23	1.74	3.18	3.00	3.06	2.64	3.51	2.71	3.95	2.69	3.22	3.00	3.00	2.90
Met*	3.94	1.94	2.57	1.24	4.09	2.00	3.84	1.98	4.00	2.00	3.96	1.79	4.13	2.00	4.21	2.09	4.19	2.00
Lys*	1.06	0.87	0.63	0.94	0.92	0.91	0.83	0.86	1.10	0.75	1.00	0.88	1.13	0.90	1.32	1.00	1.19	1.00
Ile*	3.00	1.03	1.74	0.83	2.64	1.14	2.51	1.64	2.95	1.27	3.03	1.51	3.25	1.21	3.41	1.41	3.38	1.34
Leu*	3.71	4.00	2.52	1.87	3.48	4.27	3.29	3.90	3.80	3.94	3.73	3.94	4.00	4.44	4.42	4.68	4.18	4.52
Phe*	4.21	2.47	3.42	2.25	4.09	2.56	4.00	2.13	4.06	2.00	4.29	2.00	4.33	2.68	4.51	2.78	4.50	2.71
Cys	0.05	0.00	0.00	0.00	0.05	0.05	0.04	0.05	0.05	0.00	0.04	0.00	0.05	0.05	0.05	0.07	0.05	0.05
Trp*	0.11	0.00	0.00	0.00	0.09	0.12	0.08	0.10	0.11	0.00	0.10	0.00	0.11	0.13	0.12	0.14	0.11	0.13
TEAA**	30.18	24.24	22.81	29.52	28.22	22.59	28.56	23.29	30.10	22.48	29.60	22.77	31.55	25.05	32.80	26.91	31.96	25.72

*Essential amino acid; **Total essential amino acid

Table 8. Antioxidant activity values gelatin hydrolysates produced from fish skin and scales

	DPPH				ABTS ⁺			
	mg Trolox.100 g ⁻¹ Skin	mg Trolox.100 g ⁻¹ Scale	Scavenging activity, % Skin	Scavenging activity, % Scale	mM Trolox.100 g ⁻¹ Skin	mM Trolox.100 g ⁻¹ Scale	Scavenging activity, % Skin	Scavenging activity, % Scale
A	214.08±3.26 ^{*c**}	199.65±1.85 ^d	47.29±3.11 ^{bc}	44.97±3.11 ^{bc}	16.36±1.54 ^{ab}	15.62±0.40 ^c	74.02±5.18 ^b	70.43±1.72 ^{cd}
C	141.00±1.96 ^a	154.32±3.48 ^a	35.65±3.37 ^a	37.73±2.37 ^a	14.62±1.00 ^a	13.70±1.10 ^a	65.62±2.78 ^a	61.16±1.79 ^a
P	212.00±3.16 ^c	196.91±3.81 ^d	46.99±3.12 ^{bc}	44.89±3.26 ^{bc}	16.34±1.84 ^{ab}	15.54±0.71 ^{bc}	73.92±3.56 ^b	70.05±1.80 ^{cd}
N	210.75±4.63 ^c	188.41±3.40 ^c	46.73±3.62 ^{bc}	44.54±5.10 ^{ab}	16.30±1.20 ^{ab}	15.43±0.65 ^{bc}	73.70±1.49 ^b	69.53±1.87 ^{cd}
AF	214.77±3.13 ^c	198.00±3.07 ^d	47.40±2.41 ^{bc}	43.28±2.99 ^{bc}	15.87±0.25 ^{ab}	15.19±0.51 ^{bc}	71.54±2.39 ^{ab}	68.34±2.19 ^{bc}
AC	195.52±3.98 ^b	169.83±7.32 ^b	44.35±2.97 ^b	40.27±3.67 ^{ab}	15.03±0.30 ^{ab}	14.35±1.02 ^{ab}	67.58±2.10 ^a	64.30±3.80 ^{ab}
AP	226.12±6.31 ^d	209.52±2.91 ^e	49.29±2.25 ^{bc}	47.39±3.87 ^{bc}	16.44±0.99 ^{ab}	16.04±0.25 ^c	74.39±4.91 ^b	72.46±3.25 ^{cd}
ANF	240.21±5.62 ^e	223.24±3.67 ^f	51.53±1.71 ^c	48.71±3.62 ^c	16.97±0.20 ^b	16.34±0.53 ^c	76.97±2.17 ^b	73.89±2.29 ^d
ANFC	230.00±3.32 ^d	217.08±5.70 ^f	49.92±3.76 ^{bc}	46.56±2.45 ^c	16.52±0.51 ^{ab}	15.90±0.15 ^c	74.76±3.18 ^b	71.78±2.99 ^{cd}

*The values are the mean and the standard deviation;

**Different letters in each line indicate statistical difference (p <0.05).

Conclusions

This study demonstrates that fish processing by-products, particularly fish skin and scales, can be efficiently converted into high-value collagen peptides through optimized enzymatic hydrolysis. The results show that hydrolysis efficiency is not driven by enzyme quantity but rather by the synergistic specificity of the enzymes used. Among all treatments, the Alcalase–Neutrase–Flavourzyme (ANF) combination yielded the smallest peptides, the highest antioxidant activity, and the greatest release of essential amino acids. The strong negative correlation between molecular weight and antioxidant capacity further highlights its superior performance. Overall, the ANF system represents the most effective strategy for producing bioactive collagen peptides from fish waste, offering promising potential for applications in food, nutraceutical, and pharmaceutical products.

Author Contributions

Conceptualization, S.C.R. and H.K.Y.; methodology, S.C.R., Z.B.A. and A.U.-D.; formal analysis, S.C.R., Z.B.A. and B.Ç.; investigation, S.C.R., Z.B.A. and B.Ç.; resources, F.A. and H.K.Y.; data curation, S.C.R. and B.Ç.; writing – original draft preparation, S.C.R.; writing – review and editing, Z.B.A. and H.K.Y.; visualization, S.C.R. and B.Ç.; supervision, H.K.Y.; project administration, H.K.Y. and F.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Ethical review and approval were not required for this study, as it did not involve any experiments on animals or invasive procedures on human subjects.

Informed Consent Statement

Not applicable.

Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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