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Enzymatically Produced Collagen Hydrolysates from Needlefish (*Tylosurus acus melanotus*) Skin Improve Antioxidant Activity and *In Vitro* Wound Healing

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Abstract

This study aimed to valorize needlefish (Tylosurus acus melanotus) skin as a marine by-product for producing collagen hydrolysates using alkaline protease (NCA) and papain (NCP), comparing their structure, antioxidant activity, and in vitro wound-healing potential with a commercial fish collagen hydrolysate (CCH). Hydrolysates were prepared from needlefish skin using alkaline protease and papain. Structure was evaluated by SDS–PAGE and FTIR. Antioxidant activity was assessed via DPPH, ABTS, and reducing power assays, while biocompatibility and wound healing were examined using cell viability and scratch assays with BALB/3T3 clone A31 fibroblasts at 24 and 48 h. SDS–PAGE showed faint, diffuse patterns with no bands above 37 kDa, confirming extensive degradation into low-molecular-weight peptides, especially in NCA. FTIR revealed typical collagen amide bands, with broader, less intense bands in NCA and NCP indicating structural modification. NCA showed the strongest antioxidant activity, with the lowest IC_{50} for DPPH (1.83 ± 0.06 mg/mL) and ABTS (1.34 ±

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0.02 mg/mL) and highest reducing power (90.00 ± 5.27 mg TE/g), followed by NCP and CCH. All hydrolysates were non-cytotoxic and enhanced fibroblast viability, with NCA and NCP outperforming CCH (peak at 50 μg/mL after 48 h) and promoting fibroblast migration and wound closure. Alkaline protease hydrolysis was most effective for producing bioactive needlefish skin collagen peptides with superior antioxidant capacity and fibroblast-supporting activity, highlighting potential applications in wound-healing biomaterials, nutraceuticals, and marine-based functional formulations.

Keywords:
Marine species, fish by-product utilization, hydrolyzed collagen, antioxidant, in vitro wound healing test

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1. Introduction

The needlefish (*Tylosurus acus melanotus*) is a pelagic species inhabiting surface waters and is widely distributed throughout tropical regions, particularly Southeast Asia, including Malaysia, Indonesia, and Thailand. The species can attain a maximum body length of approximately 150 cm and is characterized by an elongated body with countershaded pigmentation, comprising a bluish–green dorsal surface and a silvery ventral region that enhances camouflage in the water column (Froese & Pauly, 2024). From an economic standpoint, needlefish represents an important fishery resource in Malaysia, with an estimated annual production of approximately 760 tons (Department of Fisheries Malaysia, 2024). Despite its commercial importance, the utilization of needlefish is largely limited to its edible muscle, while other anatomical components, such as the skin, bones, head, viscera, fins, and scales, are commonly discarded during processing. These by-products may account for more than 50% of the total biomass and, when inadequately managed, pose environmental and sustainability challenges (Jaziri *et al*., 2023). Consequently, the valorisation of needlefish processing residues through a circular economy framework presents a promising strategy for converting underutilized biomass into high-value biomaterials while supporting sustainable fisheries and resource-efficient production systems.

Collagen is the most abundant structural protein in connective tissues and plays a fundamental role in maintaining tissue integrity, cellular adhesion, and extracellular matrix (ECM) organization. Due to its biocompatibility, biodegradability, and functional versatility, collagen has been extensively applied in the food, cosmetic, pharmaceutical, and biomedical sectors (Zheng *et al*., 2025). The global demand for type I collagen continues to increase in parallel with the rapid growth of functional foods, nutraceuticals, and regenerative biomaterials. In recent years, collagen hydrolysates, also referred to as collagen peptides, have attracted increasing scientific and commercial interest owing to their enhanced solubility, improved bioavailability, and superior biological functionality compared to native collagen (Zhang *et al*., 2020). Among the available production methods, enzymatic hydrolysis is widely regarded as the most effective and environmentally sustainable approach, as it enables controlled peptide generation under mild processing conditions and facilitates the release of bioactive sequences capable of modulating cellular behavior (Mora & Toldrá, 2023).

Marine-derived collagen hydrolysates produced from fish-processing by-products, including skin, cartilage, swim bladders, and bones, have been widely reported to exhibit notable antioxidant activity. Antioxidant capacity is commonly assessed using free radical scavenging and reducing assays, such as DPPH, ABTS, and FRAP, as well as through evaluations of lipid peroxidation inhibition and reactive oxygen species (ROS) suppression (Zhao *et al*., 2018). Collagen hydrolysates obtained from different fish species have demonstrated variable antioxidant efficiencies; for example, hydrolysates derived from Siberian sturgeon (*Acipenser baerii*) cartilage exhibited DPPH IC_{50} values of 1.05–1.27 mg/mL (Sheng *et al*., 2022), while those obtained from Spanish mackerel (*Scomberomorus niphonius*) skin showed stronger activity, with DPPH and ABTS IC_{50} values of 0.72–0.80 mg/mL and 0.82–0.86 mg/mL, respectively (Zhang *et al*., 2019). In addition, collagen hydrolysates from silver pomfret (*Pampus argenteus*) skin exhibited measurable ferric reducing power, reaching approximately 1.4% at 2 mg/mL in the FRAP assay (Ahmed *et al*., 2022), whereas swim bladder–derived collagen hydrolysates from grass carp (*Ctenopharyngodon idella*) displayed a DPPH IC_{50} value of 4.66 mg/mL (Dong & Dai, 2023). The antioxidant efficacy of collagen hydrolysates is strongly influenced by peptide-related factors, including molecular weight distribution, amino acid composition, and degree of hydrolysis, with low-molecular-weight peptides consistently demonstrating superior radical-scavenging and reducing

activities (Li *et al*., 2013). This enhanced antioxidant performance is particularly relevant to wound repair, as excessive oxidative stress at the wound site can impair fibroblast proliferation and migration, thereby delaying tissue regeneration (Jaziri *et al*., 2025).

Beyond their antioxidant properties, collagen hydrolysates have been increasingly recognized for their direct involvement in wound-healing mechanisms, particularly through the regulation of cell proliferation and migration. Fibroblast proliferation and migration are critical events during the proliferative phase of wound repair, as they drive ECM deposition and subsequent tissue remodelling. A growing body of in vitro evidence demonstrates that fish-derived collagen and collagen peptides significantly enhance fibroblast and keratinocyte viability, stimulate cell proliferation, and promote directional cell migration, resulting in accelerated wound closure in scratch assays, without inducing cytotoxic effects. For instance, type I collagen extracted from bigeye tuna (*Thunnus obesus*) skin significantly enhanced NIH-3T3 fibroblast growth and scratch closure rates compared to untreated controls (Lin *et al*., 2019). Similarly, collagen peptides derived from the swim bladders of giant croaker (*Nibea japonica*) effectively reduced oxidative stress–induced cellular damage in endothelial cells, indicating a cytoprotective role under wound-relevant stress conditions (Zheng *et al*., 2020). Enhanced fibroblast migration has also been reported following treatment with hydrolyzed collagen from unicorn leatherjacket (*Aluterus monoceros*) skin (Kumar *et al*., 2018), while hydrolyzed collagen from pirapitinga (*Piaractus brachypomus*) skin promoted rapid wound closure, achieving more than 80% closure within 12 h and complete closure within 24 h (Manjushree *et al*., 2023). Comparable pro-healing effects have been observed for hydrolyzed collagen from salmon skin, which significantly enhanced keratinocyte proliferation and migration, thereby facilitating the later phases of wound repair (Woonnoi *et al*., 2021). Collectively, these findings indicate that collagen hydrolysates function not only as passive structural supplements but also as bioactive functional agents capable of modulating cell-driven responses essential for effective wound repair.

Despite the growing evidence supporting the wound-healing potential of fish-derived collagen hydrolysates, systematic studies investigating the effects of different proteolytic enzymes on the physicochemical characteristics, molecular properties, and cell-regulatory functions of collagen hydrolysates derived from tropical fish skin are limited. In particular, scientific information regarding collagen hydrolysates obtained from the skin of needlefish is scarce, especially with respect to their ability to modulate fibroblast proliferation and migration. Therefore, this study aimed to evaluate needlefish skin as a sustainable source of enzymatically produced collagen hydrolysates, with a specific focus on their capacity to induce fibroblast cell migration and proliferation. This study investigated the effects of different proteolytic enzymes on the physicochemical properties, antioxidant activity, and in vitro wound-healing potential of needlefish skin collagen hydrolysates using BALB/3T3 fibroblast cells. These findings provide mechanistic insights into the role of needlefish-derived collagen hydrolysates in wound repair and support their potential application in marine-based nutraceutical and biomedical products.

2. Material and Methods

2.1. Materials

A commercial fish collagen hydrolysate (CCH) derived from type I collagen in tilapia skin was obtained from SRB Suria Trading (Kedah, Malaysia). Pepsin-soluble collagen (PSC) isolated from the needlefish skin was obtained from a previously reported study by Ramle *et al*. (2022). Proteolytic enzymes employed for collagen hydrolysis, including papain from papaya (800 kU/g; G8430) and alkaline protease (200 U/mg; B8360), were purchased from Solarbio (Beijing, China). Reagents for antioxidant capacity determination, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), were procured from Merck (Darmstadt, Germany) and Sigma-Aldrich (MO, USA). Prestained protein molecular weight standards were supplied by Bio-Rad Laboratories (CA, USA), and a multicolor low-range protein ladder was obtained from Thermo Fisher Scientific (MA, USA). BALB/3T3 clone A31 fibroblast cells and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Elabscience Biotechnology Inc. (TX, USA). Cell culture supplements, including fetal bovine serum (FBS) and trypsin–EDTA, were obtained from Gibco (NY, USA). The chemicals used for cell viability assessment, namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO), were supplied by Nacalai Tesque (Kyoto, Japan). All additional chemicals used in this study were of analytical grade.

2.2. Preparation of needlefish skin collagen hydrolysate

PSC extracted from needlefish skin was used as the starting material for the production of collagen hydrolysates. The hydrolysis procedure was performed as described by Felician *et al*. (2019), with slight modifications. Briefly, one gram of PSC was dissolved in 200 mL of ultrapure water and incubated at 50 °C in a Memmert shaking water bath (Schwabach, Germany) until complete solubilization was achieved. The resulting collagen solution was independently subjected to enzymatic hydrolysis using either alkaline protease or papaya proteinase at an enzyme concentration of 5% (w/w), yielding NCA and NCP, respectively. Hydrolysis was conducted at 50 °C for 2 h, under constant agitation. The enzyme activity was subsequently terminated by heating the reaction mixture at 95 °C for 15 min. Following cooling to ambient temperature (25 °C), the hydrolysates were centrifuged at 3000 rpm for 30 min to remove the insoluble residues. The clarified supernatants were collected, freeze-dried using a Labconco freeze-dryer (KC, USA), and stored in a freezer until further analysis.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight (MW) distribution of the hydrolysates (CCH, NCA, and NCP) was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), adapted from the protocol originally described by Laemmli (1970). For sample preparation, approximately 2.5 mg of each hydrolysate was solubilized in 1 mL of 5% (w/v) SDS and incubated in a water bath at 85 °C (Schwabach, Germany) for 1 h to ensure complete protein denaturation. The solutions were subsequently centrifuged at 8,500 × g for 5 min at room temperature (25 °C), and the clarified supernatants were recovered for electrophoresis. Each sample was combined with Laemmli loading buffer, either in the presence or absence of 10% (v/v) β-mercaptoethanol, at a volumetric ratio of 1:1, and heated at 85 °C for 3 min. Aliquots of 15 µL were then applied to polyacrylamide gels comprising a 5% stacking layer and a 7.5% resolving matrix, respectively. Protein separation was performed using a Mini-PROTEAN II electrophoresis system (CA, USA) operated at a constant voltage of 120 V per gel for approximately 3 h. Following electrophoresis, protein bands were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 prepared in a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid, after which excess dye was removed by destaining using the same solvent composition.

2.4. Fourier transform Infrared Spectroscopy (FTIR)

The functional group characteristics of CCH, NCA, and NCP were examined by Fourier transform infrared (FTIR) spectroscopy using an Agilent Cary 630 FTIR spectrometer (SC, USA), with the analytical procedure adapted from Fatiroi *et al*. (2023). For the analysis, approximately 1 mg of each sample was directly applied to the crystal surface of the instrument. Infrared spectra were collected over the spectral range of 4000 to 800 cm^{-1} with a spectral resolution of 2 cm^{-1}, averaging 32 successive scans for each sample. A background spectrum was recorded from a clean, empty crystal cell at ambient temperature and was automatically subtracted from the sample spectra. Spectral processing and interpretation were conducted using the Agilent Microlab software.

2.5. Determination of antioxidant activities

DPPH assessment: The antioxidant capacity of the hydrolysate samples was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as adapted from the procedure reported by Sheng *et al*. (2022). Briefly, aliquots of 50 µL of the extracts prepared at different concentrations were combined with 195 µL of a 0.1 mM DPPH solution in methanol in a 96-well microplate. The reaction mixtures were gently mixed for 1 min and subsequently incubated at room temperature in the absence of light for 30 min to allow radical scavenging. Following incubation, the decrease in absorbance was recorded at 517 nm using a microplate reader (Infinite 200 PRO; Tecan, Männedorf, Switzerland). Trolox, tested over a concentration range of 6.25–100 µg/mL, was used as the reference antioxidant. The half-maximal inhibitory concentration (IC_{50}), representing the extract concentration required to neutralize 50% of the DPPH radicals, was determined through regression analysis of the scavenging activity data.

ABTS Assay: The radical scavenging ability of CCH, NCA, and NCP was determined using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, adapted from the method described by Zhang *et al*. (2019). The ABTS radical cation ($ABTS^{\bullet+}$) was prepared by combining 5 mL of a 7 mM ABTS solution with 88 µL of a 140 mM potassium persulfate solution (1:0.35, v/v), followed by incubation in the dark at ambient temperature for 16 h to allow complete radical formation. Before use,

the ABTS•$^{+}$ solution was diluted with distilled water to achieve an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated to 30 °C. For the assay, 100 µL of the extract was mixed with an equal volume of ABTS•$^{+}$ solution in a 96-well microplate and allowed to react for 6 min at room temperature. Absorbance was measured at 734 nm using a microplate reader. Trolox, tested at concentrations ranging from 6.25 to 100 µg/mL, was used as the reference standard. The half-maximal inhibitory concentration (IC_{50}), defined as the sample concentration required to quench 50% of the ABTS radicals, was calculated from the regression analysis of the scavenging activity.

FRAP Assay: The reducing potential of the hydrolysates was assessed using the ferric reducing antioxidant power (FRAP) assay, adapted from the protocol reported by Egner *et al*. (2025). The FRAP working solution was freshly prepared by combining sodium acetate buffer (38 mM, pH 3.6), ferric chloride (20 mM), and 2,4,6-tripyridyl-s-triazine (TPTZ, 10 mM) dissolved in 40 mM hydrochloric acid, mixed in a volumetric ratio of 10:1:1. For the analysis, 20 µL of each extract was added to 180 µL of freshly prepared FRAP reagent in a 96-well microplate. The reaction mixtures were incubated at 37 °C for 40 min in the dark to allow the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. Following incubation, the absorbance was measured at 593 nm using a microplate reader. Antioxidant capacity was quantified by comparison with a Trolox calibration curve and expressed as milligrams of Trolox equivalent per gram of extract (mg TE/g extract).

2.6. BALB/3T3 cell culture

The BALB/3T3 clone A31 fibroblast cell line was used for all in vitro experiments using cells at passages 8–10. Cells were propagated in T25 culture flasks containing 5 mL of complete growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (100 U/mL), as described by Migone *et al*. (2022). Cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in a ThermoFisher Scientific incubator (MA, USA), and the culture medium was replaced every 2–3 days until the cells reached approximately 70–80% confluence. Upon reaching the desired confluence, the cells were enzymatically detached by adding 1 mL of trypsin–EDTA solution and incubating for 3 min under standard culture conditions. The resulting cell suspension was transferred to a 15 mL centrifuge tube, and complete medium was added to neutralize the trypsin activity. Cells were then pelleted by centrifugation at 1000 × g for 5 min, after which the supernatant was discarded, and the cell pellet was resuspended in fresh complete medium. Cell morphology and viability were routinely examined using an inverted microscope (OlympusT, YO, Japan).

2.7. Determination of cell viability

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Prior to cell seeding, BALB/3T3 fibroblasts were enumerated using a hemocytometer. Cells were then plated into 96-well culture plates at a density of 1×10^4 cells per well and incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2 to permit cell adherence. Following attachment, the cells were exposed to collagen hydrolysates at concentrations of 0, 6.25, 12.5, 25, 50, and 100 µg/mL and incubated for an additional 24 h under identical culture conditions. After treatment, 50 µL of MTT reagent (0.5 mg/mL prepared in 1× phosphate-buffered saline) was added to each well, and the plates were incubated for 6 h to allow the reduction of MTT into insoluble formazan crystals by the metabolically active cells. The culture medium was then carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The plates were gently agitated and incubated for 15 min, after which the absorbance was measured at 570 nm using an Infinite 200 PRO microplate reader (ZH, Switzerland), following the method described by Migone *et al*. (2022).

2.8. Determination of cell migration

Cell migration was evaluated using an in vitro scratch (wound healing) assay adapted from the method described by Manjushree *et al*. (2023) with slight modifications. BALB/3T3 fibroblast cells were seeded into sterile 24-well culture plates at a density of 1×10^5 cells per well and maintained at 37 °C in a humidified incubator containing 5% CO_2 for 24 h until a confluent cell monolayer was formed. A straight and consistent scratch was introduced across the cell monolayer using a sterile 100 µL pipette tip. Detached cells and debris were carefully removed by washing the wells twice with phosphate-buffered saline (PBS). Following scratch formation, the cells were incubated in serum-free medium as a negative control or treated with collagen hydrolysates at concentrations ranging from 6.25 to 100

μg/mL. Medium supplemented with 10% fetal bovine serum (FBS) was used as a positive control. Images of the wound area were acquired immediately after scratching (0 h) and after 24 and 48 h of incubation using an inverted microscope with a 4× objective lens. The extent of wound closure was quantified using ImageJ software by determining the reduction in the scratch area over time. The percentage of scratch closure was calculated using the following equation:

$$\text{Wound closure (\%)} = [(W_0 - W_t) / W_0] \times 100$$

where W_0 denotes the initial wound area at 0 h and W_t represents the wound area measured at the specified time.

2.9. Statistical Analysis

All experimental data are presented as the mean ± standard deviation (SD) of a minimum of three independent replicates. Statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test to assess differences between treatment groups. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the SPSS software (version 29.0; IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. SDS-PAGE analysis

The SDS–PAGE profiles of commercial fish collagen hydrolysate (CCH) and needlefish skin collagen hydrolysates prepared using alkaline protease (NCA) and papain (NCP) are presented in Figure 1. All hydrolysate samples showed faint and diffuse electrophoretic patterns, with most of the staining intensity concentrated near the lower region of the gel. This indicates that the collagen molecules were extensively cleaved into lower-molecular-weight peptides. The absence of clear bands above 37 kDa suggests that the large collagen chains were effectively degraded during hydrolysis. NCA exhibited a greater proportion of low-molecular-weight peptides than NCP and CCH, as indicated by the more diffuse migration pattern toward the lower part of the gel. This suggests that alkaline protease produced more extensive peptide chain cleavage than papain. SDS–PAGE analysis provides important evidence regarding the molecular weight distribution of collagen and its hydrolysates. Native type I collagen typically exhibits two major α-chains at approximately 100–130 kDa, along with higher-molecular-weight β- and γ-components formed through cross-linked collagen chains. The disappearance of these bands in CCH, NCA, and NCP indicates extensive degradation of the collagen structure into smaller peptide fragments. This result confirms that the samples were no longer composed of intact collagen molecules but mainly consisted of hydrolyzed collagen peptides. A similar finding was reported by Schmidt *et al*. (2020), who observed that collagen hydrolyzed using Alcalase and Flavourzyme showed the disappearance of medium- to high-molecular-weight bands in SDS–PAGE, confirming the conversion of collagen molecules into smaller peptide fractions.

The apparent difference between NCA and NCP suggests that enzyme specificity plays an important role in determining the molecular profile of the hydrolysates. Alkaline protease may have cleaved collagen chains more extensively, resulting in smaller peptide fragments. In contrast, papain hydrolysis may have produced slightly larger peptide fractions because of its different cleavage preference. These findings are consistent with previous studies reporting that alkaline proteases, particularly Alcalase, are highly effective in degrading protein substrates into low-molecular-weight peptides. Forghani *et al*. (2012) reported that black sea cucumber (*Stichopus horrens*) hydrolysed using Alcalase showed peptide fractions mainly distributed below 20 kDa compared with hydrolysates produced using other proteolytic enzymes. Similarly, Ahmadifard *et al*. (2016) demonstrated that rice bran protein concentrate hydrolyzed using Alcalase exhibited greater protein degradation than papain-treated samples, resulting in lower molecular weight peptide distributions. Therefore, the more diffuse low-molecular-weight pattern observed in NCA supports the strong proteolytic efficiency of alkaline protease in producing smaller collagen-derived peptides than that of pepsin.

These enzyme-dependent differences are relevant because peptide size distribution strongly influences the physicochemical and biological properties of collagen hydrolysates, including their solubility, antioxidant activity, cellular uptake, and bioactivity (González-Serrano *et al*., 2022). Smaller peptide fragments are generally more soluble and may interact more efficiently with biological systems than larger protein fragments (Chang *et al*., 2024; Sulistyo *et al*., 2025). Thus, the lower molecular weight profile of NCA may partly explain its stronger antioxidant activity, fibroblast viability-enhancing effect,

and wound closure activity observed in subsequent assays. The SDS–PAGE profile of NCA was comparable to that of CCH, indicating that the alkaline protease-derived needlefish collagen hydrolysate had a degree of degradation approaching that of the commercial fish collagen hydrolysate. This finding is important because it suggests that needlefish skin, a fish-processing by-product, can be transformed into a collagen hydrolysate with molecular characteristics approaching those of commercial collagen products. However, unlike commercial hydrolysates, the enzymatic process applied to needlefish skin can be controlled to generate specific peptide profiles with targeted biological functions. Taken together, SDS–PAGE analysis demonstrated that enzymatic hydrolysis using alkaline protease and papain effectively degraded needlefish skin collagen into lower-molecular-weight peptides. The absence of intact collagen chains and the presence of diffuse low-molecular-weight patterns support the successful production of collagen hydrolysates.

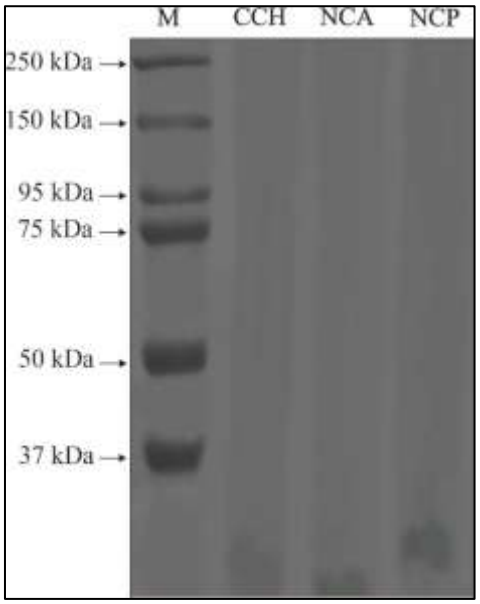


Figure 1. SDS–PAGE profiles of needlefish skin hydrolysates on a 7.5% resolving gel. Lane M, Precision Plus Protein Dual Color Standard; lane CCH, commercial fish collagen hydrolysate; lane NCP, needlefish collagen hydrolysed with papain; lane NCA, needlefish collagen hydrolysed with alkaline protease.

3.2. IR-spectra analysis

FTIR spectroscopy is commonly used to identify the functional groups and structural changes in collagen hydrolysates. Fig. 2 shows the FTIR spectra of fish collagen hydrolysates, in which all samples displayed typical absorption bands of collagen-derived materials, including amides A, B, I, II, and III. The broad amide A band observed at approximately 3300 cm^{-1} indicates N–H stretching vibrations, which may be influenced by hydrogen bonding between the peptide chains and water molecules (Sai & Babu, 2001). The amide B band, observed at approximately 2920–2940 cm^{-1}, reflects the asymmetrical stretching of CH_2 groups and is commonly associated with the side chains and backbone structure of collagen peptides (Hsu *et al*., 2005). The amide I band is one of the most important spectral regions for evaluating collagen structure because it is highly sensitive to changes in the secondary structure of proteins. In the present study, the amide I band was detected at approximately 1650–1660 cm^{-1} in all hydrolysate samples, suggesting the presence of peptide carbonyl groups originating from collagen chains, as this band is mainly associated with C=O stretching vibrations along the polypeptide backbone (Payne & Veis, 1988). The amide II and amide III bands further confirmed the presence of peptide bonds through N–H bending coupled with C–N stretching vibrations, which were observed at approximately 1540–1560 cm^{-1} and 1230–1240 cm^{-1}, respectively (Jackson *et al*., 1995). These results demonstrate that enzymatic hydrolysis maintained the basic collagen peptide structure while modifying the molecular arrangement of the hydrolysates.

The broader and less intense amide bands observed in NCA and NCP compared with CCH indicate that enzymatic hydrolysis altered the structural organization of the needlefish skin collagen. Hydrolysis by alkaline protease and papain cleaves peptide bonds, reduces molecular size, and disrupts intermolecular interactions, including hydrogen bonding and the formation of ordered collagen structures. Consequently, the reduction in amide band intensity may reflect the breakdown of larger collagen

molecules into smaller peptide fragments. This spectral pattern is consistent with the expected transformation of collagen into collagen hydrolysates and agrees with Schmidt *et al*. (2020), who reported that enzymatic hydrolysis using Alcalase and Flavourzyme reduced the intensity of amide bands and promoted structural changes in collagen hydrolysates. The more pronounced decrease in the amide band intensity in NCA suggests that alkaline protease may have produced a greater extent of peptide chain cleavage than papain. This finding is consistent with previous reports showing that collagen hydrolysates prepared using alkaline protease exhibited a lower amide band area than those produced using other proteases, indicating partial degradation of collagen helical arrangements during hydrolysis (Schmidt *et al*., 2020).

The decrease in the amide III region may also indicate a partial disruption of the collagen triple-helical structure. The amide III band near 1240 cm^{-1} is sensitive to changes in the triple-helical arrangement of collagen molecules, whereas the band around 1450–1456 cm^{-1} is associated with pyrrolidine ring vibrations of proline and hydroxyproline residues (Bet *et al*., 2001). Therefore, the reduced intensity of the amide III band in the hydrolyzed samples, particularly in NCA, suggests that alkaline protease hydrolysis partially denatured the ordered collagen structures. A similar structural disruption was reported by Chi *et al*. (2016), who found that trypsin hydrolysis destroyed the triple helical structure of collagen from skate, red stingray, and scalloped hammerhead cartilages. These enzyme-dependent differences in peptide structure are important because molecular size, amino acid sequence, and functional groups can influence the biological activity of collagen hydrolysates.

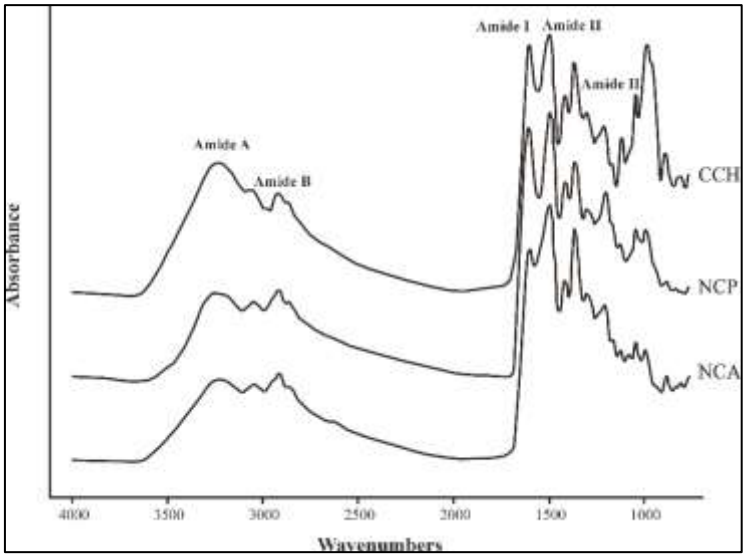


Figure 2. FTIR spectra of needlefish skin hydrolysates: CCH, commercial fish collagen hydrolysate; NCP, needlefish collagen hydrolyzed with papain; NCA, needlefish collagen hydrolyzed with alkaline protease.

3.3. Antioxidant properties

The antioxidant properties of the needlefish skin collagen hydrolysates are presented in Table 1. The positive control, ascorbic acid, showed the strongest radical-scavenging activity ($p < 0.05$), with the lowest IC_{50} values for both DPPH and ABTS assays, namely, 0.05 ± 0.01 mg/mL and 0.13 ± 0.01 mg/mL, respectively. Among the collagen hydrolysate samples, NCA exhibited the highest antioxidant activity ($p < 0.05$), as indicated by the lowest IC_{50} values for DPPH and ABTS radicals. The IC_{50} values of NCA were 1.83 ± 0.06 mg/mL for DPPH and 1.34 ± 0.02 mg/mL for ABTS, which were significantly lower than those of NCP and CCH. NCP exhibited intermediate antioxidant activity, with IC_{50} values of 3.02 ± 0.13 mg/mL for DPPH and 4.31 ± 0.30 mg/mL for ABTS. These values were higher than those of NCA but lower than those of CCH, indicating that papain-derived needlefish collagen hydrolysate possesses a moderate radical-scavenging capacity. In contrast, CCH exhibited the weakest antioxidant activity ($p < 0.05$) among the hydrolysate samples, with IC_{50} values of 3.34 ± 0.11 mg/mL and 5.30 ± 0.31 mg/mL for DPPH and ABTS, respectively. A similar trend was observed in the reducing power assay expressed as mg of TE/g of extract. NCA showed the highest reducing capacity ($p < 0.05$), reaching 90.00 ± 5.27 mg TE/g extract, followed by NCP at 46.67 ± 4.28 mg TE/g extract and CCH at 12.19 ± 1.46 mg TE/g. These results indicate that alkaline protease hydrolysis produces collagen

hydrolysates with superior electron-donating capacity compared to papain hydrolysis and commercial collagen hydrolysates.

The antioxidant activity of collagen hydrolysates is strongly influenced by their peptide profiles generated during enzymatic hydrolysis. In the present study, NCA exhibited the strongest radical-scavenging activity and reducing power among the hydrolysate samples, suggesting that alkaline protease was more effective than papain in generating antioxidant peptides from needlefish skin collagen. The lower IC_{50} values of NCA in both the DPPH and ABTS assays indicate that this hydrolysate required a lower concentration to neutralize 50% of free radicals. This result suggests that NCA contains peptide fractions with a higher radical-quenching efficiency than those of NCP and CCH. Similar antioxidant potential has been reported for collagen hydrolysates from marine fish by-products. Chen *et al*. (2018) reported that collagen hydrolysate from milkfish (*Chanos chanos*) scale showed strong DPPH and ABTS radical-scavenging activities, while Wang *et al*. (2020) demonstrated that purified peptides from redlip croaker (*Pseudosciaena polyactis*) scale collagen hydrolysate exhibited potent DPPH scavenging activity, lipid peroxidation inhibition, and protection against oxidative DNA damage. These findings support the present results that fish collagen hydrolysates can serve as promising sources of antioxidant peptides.

The superior antioxidant activity of NCA may be associated with differences in peptide size, amino acid composition, and sequence. Enzymatic hydrolysis breaks collagen into smaller peptides and exposes amino acid residues that contribute to its antioxidant activity. Peptides containing hydrophobic amino acids, aromatic residues, and imino acids, such as proline and hydroxyproline, may enhance radical-scavenging activity by improving interactions with free radicals and donating hydrogen atoms or electrons. This explanation is consistent with that of Pan *et al*. (2019), who reported that low-molecular-weight collagen peptides with specific amino acid sequences exhibited marked DPPH and ABTS radical-scavenging activities. Similarly, Zheng *et al*. (2020) found that collagen peptides from giant croaker (*Nibea japonica*) swim bladders exhibited strong DPPH and ABTS radical-scavenging activities and protected cells against H_2O_2-induced oxidative damage by enhancing endogenous antioxidant enzymes. Therefore, the strong DPPH and ABTS scavenging activities of NCA may reflect the formation of peptide sequences with favorable antioxidant properties.

The ABTS assay generally detects both hydrophilic and lipophilic antioxidant compounds, whereas the DPPH assay is more sensitive to compounds with stronger activity in relatively nonpolar systems. The consistently low IC_{50} values of NCA in both assays indicate that alkaline protease-derived collagen hydrolysate has a broad radical-scavenging ability. This finding is further supported by the reducing power result, where NCA showed the highest value of 90.00 mg TE/g of extract. A high reducing power indicates that the hydrolysate can act as an electron donor, converting oxidized intermediates into more stable products, thereby interrupting oxidative chain reactions. Comparable antioxidant activity has also been reported by Devita *et al*. (2021), who demonstrated that bigeye tuna (*Thunnus obesus*) skin collagen hydrolysate possessed antioxidant activity, further confirming the potential of tropical marine fish skin as a source of bioactive collagen peptides. The lower antioxidant activity of NCP compared to of that NCA may be attributed to the different cleavage specificities of papain. Although papain hydrolysis produces peptides with antioxidant activity, the resulting peptide profile may be less optimal for radical scavenging and electron donation than that produced by alkaline protease. Nevertheless, NCP exhibited better antioxidant activity than CCH, indicating that needlefish skin collagen hydrolysate prepared using papain retained relevant antioxidant potential. This study confirms that needlefish skin is a promising raw material for producing bioactive collagen peptides.

Table 1. Antioxidant properties of needlefish skin collagen hydrolysates prepared using different proteases

Samples	IC_{50} for DPPH (mg/mL)	IC_{50} for ABTS (mg/mL)	mg TE/g extract
PC	0.05 ± 0.01^{a}	0.13 ± 0.01^{a}	-
CCH	3.34 ± 0.11^{d}	5.30 ± 0.31^{d}	12.19 ± 1.46^{c}
NCA	1.83 ± 0.06^{b}	1.34 ± 0.02^{b}	90.00 ± 5.27^{a}
NCP	3.02 ± 0.13^{c}	4.31 ± 0.30^{c}	46.67 ± 4.28^{b}

The results are presented as mean ± standard deviation (n = 3). Different letters (within a bar) indicate significant differences (one-way ANOVA, Tukey's HSD test, $p < 0.05$). PC: Positive control (ascorbic acid); CCH: Commercial fish collagen hydrolysate; NCA: needlefish skin collagen hydrolyzed using alkaline protease; NCP: needlefish skin collagen hydrolyzed using papain.

3.4. Cell viability of fibroblast BALB/3T3 clone A31

The cytocompatibility of the needlefish skin collagen hydrolysates was evaluated using BALB/3T3 clone A31 fibroblasts after 24 and 48 h of treatment (Figure 3). All collagen hydrolysate samples increased fibroblast viability above that of the serum-free control, indicating that none of the tested concentrations exhibited cytotoxic effects. In the NCA treatment, fibroblast viability increased significantly ($p < 0.05$) at all concentrations compared to that in the untreated control, reaching approximately 145–165% after 24 h and 155–185% after 48 h. The highest response was observed at 50 µg/mL after 48 h, which approached the proliferative response induced by the 10% FBS positive control ($p > 0.05$). A similar pattern was observed in fibroblasts treated with the NCP. Cell viability increased in a concentration- and time-dependent manner, particularly after 48 h of incubation. At 24 h, NCP promoted fibroblast viability to approximately 135–160%, whereas after 48 h, the viability increased to approximately 155–185%. The 50 µg/mL NCP treatment showed the strongest stimulatory effect and was statistically comparable to the highest-response groups, suggesting that the papain-derived needlefish collagen hydrolysate supported fibroblast metabolic activity and/or proliferation. Commercial collagen hydrolysate (CCH) also enhanced BALB/3T3 viability, although the response was lower than that observed for NCA and NCP. CCH increased cell viability to approximately 110–150% after 24 h and 115–150% after 48 h, with the highest response generally observed at 50 µg/mL concentration. At 100 µg/mL, the stimulatory effect slightly declined, suggesting that the commercial hydrolysate may have reached a saturation point or contained peptide fractions with lower bioactivity at higher concentrations.

The increase in BALB/3T3 viability following treatment with NCA, NCP, and CCH indicates that collagen hydrolysates are biocompatible and do not exert cytotoxic effects on fibroblast cells. In cytotoxicity assessment, a material is generally considered non-cytotoxic when cell viability remains ≥70% relative to the control; in the present study, all treatments produced viability values well above this threshold. This finding suggests that needlefish skin collagen hydrolysates are safe for fibroblasts exposure within the tested concentration range. Comparable cytocompatibility has been reported for acid-solubilized collagen from flounder fish skin, where HFF-1 and L929 cells maintained at least 70% viability after collagen treatment (Sousa *et al*., 2023). In addition, hydrolyzed collagen from pirapitinga skin increased the viability of L292 mouse fibroblast cells and was considered non-toxic based on MTT assay results (Manjushree *et al*., 2023). These findings support the present results that fish collagen-derived materials can maintain or enhance skin cell viability under in vitro conditions.

The higher fibroblast viability observed after 48 h than after 24 h suggests that the collagen hydrolysates exerted a time-dependent stimulatory effect. Fibroblasts play a central role in the proliferative phase of wound healing through migration, extracellular matrix synthesis, collagen deposition and tissue remodeling. Therefore, the ability of collagen hydrolysates to enhance fibroblast viability is biologically relevant, as fibroblast proliferation is essential for granulation tissue formation and wound closure (Benito-Martínez et al., 2022). Similar findings were reported for type I collagen from bigeye tuna skin, which enhanced NIH-3T3 fibroblast growth and improved scratch closure compared to the control (Lin *et al*., 2019). Similarly, narrow-barred Spanish mackerel skin collagen-based films increased 3T3 cell growth and proliferation, which was partly attributed to improved cell attachment to the collagen-based matrix (Gharahgheshlagh *et al*., 2023).

The superior activity of NCA and NCP compared to that of CCH may be associated with differences in the peptide profiles generated by enzymatic hydrolysis. Alkaline protease and papain may release specific low-molecular-weight collagen peptides enriched with glycine, proline, and hydroxyproline-containing sequences, which are commonly associated with collagen-derived activities. Previous studies have shown that the molecular weight distribution and amino acid sequence of fish collagen peptides strongly influence their biological activity, including fibroblast proliferation. Mechanistically, collagen-derived peptides may act as nutritional substrates and bioactive signaling molecules. Collagen peptides from giant croaker swim bladder have been reported to protect HUVECs against H_2O_2-induced oxidative damage, indicating that fish collagen peptides can support cellular homeostasis under stressful conditions (Zheng *et al*., 2020). Furthermore, collagen-coated silk fibroin nanofibers increased

fibroblast viability and collagen production in BALB/3T3 clone A31 cells, confirming the responsiveness of this cell line to collagen-based biomaterials (Selvaraj *et al*., 2023).

Collectively, these findings demonstrate that needlefish skin collagen hydrolysates, particularly those produced using alkaline protease and papain, effectively enhance BALB/3T3 fibroblast viability. The viability-enhancing effect observed in this study is consistent with previous evidence showing that fish collagen and collagen-derived biomaterials support fibroblast growth, cellular biocompatibility, and wound repair-related cellular responses. Therefore, needlefish skin can be considered a valuable marine by-product for producing bioactive collagen peptides with potential applications as wound-healing biomaterials.

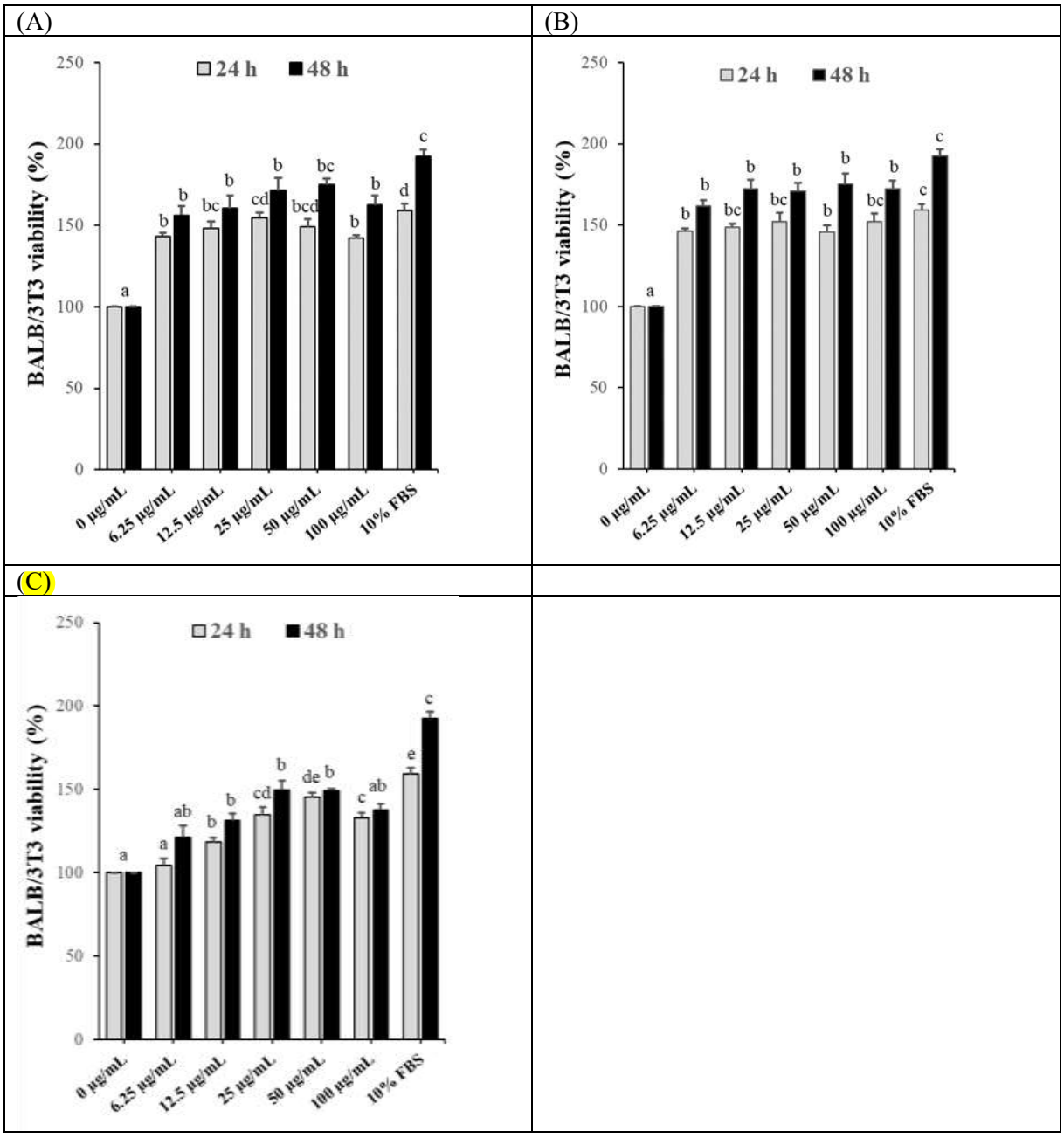


Figure 3. BALB/3T3 fibroblast viability after 24 and 48 h of treatment with needlefish skin collagen hydrolysates.

Cells treated with 0 µg/mL in serum-free and 10% FBS-supplemented media served as the negative and positive controls, respectively. (A) NCA, needlefish collagen hydrolyzed with alkaline protease; (B) NCP, needlefish collagen hydrolyzed with papain; and (C) CCH, commercial fish collagen hydrolysate. (B) Data are presented as mean ± standard deviation (SD) (n = 3). Different letters indicate significant differences (one-way ANOVA, Tukey's HSD, $p < 0.05$).

3.5. In vitro wound closure rate

The in vitro wound closure activity of needlefish skin collagen hydrolysates was evaluated using a scratch assay on BALB/3T3 clone A31 fibroblasts after 24 and 48 h of treatment (Figure 4). At 0 h, all treatment groups showed a clearly defined scratch area, indicating that the wound gap was uniformly generated prior to treatment. After 24 h of incubation, partial closure of the scratch area was observed in all groups, with a more evident reduction in the wound gap in collagen hydrolysate-treated cells than in the negative control. After 48 h, fibroblast migration became more pronounced, particularly in the positive control and cells treated with needlefish collagen hydrolysates. The negative control exhibited the lowest wound closure activity, indicating limited fibroblast migration under serum-free conditions. In contrast, the positive control containing 10% FBS exhibited the highest wound closure response, confirming the important role of serum-derived growth factors and nutrients in stimulating fibroblast migration in wound healing. Treatment with needlefish skin collagen hydrolysates enhanced wound closure compared to the negative control, demonstrating their ability to support fibroblast movement into the scratched area.

Among the hydrolysate treatments, NCA consistently promoted wound closure across all tested concentrations. The wound gap became visibly narrower after 24 h and further decreased after 48 h, indicating that the alkaline protease-derived collagen hydrolysate effectively supported fibroblast migration over time. NCP also promoted wound closure, with a clear improvement in scratch closure after 48 h compared to 24 h. This suggests that papain-derived collagen hydrolysate may provide bioactive peptide fractions that stimulate fibroblast motility during wound healing. The commercial collagen hydrolysate also enhanced fibroblast migration, but the wound closure response appeared lower and less consistent than that observed with NCA and NCP treatments. Moderate concentrations of CCH showed better wound closure than the lowest concentration, whereas the highest concentration did not further improve the response. The scratch assay results indicated that needlefish skin collagen hydrolysates, particularly those produced using alkaline protease and papain, exhibited promising in vitro wound-healing activity by promoting BALB/3T3 fibroblast migration.

Fibroblast migration is a key cellular event in the proliferative phase of wound healing. After tissue injury, fibroblasts migrate to the wound area, proliferate, and contribute to extracellular matrix deposition, collagen synthesis, and granulation tissue formation (Hossian & Mattheolabakis, 2020). Therefore, the ability of a material to enhance fibroblast migration in a scratch assay is commonly used as an early indicator of wound healing potential. This interpretation is consistent with previous in vitro studies showing that fish collagen and collagen hydrolysates improve cell migration and scratch closure. For instance, type I collagen from bigeye tuna (*Thunnus obesus*) skin enhanced NIH-3T3 fibroblast growth and produced better scratch closure than that in the control group (Lin *et al*., 2019). Similarly, hydrolyzed collagen from Nile tilapia (*Oreochromis niloticus*) skin promoted higher scratch closure in HaCaT cells, particularly at 50.0 μg/mL (Hu *et al*., 2017). In the present study, the limited wound closure observed in the negative control group suggests that serum deprivation restricted fibroblast migration, whereas the strong response in the positive control group confirmed that the assay system was responsive to pro-migratory stimulation.

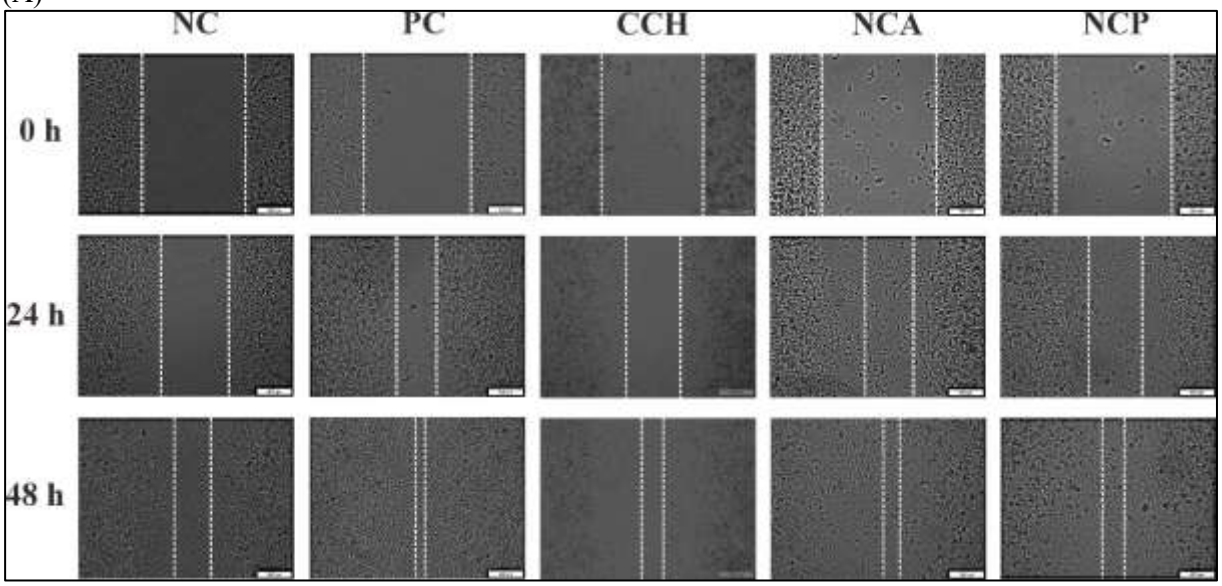
The enhanced wound closure observed after treatment with NCA and NCP indicates that the needlefish skin collagen hydrolysates contain bioactive peptides capable of supporting fibroblast migration. Collagen hydrolysates contain low-molecular-weight peptides rich in glycine, proline, and hydroxyproline, which may contribute to cellular activities related to wound repair (Li *et al*., 2024). These peptides may act as nutritional substrates and bioactive signals that influence fibroblast behavior, including adhesion, proliferation, migration, and extracellular matrix remodeling (Harris *et al*., 2021). Comparable findings were reported for hydrolyzed collagen from unicorn leatherjacket (*Aluterus monoceros*) skin, where a concentration of 0.2 mg/mL promoted greater cell migration than other treatments (Kumar *et al*., 2019). Manjushree *et al*. (2023) also reported that hydrolyzed collagen from pirapitinga (*Piaractus brachypomus*) skin showed non-toxic behavior in fibroblast cells and produced remarkable wound closure, reaching more than 80% at 12 h and complete closure within 24 h. These studies support our findings that fish skin collagen hydrolysates can enhance wound repair-related cellular responses.

The stronger activity of NCA and NCP compared to CCH may be related to differences in enzymatic hydrolysis and peptide composition. Alkaline protease and papain have different cleavage specificities, generating distinct peptide profiles with different molecular sizes and amino acid sequences. These differences may influence the interactions between collagen-derived peptides and fibroblast cells. The more consistent wound closure response observed in the NCA and NCP treatments suggests that

controlled enzymatic hydrolysis of needlefish skin collagen may produce peptide fractions with higher biological relevance for wound repair than commercial collagen hydrolysates. This finding is consistent with previous reports showing that collagen-derived materials can stimulate cell migration in wound-healing models. Felician *et al*. (2019) reported that jellyfish (*Rhopilema esculentum*) collagen significantly enhanced scratch closure in human umbilical vein endothelial cells after 48 h of treatment. In addition, Migone *et al*. (2022) demonstrated that jellyfish-derived polysaccharides supported BALB/3T3 clone A31 cell migration and proliferation, achieving an effective scratch repair of approximately 80% within 2 days.

The absence of a clear linear dose-response pattern, especially in NCA and NCP treatments, suggests that fibroblast migration may not depend solely on the peptide concentration. Once an effective concentration is reached, additional increases in collagen hydrolysate concentration may not necessarily result in greater wound closure rates. This phenomenon may occur because cellular responses can reach a saturation point, particularly when peptide-mediated signaling, cell surface interactions, or nutrient uptake reach an optimal level. Therefore, the biological activity of collagen hydrolysates is likely influenced by peptide quality, molecular weight distribution, and sequence specificity, rather than concentration alone. These findings demonstrate that needlefish skin collagen hydrolysates can enhance in vitro wound closure by promoting fibroblast migration. These results support the potential use of needlefish skin, a fish-processing by-product, as a sustainable source of bioactive collagen peptides for wound-healing applications.

(A)



(B)

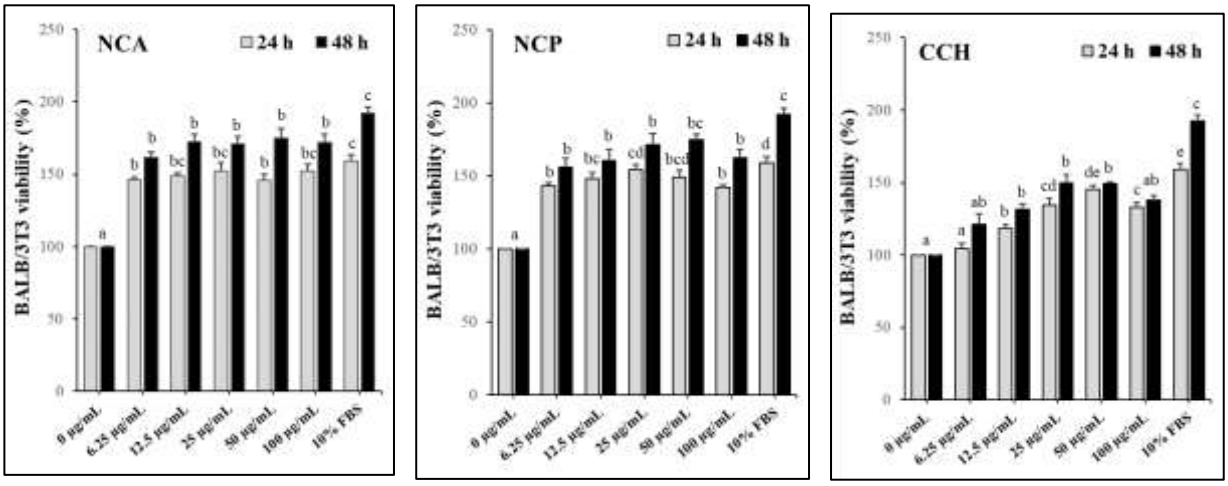


Figure 4. BALB/3T3 fibroblast cell migration after 24 and 48 h of treatment with needlefish skin collagen hydrolysates.

(A) Representative photographs of scratch test of different hydrolysates treated at 0, 24, and 48 h. (B) Cell migration rates at 24 h and 48 h. Cells treated with 0 µg/mL in serum-free medium and 10% FBS-supplemented medium served as the negative and positive controls, respectively. (A) NCA, needlefish collagen hydrolysed with alkaline protease; (B) NCP, needlefish collagen hydrolysed with papain; (C) CCH, commercial fish collagen hydrolysate. Data are presented as mean ± SD (n = 3). Different letters indicate significant differences (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

4. Conclusions

This study demonstrated that needlefish (*Tylosurus acus melanotus*) skin can be effectively valorised into bioactive collagen hydrolysates through enzymatic hydrolysis using alkaline protease and papain. SDS–PAGE confirmed the extensive degradation of collagen into lower-molecular-weight peptides, while FTIR analysis showed that the hydrolysates retained the characteristic collagen-derived amide bands, despite structural modifications induced by enzymatic treatment. Among the hydrolysates, alkaline protease-derived collagen hydrolysate (NCA) exhibited the most pronounced molecular degradation, suggesting that alkaline protease has a greater capacity to generate smaller peptide fractions than papain. Enzymatic treatment significantly influenced the biological properties of the hydrolysates. NCA exhibited the strongest antioxidant activity, as indicated by the lowest IC_{50} values for DPPH and ABTS radical scavenging and the highest reducing power compared with NCP and CCH. In cell-based assays, all hydrolysates were non-cytotoxic to BALB/3T3 clone A31 fibroblasts and enhanced cell viability above that of the serum-free control. NCA and NCP exhibited stronger fibroblast-supporting effects than CCH, particularly after 48 h of treatment, indicating that enzymatically generated needlefish collagen peptides may promote fibroblast metabolic activity and proliferation. Furthermore, the scratch assay demonstrated that NCA and NCP enhanced fibroblast migration and wound closure, supporting their potential role in stimulating cellular events associated with wound healing. In conclusion, alkaline protease hydrolysis was the most effective treatment for producing needlefish skin collagen hydrolysates with superior antioxidant capacity and wound-healing-related bioactivity. These findings highlight the potential of needlefish skin as a promising marine by-product for developing value-added collagen peptides for functional foods, nutraceuticals, cosmeceuticals, and wound-healing biomaterials. Future studies should focus on peptide fractionation and LC–MS/MS-based sequence identification to determine the specific peptides responsible for observed bioactivity.

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Conflic of Interest
The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this study.

Author Contribution
A.A.J. conceived and designed the study, conducted the investigation, performed the formal analysis, curated the data, drafted the manuscript, and revised the final version. R.N. supervised the study, contributed to the methodology, validated the results, and revised the manuscript accordingly. M.G.A. contributed to the investigation, data analysis, and manuscript revisions. D.A. contributed to the experimental work, data curation, and manuscript revisions. M.A.Z.B. contributed to the cell-based assay methodology, validation, and manuscript revisions. M.T.M.L. provided scientific input, resources, supervision, and manuscript revisions. I.M.A.Z. contributed to the sample preparation, investigation, and data analysis. M.A.A. contributed to the supervision, resources, and manuscript revision. R.A.M.M. contributed to the investigation, formal analysis, and interpretation of the data. N.H.S. contributed to the methodology, validation, resources, and manuscript revision. N.H. supervised the research, contributed to the conceptualization, provided scientific guidance, and revised the final manuscript. All the authors have read and approved the final version of the manuscript.

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